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INCREASED ACTIVATION OF p38 MAPK IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Teresa Renda[1,2], Simonetta Baraldo[3], Girolamo Pelaia[1], Erica Bazzan[3], Graziella Turato[3], Alberto Papi[4], Piero Maestrelli[5], Rosario Maselli[1], Alessandro Vatrella[2], Leonardo Fabbri[6], Renzo Zuin[3], Serafino Antonio Marsico[2], Marina Saetta[3].

[1]University "Magna Græcia" of Catanzaro, Department of Experimental and Clinical Medicine, Catanzaro, Italy.

[2]Second University of Naples, Department of Cardiothoracic and Respiratory Sciences, Naples, Italy.

[3]University of Padova, Department of Cardiac, Thoracic and Vascular Sciences, Padova, Italy.

[4]University of Ferrara, Department of Clinical and Experimental Medicine, Ferrara, Italy.

[5]University of Padova, Department of Environmental Medicine and Public Health, Padova, Italy.

[6]University of Modena and Reggio Emilia, Department of Oncology, Haematology and Respiratory Disease, Modena, Italy.

Corresponding author: Marina Saetta, University of Padova, Department of Cardiac, Thoracic and Vascular Sciences, Section of Respiratory Diseases, via Giustiniani 3, Padova, Italy.

email: marina.saetta@unipd.it, tel: 0039 049 8218515, fax: 0039 049 8213110

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Abbreviations

AP-1: Activator Protein-1

COPD: Chronic Obstructive Pulmonary Disease

CXCR3: Chemokine (C-X-C motif) receptor 3

DLCO: Diffusing Lung Capacity for carbon monoxide

ERK: Extracellular signal-regulated kinases

FEV_1: Forced Expiratory Volume in the first second

FEV_1/FVC: the ratio of Forced Expiratory Volume in the first second to the Forced Vital Capacity

HDAC: Histone Deacetylase

GOLD: Global Initiative for Chronic Obstructive Lung Disease, *guidelines*

GR: Glucocorticoid Receptor

IFN-γ: Interferon-γ

IL-1β: Interleukin-1β

IL-8: Interleukin-8

JNK: c-jun NH_2-terminal kinases

MAPK: Mitogen-activated protein kinases

NF-κB: Nuclear factor-κB

Phospho-p38: phosphorylated-p38

RV: Residual Volume

TNF-α: Tumour Necrosis Factor-α

ABSTRACT

Inflammation, oxidative stress and apoptosis, which are involved in COPD pathogenesis, may activate the p38 subgroup of mitogen-activated protein kinases (MAPK). Therefore, the aim of this study was to evaluate the expression of the phosphorylated, active form of p38 MAPK in lungs of COPD patients.

Surgical specimens were obtained from 18 smokers with COPD at different stages of disease severity, 9 smoking and 8 nonsmoking subjects with normal lung function. Phospho-p38+ cells were quantified by immunohistochemistry in both alveolar spaces and alveolar walls. Moreover, a Western blot analysis of phosphorylated p38 and total p38α expressed by alveolar macrophages was performed.

Phospho-p38+ alveolar macrophages and phospho-p38+ cells in alveolar walls were increased in patients with severe and mild/moderate COPD when compared with smoking and nonsmoking controls. Moreover, they were inversely correlated to values of FEV_1 and FEV_1/FVC. Western blot analysis showed that phosphorylated p38, but not the total α isoform, was specifically increased in alveolar macrophages from COPD patients.

Activation of the p38 MAPK pathway appears to be involved in the pathogenesis of COPD. Therefore, our findings suggest that this MAPK may be a suitable pharmacologic target for therapeutic intervention in COPD.

INTRODUCTION

Cigarette smoke plays a crucial role in the pathogenesis of COPD. Much of the information concerning COPD pathogenesis comes from studies performed in animal models or experimental *in vitro* systems, which do not always directly reflect what is occurring in human disease. However, it is currently well known that oxidants and oxidative stress due to cigarette smoking promote lung inflammation which is mediated, at least in part, by activation of the transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), coordinating the expression of several genes thought to be important in COPD such as interleukin-8 (IL-8) and tumour necrosis factor-α (TNF-α) [1-3]. These pro-inflammatory cytokines and chemokines, together with interleukin-1β (IL-1β), strongly activate the p38 subgroup of mitogen-activated protein kinases (MAPK), a family of signal transducing enzymes which also includes extracellular signal-regulated kinases (ERK) and c-jun NH_2-terminal kinases (JNK) [4]. MAPK are hierarchically organized into three modules operating via a phosphorylation cascade that converts them into activated forms (phospho-MAPK), thereby enabling their interaction with cytoplasmic substrates and translocation to the nucleus, where many MAPK targets such as transcription factors and histones are located [5]. The ERK pathway is especially stimulated by G-protein coupled receptors and growth factors involved in cell proliferation, differentiation and survival, whereas JNK and p38 modules are mainly activated by cytokines implicated in inflammation and apoptosis [6]. Within the MAPK family, both JNK and p38 subgroups are involved in mediating proinflammatory responses, though p38 seems to play a prominent role in COPD. Indeed, p38 activation in key inflammatory cell types correlates with disease initiation and progression [7]. p38 also appears to be the most effective MAPK in stabilising, at post-transcriptional level, the mRNAs for cytokines and chemokines relevant to COPD pathogenesis, such as TNF-α, IL-1β, IL-6, and IL-8 [8]. Conversely, p38 inhibitors are able to attenuate disease severity in animal models of COPD; therefore, p38 currently represents the main focus of novel anti-inflammatory therapies based on MAPK inhibition [9]. Four different isoforms of p38 MAPK are currently known, which are identified as α, β, γ, and δ, respectively, and

