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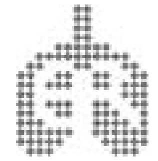

Decreased macrophage release of TGF-β and TIMP-1 in chronic obstructive pulmonary disease

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ABSTRACT: The present study tested the hypothesis that alveolar macrophages (AM) from patients with chronic obstructive pulmonary disease (COPD) release more pro-inflammatory and/or less anti-inflammatory mediators than those from smokers with normal lung function and never-smokers.

AM were sorted by flow cytometry from bronchoalveolar lavage fluid in 13 patients with COPD (mean±SEM 67±2 yrs, forced expiratory volume in one second (FEV1) 61±4% reference), 16 smokers with normal lung function (55±2 yrs, FEV1 97±4% reference) and seven never-smokers (67±7 yrs, FEV1 94±4% reference). After sorting, AM were cultured (with and without lipopolysaccharide stimulation) after 4 h and 24 h, and the concentrations of leukotriene B_4 (LTB_4), transforming growth factor (TGF)-β_1 and tissue inhibitor of metalloproteinase (TIMP)-1 were quantified in the supernatant by ELISA. The production of reactive oxygen intermediates (ROI) in freshly isolated AM was determined by flow cytometry.

LTB_4 secretion and ROI production were not different between groups. In contrast, AM from COPD patients released significantly less TGF-β_1 and TIMP-1 than those from smokers with normal lung function and nonsmokers.

In conclusion, these observations are compatible with reduced anti-inflammatory and anti-elastolytic capacity in chronic obstructive pulmonary disease, which is likely to contribute to the pathogenesis of the disease.

KEYWORDS: Chronic obstructive pulmonary disease, leukotrienes, oxidative stress, tissue inhibitor of metalloproteinase-1, transforming growth factor-β_1

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The inflammatory response to tobacco smoking is an essential pathogenic factor in chronic obstructive pulmonary disease (COPD) [1]. A variety of inflammatory cells, including alveolar macrophages (AM), polymorphonuclear (PMN) leukocytes and lymphocytes [2], and mediators such as cytokines, oxidants and matrix metalloproteinases (MMPs) participate in this response [2]. Given that tobacco smoking elicits an inflammatory response in all smokers [3], but only some of them develop COPD [4], it is likely that its type, intensity, localisation and/or failure to resolve after smoking cessation may contribute to the pathogenesis of the disease [5].

AM are ideally suited to control the degree and duration of the inflammatory response elicited by tobacco smoking because they are the major immunocompetent cell population in the lower human respiratory tract, where they act as sentinel cells [6]. In response to an inflammatory stimulus such as smoking, AM rapidly release the following: 1) cytokines, such as interleukin (IL)-8 and tumour necrosis factor-α, which act in further recruiting PMNs that can, in turn, release more inflammatory mediators [7]; 2) leukotriene B_4 (LTB_4), a potent chemoattractant for other inflammatory cells [7]; 3) reactive oxygen intermediates (ROI) [8, 9], which can contribute towards enhancing the inflammatory response; and 4) MMP-1 and MMP-9, which can degrade the interstitial matrix and cause emphysema [7]. Conversely, AM also contribute to the resolution of the inflammatory response because they are capable of releasing several anti-inflammatory molecules, such as transforming growth factor (TGF)-β and tissue inhibitors of MMPs (TIMPs) [6, 7]. Previous studies in COPD have reported an abnormal release of IL-8, MMP-1 and MMP-9 by AM in these patients [9–11]. In the present investigation, the hypothesis that AM harvested

from patients with COPD release more pro-inflammatory mediators (LTB_4, ROI) and/or less anti-inflammatory molecules (TGF-β_1, TIMP-1) than those obtained from smokers with preserved lung function was tested.

METHODS

Subjects and ethics

The current authors studied 13 patients with COPD (four current and nine ex-smokers) as defined by the Global Initiative for Obstructive Lung Disease (GOLD) criteria [1], 16 active smokers without chronic bronchitis or dyspnoea and with normal lung function, and seven never-smokers. All required bronchoscopic evaluation of a solitary pulmonary nodule or a haemoptysis episode. All patients with COPD were clinically stable and had not had any exacerbations for at least the 3 months that preceded inclusion in the study. The patients were treated with inhaled bronchodilators (salmeterol and/or salbutamol and/or ipratropium bromide), and four patients were treated with inhaled corticosteroids (ICS), but none received oral steroids. Patients with other chronic lung diseases (*e.g.* asthma, bronchiectasis and interstitial lung diseases) and cardiac, hepatic or renal failure were excluded. All participants gave signed, informed consent. The Ethics Committee of Comite d'Etica de les Illes Balears (Palma Mallorca, Spain) approved the study protocol.