seem to be characterised by different functional patterns and cell expression profiles. In particular, lung macrophages predominantly express the α isoform; these cells also express p38δ, but not p38β or p38γ [10].

The initial inflammatory events leading to COPD pathogenesis probably represent a reaction of the airways to non-specific damaging agents. However, COPD is associated with an abnormal lung inflammation that persists even in patients who have stopped smoking [11]. It can be hypothesised that some inappropriate and self-amplifying pathogenic circuits may prevent the resolution of the inflammatory response in susceptible individuals. Within this biological context, activation of a key proinflammatory signalling pathway, such as p38 MAPK, could play a crucial role. Therefore, we decided to investigate the expression of the phosphorylated, active form of p38 in peripheral lung tissue as well as in primary cultures of alveolar macrophages obtained from smokers with COPD, in comparison with both smoking and non smoking controls. Preliminary results of this study have been previously reported in abstract form [12].

METHODS

Subject characteristics

Four groups of subjects undergoing lung resection for solitary peripheral carcinoma or lung volume reduction surgery (LVRS) were recruited to the study: 7 smokers with severe/very severe COPD (Gold Stages 3 and 4), 11 smokers with mild/moderate COPD (Gold Stages 1 and 2), 9 smoking and 8 nonsmoking controls (asymptomatic and with normal lung function). 5 of the severe/very severe COPD subjects underwent LVRS, while 2 underwent resection for lung cancer. All the patients with mild/moderate disease, the control smokers and the non-smokers underwent resection for lung cancer. Pulmonary function tests were performed during the week before surgery. According to the recent guidelines, fixed airway obstruction was defined as a ratio of forced expiratory volume in one second to forced vital capacity (FEV_1/FVC) of less than 70% predicted [13]; all patients had a reversibility of less than 12% after inhalation of 400 μg of salbutamol.

Chest radiographs were obtained within 48 hours of lung function studies. In a subset of patients, scores for emphysema were calculated on the basis of criteria for evaluation of roentgenographic signs of emphysema as previously described [14]. Briefly, these included signs of overinflation, i.e., depression and flattening of hemidiaphragms (evaluated on both the posteroanterior and the lateral views), and increased retrosternal space, which were individually scored from 0 to 3. For the evaluation of pulmonary vascular abnormalities, the posteroanterior film was divided in four quadrants at the level of the carina. Each quadrant was analyzed for the presence of different vascular abnormalities, which were assigned an individual score from 0 to 0.5. The partial scores from the four quadrants were summed and combined with the overinflation score to yield a cumulative score that ranged from 0 to 16.

During the month preceding the study, patients with COPD had no exacerbations, which are defined as increased dyspnea associated with a change in the quality and quantity of sputum, leading the subject to seek medical attention [15]. All the subjects had been free of acute upper respiratory tract infections and none had received bronchodilators within the previous 48 hrs. They were non-atopic (had negative skin tests for common allergen extracts) and had no past history of asthma or allergic rhinitis.

The study conformed to the Declaration of Helsinki, and informed written consent was obtained for each subject undergoing surgery or bronchoscopy.

Immunohistochemistry

Tissue blocks were taken from the subpleural parenchyma of the lobe obtained at surgery, avoiding areas involved by tumor. Samples were fixed in 4% formaldehyde and embedded in paraffin, as described previously [16], using a rabbit monoclonal primary antibody against phospho-p38 MAPK (dilution 1:100; Cell Signaling Technology Inc., USA), the avidin-biotin complex (ABC-complex kit, Dako), and detection by Fast Red substrate staining (Dako).

Phospho-p38 immunostaining was quantified in both alveolar walls and alveolar macrophages as follows. For cell counts in the lung parenchyma, we examined only the alveolar walls with a single layer of cells, to avoid bias caused by technical artifacts such as adjacent alveolar walls [17]. The number of positively stained cells within the alveolar walls was computed using a light microscope (Olympus BX41), connected to a video recorder linked to a computerized image system (Software: Image Pro Plus, 2005 Media Cybernetics Inc). Briefly, at a magnification of x630, the length of the alveolar walls was measured, and the number of positive phospho-p38 MAPK cells within these alveolar walls was counted. Ten fields, randomly distributed across the slide, were evaluated for each subject and the result was expressed as the number of positive cells/mm of alveolar wall as previously described [16]. Briefly, for evaluation of phospho-p38 expression in the alveolar macrophages, at least 100 macrophages inside the alveolar spaces were evaluated. Alveolar macrophages were defined as mononuclear cells with a well represented cytoplasm, present in the alveolar spaces and not attached to the alveolar walls. Results were expressed as number of positive macrophages per high power field (hpf). To correct for possible differences in absolute number of macrophages, results were also expressed as percentage of positive macrophages over total macrophages [17].

Moreover, we performed immunohistochemical analysis of inflammatory cells infiltrating the alveolar walls. Briefly, mouse monoclonal antibodies were used for identification of neutrophils (anti-elastase M752, Dako Ltd, High Wycombe, UK), macrophages (anti-CD68 M814, Dako), and CD8+ cells (anti-CD8 M7103, Dako) as previously described [18]. Ten fields, randomly distributed across the slide, were evaluated for each subject and the result was expressed as the number of positive cells/mm of alveolar wall as previously described [16].

The cases were coded and the measurements made without knowledge of clinical and functional data.

Cell cultures and Western blotting

Cells from two patients with COPD, one smoking and one non-smoking control (both with normal lung function) were obtained at bronchoscopy, collected in sterile saline solution and centrifuged at 500 x *g* for 5 min [19]. The supernatant was removed, cellular pellets were first washed and then resuspended in 10 ml of RPMI medium with added antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin; Sigma) and Fungizone (1 μg/ml; GIBCO BRL, Gaithersburg, MD). Cell types were determined by morphology using Diff-Quick staining (Baxter, Miami, FL); macrophages were >95% in each cell preparation. Finally, cells were diluted to 1 x 10^6 macrophages/ml, plated onto 6-well plates (1 ml per well) and allowed to incubate for 2 to 4 hrs to facilitate attachment. Subsequently, the medium was removed, and fresh medium was added. The cells were incubated overnight at 37^{o}C with 5% CO_2.

After culture, cells were trypsinised for 5 min, resuspended in supplemented RPMI, centrifuged at 4^{o}C for 5 min, and then lysed for Western blotting in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 10% glycerol, 4 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 50 mM Hepes, pH 7.4, plus PPIM, 25 mM β-glycerophosphate, 1mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Protein extracts were then separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia, Little Chalfont, UK). Immunoblotting was performed using the same anti-phospho-p38 monoclonal antibody used for immunohistochemistry. After being "stripped", the membranes were re-probed with an anti-p38α antibody (Autogen Bioclear, Calne, Wiltshire, UK). Antibody binding was visualized by enhanced chemiluminescence (ECL-Plus; Amersham Pharmacia).

Statistical Analysis

Group data were expressed as mean and standard error (SEM), or as median and range when appropriate. Differences between groups were analysed using the following tests for multiple comparisons: the analysis of variance (ANOVA) for clinical data, and the Kruskall-Wallis test for histological data. The Mann-Whitney U test was carried out after the Kruskall-Wallis test when appropriate. Spearman's rank correlation coefficient test was used to examine the association between histological parameters and functional data. Probability values of $p < 0.05$ were accepted as significant.

RESULTS

Clinical findings

TABLE 1. CLINICAL CHARACTERISTICS OF THE SUBJECTS*

	Smokers with COPD		**Subjects with normal lung function**	
	Severe-very severe	*Mild-moderate*	*Smokers*	*Non smokers*
Subjects	5 M:2 F	10 M: 1 F	9 M	3 M:5 F
Age, yrs	65 ± 4	65 ± 3	65 ± 3	63 ± 4
Smoking history (pk-yrs)	47 ± 11	43 ± 5	47 ± 9	-
FEV_1 , % pred	33 ± 4†	74 ± 5‡	101 ± 4	104 ± 6
Ex/current smokers	4/3	3/8	6/3	-
FEV_1/FVC (%)	39 ± 5†	65 ± 1‡	80 ± 2	79 ± 2
Emphysema Score	10 ± 1	4 ± 1	-	-
DLCO%	32 ± 8	90 ± 15	-	-
RV, % pred	181 ± 25	94 ± 18	-	-
PaO_2, mmHg	68 ± 6§	81 ± 2	89 ± 3	89 ± 7
$PaCO_2$, mmHg	39 ± 2	41± 1	41 ± 4	38 ± 1

* Values are expressed as mean ± SEM; measurements of RV and emphysema score were available in 10 subjects; DLCO was available in 7 subjects; 5 of the severe/very severe COPD subjects underwent LVRS, while 2 underwent resection for lung cancer. All the patients with mild/moderate disease, the control smokers and the non-smokers underwent resection for lung cancer.

† Significantly reduced as compared to mild/moderate COPD, control smokers and nonsmokers ($p<0.0001$ for all comparisons).

‡ Significantly reduced as compared to control smokers and nonsmokers ($p<0.002$ for all comparisons).

§ Significantly reduced as compared to mild/moderate COPD, control smokers and nonsmokers ($p<0.05$ for all comparisons).

Table 1 shows the clinical characteristics of the subjects examined. The 4 groups of subjects were similar with regard to age and the 3 groups of smokers had similar values of pack-years. The 3 groups of smokers included both current smokers and ex-smokers, and all ex-smokers had quit smoking for more than 1 year. As expected from the selection criteria, the values of FEV_1 (% predicted) and FEV_1/FVC ratio (%) were different in the four groups ($p<0.0001$): smokers with severe COPD had a significantly lower FEV_1 (% predicted) and FEV_1/FVC ratio (%) than smokers with mild/moderate COPD, smokers with normal function and nonsmokers ($p<0.0001$ for all comparisons). Moreover, smokers with mild/moderate COPD had a significantly lower FEV_1 (% predicted) and FEV_1/FVC ratio (%) than smokers with normal function ($p<0.0005$) and nonsmokers ($p<0.002$). In smokers with COPD, the average response to bronchodilator was 5%. The four groups of subjects also differed in arterial oxygen tension values (PaO_2) ($p=0.008$). Indeed, smokers with severe COPD had lower PaO_2 values as compared to smokers with mild/moderate COPD ($p=0.03$), smoking ($p=0.005$) and nonsmoking controls ($p=0.04$), while no significant differences were observed among the other three groups of subjects examined. Moreover, there were no significant differences in arterial carbon dioxide tension ($PaCO_2$) among the 4 groups of subjects. The average values of RV (% predicted) and radiological emphysema score were higher in smokers with severe COPD than in smokers with mild/moderate COPD, whereas the opposite was true with regard to the average percentage values of DLCO. However, since measurements of residual volume (together with emphysema score) and DLCO% were performed only in a subset of patients, a formal statistical analysis was not applied to these parameters.

Immunohistochemical findings

The results of phospho-p38 quantification in alveolar macrophages and alveolar walls are shown in figures 1, 2 and 3. The median numbers and relative ranges of cells expressing phospho-p38 are shown in table 2.

TABLE 2. PHOSPHO-p38 IMMUNOSTAINING QUANTIFICATION IN LUNG PARENCHYMA *

	Smokers with COPD		**Subjects with normal lung function**	
	Severe-very severe	*Mild-moderate*	*Smokers*	*Non smokers*
Phospho-p38+ macrophages,%	53†	33‡	3	3
	(13-70)	(0-63)	(0-8)	(0-6)
Phospho-p38+macrophages/hpf,n°	5.1†	3.3‡	0.2	0.1
	(1.3-8.3)	(0-10.5)	(0-1)	(0-0.3)
Phospho-p38+ cells/mm of alveolar wall	8.5†	3.8‡	0	0
	(1-12)	(0-16.8)	(0-0.2)	-

* Values are expressed as median with range shown in parentheses

† Significantly increased as compared to control smokers and nonsmokers ($p<0.001$ for all comparisons)

‡ Significantly increased as compared to control smokers and nonsmokers ($p<0.05$ for all comparisons)

Phospho-p38+ macrophages (%) were increased in patients with severe COPD as compared with smokers with normal lung function ($p=0.0008$) and nonsmokers ($p=0.001$). Phospho-p38+ macrophages were also increased in smokers with mild/moderate COPD as compared with both smoking ($p=0.01$) and nonsmoking controls ($p=0.02$). No significant difference was detected between smokers with severe COPD and those with mild/moderate COPD, nor between smokers with normal lung function and nonsmokers. Similar findings were obtained when we expressed the results as number of phospho-p38+ macrophages/hpf (table 2).

Significant differences among the 4 groups of subjects examined were observed also in phospho-p38+ cells in alveolar walls ($p< 0.0001$). Indeed, the number of phospho-p38+ cells/mm was increased in the group of severe COPD patients as compared with smoking ($p=0.0005$) and nonsmoking controls ($p=0.0004$). The phospho-p38+ cells within alveolar walls were also increased in smokers with mild/moderate COPD as compared with both smoking ($p=0.006$) and nonsmoking controls ($p=0.0005$). Once more, no significant difference was observed between smokers with severe COPD and those with mild/moderate COPD, nor between smokers with normal lung function and nonsmokers (table 2).

When we examined the different inflammatory cell subtypes, we found that the number of CD8+ T cells infiltrating the alveolar walls, but not that of macrophages and neutrophils, was significantly different among the four groups of subjects examined ($p=0.002$). In particular, CD8+ T cells were increased in both smokers with severe COPD [median (range): 8.3(6.9-14.4) cells/mm] and smokers with mild/moderate COPD [5.2 (1.8-13) cells/mm] as compared to control smokers [2.4 (1.4-2.6) cells/mm; $p=0.004$ and $p=0.03$, respectively] and nonsmokers [2 (0-3) cells/mm; $p=0.008$ and $p=0.03$, respectively]. No significant difference was observed between smokers with severe COPD and those with mild/moderate COPD, nor between smokers with normal lung function and nonsmokers.

The total length of alveolar walls examined was 4.3 ± 0.4 mm (average ± SEM) in smokers with severe COPD, 5.3 ± 0.4 mm in smokers with mild/moderate COPD, 6.6 ± 0.4 mm in smokers with normal lung function, and 6.8 ± 0.2 mm in nonsmokers.

When we considered all the smoking subjects as one group, inverse correlations were observed between phospho-p38+ macrophages and FEV_1 values (% predicted; $p=0.008$, $r=-0.52$) or FEV_1/FVC ratio (%; $p=0.006$, $r=-0.54$). Inverse correlations were also found between phospho-p38+ cells within alveolar walls and the values of either FEV_1 (% predicted; $p=0.001$, $r=-0.65$) or FEV_1/FVC ratio ($p=0.0003$, $r=-0.71$). Furthermore, there was a negative correlation between the number of phospho-p38+ cells within alveolar walls and PaO_2 values ($p=0.04$, $r=-0.41$).

When we considered the relationship with inflammatory cells, phospho-p38+ cells in alveolar walls showed a significant positive correlation with the numbers of CD8+ cells infiltrating the alveolar walls ($p = 0.0006$; $r = 0.75$), but not with neutrophils nor with macrophages.

Finally, phospho-p38 expression was not correlated with cumulative smoking history and was not influenced by current smoking status.

Western immunoblot analysis

A markedly increased phosphorylation of p38 MAPK was detected in cell protein extracts of alveolar macrophages obtained from the two COPD patients when compared with smoking and non smoking controls (figure 4). By contrast, no differences were observed with regard to the total p38α MAPK expressed by alveolar macrophages in the four subjects examined (figure 4).

DISCUSSION

The present study shows an increase in the phosphorylated form of p38 MAPK in both alveolar macrophages and alveolar walls of smokers with COPD. Western blot analysis confirmed the increased phospho-p38 expression in alveolar macrophages from COPD patients, in the absence of any change in total p38α levels. Furthermore, phospho-p38 expression was related to the degree of lung function impairment and to the number of CD8 T-lymphocytes infiltrating the alveolar walls. To the best of our knowledge, this is the first evidence obtained in human lung tissue of the potential role played by p38 activation in the inflammatory process associated with COPD.

In our study, for both immunohistochemistry and Western blot experiments, an anti-phospho-p38 monoclonal antibody was used that specifically recognises two phosphorylation sites (Thr180 and Tyr182) of p38. Since dual phosphorylation involving these amino acid sites occurs in the p38α and p38β isoforms only and expression of the β isoform seems to be restricted to T-cells [20], we can reasonably argue that the phospho-p38 detected in lung macrophages is predominantly

the α isoform. Therefore, phosphorylation-dependent activation of p38α is probably a leading molecular event in signalling mechanisms underlying the perpetuation of the inflammatory process in COPD. This is also confirmed by the ability of p38α and β inhibitors to suppress cytokine production in animal models of COPD [7]. *In vitro,* an increased p38 MAPK phosphorylation has been recently shown by Smith and colleagues in lipopolysaccharide (LPS)-stimulated lung macrophages [10], whereas in our study we detected high levels of phospho-p38 expression in alveolar macrophages and alveolar walls *in vivo*.

It is well known that alveolar macrophages play a central role in the pathogenesis of COPD by continuously releasing proteolytic enzymes leading to destruction of lung tissue. These cells may also orchestrate the inflammatory response by secreting large amounts of cytokines and chemokines. p38 MAPK activation is a crucial step in the synthesis of multiple inflammatory mediators and, in particular, LPS-induced release of TNF-α from human lung macrophages is dependent on p38-mediated stabilisation of TNFα-mRNA [10]. Moreover, p38 inactivation caused by p38α/β inhibitors significantly reduces TNF-α production.

Our results also refer to other cell types involved in COPD pathogenesis. In this regard, T cells are important players in the immune response in COPD and a shift toward a type 1 profile with increased CXCR3 expression and release of TNF-α and IFN-γ has been described in this disease [21,22]. Of interest, there is recent evidence that, in human bronchial epithelial cells, CXCR3-induced chemotaxis is critically dependent on signalling by the p38 MAPK pathway [23]. Moreover, in our study, we observed a positive correlation between the numbers of phospho-p38 positive cells and $CD8^+$ cells within the alveolar walls. Altogether these results suggest that there is a strict interplay between the p38 pathway and T lymphocytes activation, which may result in perpetuation of the inflammatory response. To the best of our knowledge, it is not yet known which of the p38 isoenzymes are expressed by $CD8^+$ T lymphocytes. However, IL-2 production by these cells can be suppressed by a selective inhibitor of p38α and β [24], that thus appear to be the more

relevant isoforms to $CD8^+$ T cell functions [20]. Dual phosphorylation of p38α and p38β has been detected in both $CD4^+$ and $CD8^+$ T cells [25]. Availability of more specific antibodies recognising the different p38 isoforms, as well as the development of p38 inhibitors with greater selectivity, could probably contribute to better characterise the p38 subtypes preferentially expressed by $CD8^+$ T lymphocytes. This expression pattern may have relevant functional implications because, for example, p38β is a much more potent stimulator, when compared to the α isoform, of activating transcription factor 2 (ATF2) [25].

Our present findings obtained in human lung samples confirm and extend previous studies performed in experimental models of smoking-induced pulmonary inflammation. Marwick and colleagues examined rat lungs exposed to cigarette smoke and detected enhanced p38 phosphorylation, associated with increased activation of the transcription factors NF-κB and AP-1 [26]. Furthermore, they observed significant chromatin rearrangement with hyperacetylation of histone 4 and phospho-acetylation of histone 3, together with decreased histone deacetylase activity. Ito and coworkers recently investigated the expression and activity of histone deacetylase (HDAC) in peripheral lung tissue of COPD patients, and showed a progressive impairment of HDAC activity which was paralleled by significant increases in IL-8 mRNA and histone 4 acetylation [27]. Interestingly, the IL-8 gene is transcriptionally regulated by NF-κB and its activation is controlled, at least in part, by p38 MAPK-mediated histone H3 phosphorylation on Serine 10 [28]. In light of all these considerations, it can be proposed that phosphorylation-dependent activation of p38 MAPK may contribute, together with histone modifications, to the onset and progression of lung inflammation. This interaction may become important when considering the poor response to steroids observed not only in patients with COPD, but also in smoking asthmatic patients. Several mechanisms have been proposed to explain steroid resistance; among these, it is known that activated p38 MAPK phosphorylates glucocorticoid receptors (GR) *in vitro*, thereby reducing corticosteroid binding affinity [29]. As a result, the transcriptional activity of genes regulated by corticosteroids will be impaired, with a consequent reduction of MKP-1 (MAP kinase phosphatase-

1) expression, which is indeed induced by activated GR [30]. Therefore, it is possible that in moderate to severe COPD, an excessive phospho-p38 activity can implement a feed-forward circuit that further amplifies the role of MAPK pathways. In such a situation, p38 activation will be even more sustained because of a defective dephosphorylation due to MKP-1 down-regulation.

In our study, p38 activation in both macrophages and alveolar walls was not upregulated by tobacco smoking itself, since a similar immunoreactivity was present in smokers with normal lung function and non smoking controls. Furthermore, phospho-p38 expression was not influenced by current smoking status or cumulative smoking history. This scenario is different from that observed in animal models, where exposure to tobacco smoke was sufficient to enhance p38 phosphorylation [26]. By contrast, in humans p38 stimulation is strictly related to the presence of the disease, because once fixed airflow limitation has been established, p38 MAPK activation persists even after smoking cessation. It is important to note that the inflammatory response as well persists in smokers with COPD long after smoking cessation [11]. Altogether, these observations support the view that, beyond cigarette smoking, a complex interplay of different factors (including genetic predisposition, air pollution and respiratory infections) promotes COPD, which is in fact characterised by high variability of phenotypic expressions.

A possible limitation of our study is that it was performed on surgical specimens mainly taken from patients with lung carcinoma, and the presence of the cancer itself may have possibly influenced MAPK activation. We tried to avoid this bias by taking into consideration only histologically normal areas, characterised by being free of tumor and distant from affected areas. Moreover, since lung cancer was equally present in mild/moderate COPD, in smoking and non-smoking controls, we are rather confident that our finding of an increased phosphorylated p38 in COPD is valid. Finally, our results were further validated as p38 activation was also observed in patients not affected by cancer (i.e. those with severe emphysema undergoing lung volume reduction surgery). We should acknowledge that, ideally, a true control group of nonsmokers without concomitant lung cancer is required in this kind of studies; however, it is extremely difficult

to have access to surgical specimens from normal people in whom a complete clinical and functional characterisation has been performed.

In conclusion, our study demonstrated an increased expression of phospho-p38 in alveolar spaces and alveolar walls of smokers with COPD, suggesting that activation of p38 MAPK pathway is a crucial step in the pathogenesis of the disease. These findings support the relevance of p38 MAPK as a suitable molecular target for the development of new and more effective therapeutic strategies for COPD.

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FIGURE LEGENDS

Figure 1. Individual counts for phospho-p38+ macrophages in the alveolar spaces of smokers with severe COPD, smokers with mild-moderate COPD, smoking and non smoking subjects with normal lung function. The results are expressed as percentage of alveolar macrophages. *Horizontal bars* represent median values.

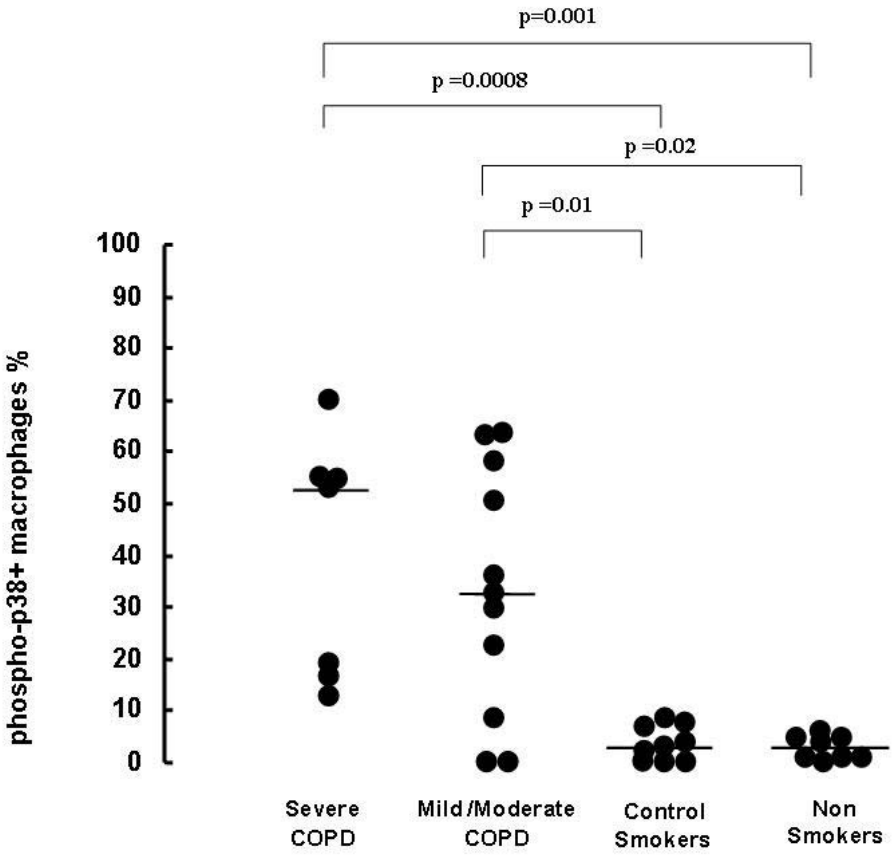

Figure 2. Individual counts for phospho-p38+ cells within alveolar walls of smokers with severe COPD, smokers with mild-moderate COPD, smoking and non smoking subjects with normal lung function. The results are expressed as numbers of cells per mm of alveolar wall. *Horizontal bars* represent median values.

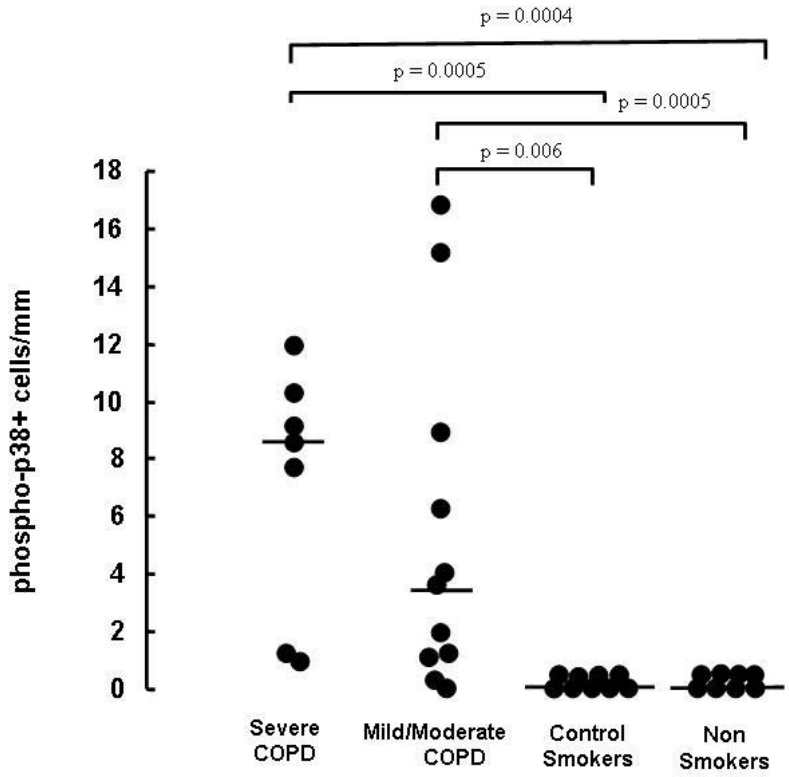

Figure 3. Microphotographs from a smoker with COPD (A) and a smoker with normal lung function (B) showing phospho-p38+ cells within alveolar wall and phospho-p38+ alveolar macrophages. Immunostaining with anti-phospho-p38 monoclonal antibody. *Arrow* indicates a phospho-p38+ alveolar macrophage and *arrow head* indicates a phospho-p38+ cell within the alveolar wall. Original magnification: x630.

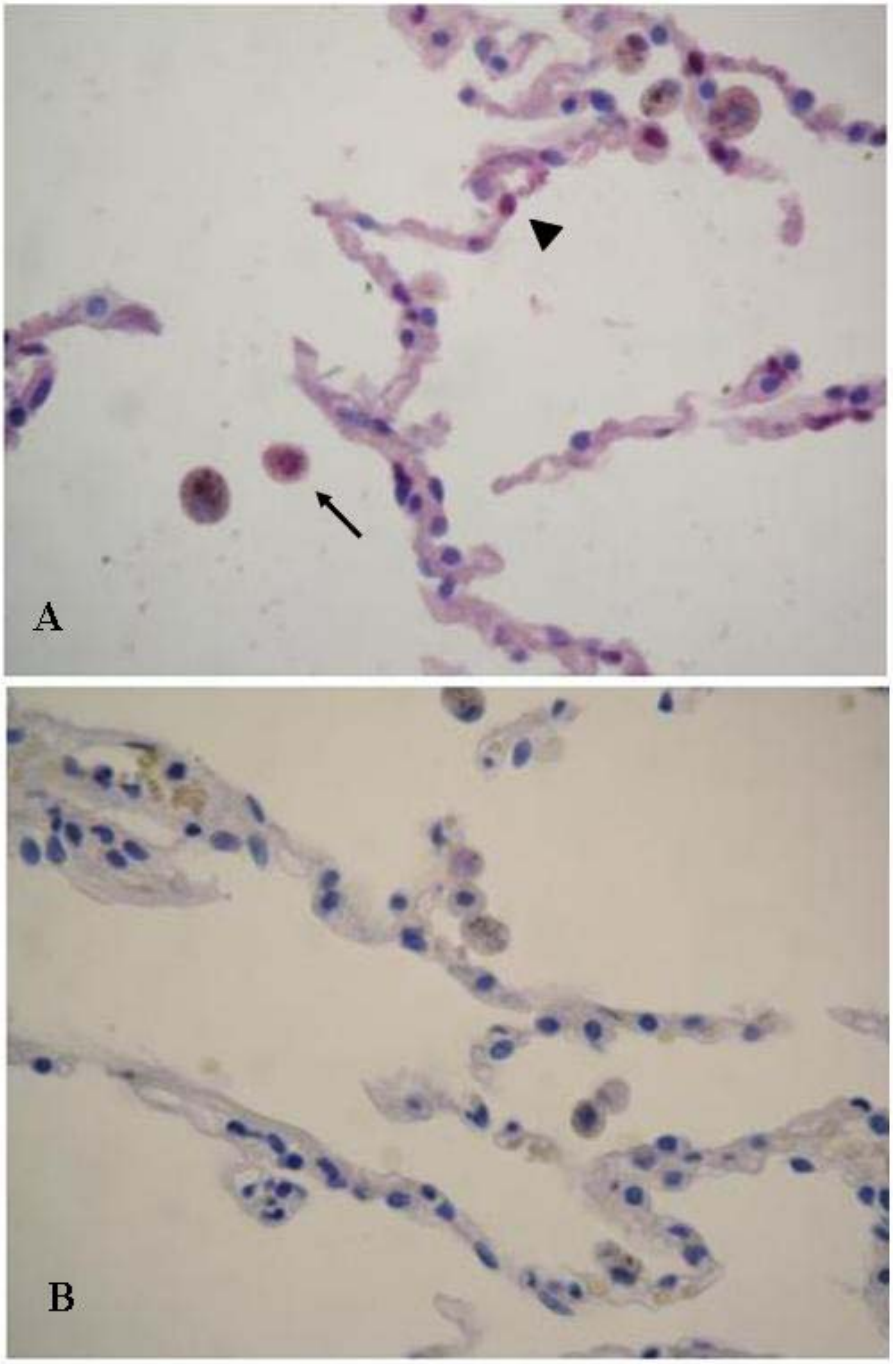
A
B

Figure 4. Ωεστερν ιμμυνοβλοτ αναλψσισ οφ πηοσπηο−π38 ανδ π38α expressed by human alveolar macrophages obtained from two patients with COPD, a smoker (S) and a non smoker (NS) (both with normal lung function).