Lung function

A forced spirometry (GS; Warren E. Collins, Braintree, MA, USA) was obtained in all participants [12]. Spirometric reference values were those of a Mediterranean population [13]. Exhaled CO concentrations (ToxCO; Bedfont, Rochester, UK) were recorded before bronchoscopy to exclude active smoking.

Bronchoscopy

Bronchoscopy was performed with a flexible fibreoptic bronchoscope (Pentax 15v; Pentax, Tokyo, Japan). Active smoking subjects refrained from smoking for at least 12 h before bronchocopy. This was checked by the exhaled CO concentration, which was <10 ppm in all cases. Bronchoalveolar lavage (BAL) was performed following the standard methodology. Eight 25-mL aliquots of sterile saline solution were instilled in one pulmonary segment of one of the lower pulmonary lobes not containing any nodule. Aliquots were placed in plastic recipients and immediately centrifuged at $200 \times g$ for 10 min at 4℃. The cell pellet was washed once in calcium- and magnesium-free Hank's balanced saline solution (Gibco BRL, Eggenstein, Germany) and resuspended in minimal essential medium (Gibco BRL) until sorting. Cells were counted with a haemocytometer. Viability determined by trypan blue exclusion was always $>95\%$.

The presence of airway bacterial infection was excluded by using a protected specimen brush (PSB; Mill-Rose Lab, Mentor, OH, USA) to sample bronchial secretions sterilely before BAL [14]. The culture of PSB samples yielded $<10^3$ colony forming units$\cdot$mL^{-1} in all participants.

Sorting of alveolar macrophages

AM were sorted in BAL samples using a Coulter Epics Altra HyPerSort^{TM} System (Beckman Coulter, Izasa, Spain). Calibration of the optical system was performed using flow-check fluorospheres (Beckman Coulter). Nozzle orifice, flow rate and sorting strategy were set up as previously described [15]. Briefly, a 100-µm diameter flow cell tip was used and the flow rate was adjusted to 400–500 events$\cdot$s^{-1}. AM were gated on a forward scatter (linear scale) *versus* green fluorescence (logarithmic scale) to discriminate them, with a high degree of autofluorescence from neutrophils and lymphocytes. Cell suspensions, before and after sorting, were kept in polypropylene tubes and maintained on ice. After sorting, cells were counted in a haemocytometer and viability was determined by trypan blue exclusion. Cytospin preparations were prepared for morphological differentiation of BAL cells following Papanicolau staining. The purity of sorted AM was always $>96\%$ as analysed by flow cytometry and visual quantification. Cell viability by trypan blue exclusion was always $>95\%$.

Macrophage culture

Sorted AM were suspended at $5 \times 10^5$$\cdot$mL^{-1} in endotoxin-free RPMI 1640/10% foetal calf serum, supplemented with 2 mM L-glutamine, 25 U$\cdot$mL^{-1} penicillin and 25 µg$\cdot$mL^{-1} streptomycin. Cells were separated into aliquots and cultured in 24-well polystyrene plates (Nunc, Wiesbaden, Germany) at 37℃ and 5% CO_2 for 4 h and 24 h, with or without lipopolysaccharide (LPS (O55:B5), 100 ng$\cdot$mL^{-1} (final concentration); *Escherichia coli* LPS; Sigma Chemicals CO, St. Louis, MO, USA). At these time points, the supernatant was collected and stored cell free at -80℃ until analysed.

Measurement of TGF-β, TIMP and LTB_4

The cell culture supernatant concentration of TGF-β_1, TIMP-1 and LTB_4 was measured using a commercially available sandwich ELISA kits (Amersham Pharmacia Biotech, Chalfont, UK). Biologically active TGF-β_1 was measured in previously acidified samples (0.02 mL of 1-M hydrochloric acid was added to 100 µL of samples to achieve pH 1–2). LTB_4 was measured using an enzyme-immunoassay system according to the manufacturer's directions (Amersham Pharmacia Biotech). The detection limits of TGF-β_1, TIMP-1 and LTB_4 assays were 6 pg$\cdot$mL^{-1}, 15.6 pg$\cdot$mL^{-1} and 1.25 pg$\cdot$mL^{-1}, respectively.

Measurement of ROI

Production of ROI by fresh AM was determined by flow cytometry through the formation of the fluorescent compound rhodamine-123 from dihydrorhodamine-123 (DHR; Molecular Probes, Eugene, OR, USA). In brief, two 200-µL samples of the macrophage suspension ($5 \times 10^5$$\cdot$mL^{-1}; Falcon No. 2052; Beckton-Dickinson, Lincoln Park, NJ, USA) were incubated in polypropylene tubes with 10 µL of a DHR solution (100 µg$\cdot$mL^{-1}) for 10 min at 37℃. One sample was used to assess the spontaneous ROI production, while the other was mixed with 20 µL of LPS (10 ng$\cdot$mL^{-1}, final concentration). Both samples were incubated for 30 min at 37℃. At the end of the second incubation period, 500 µL of cold PBS was added to both samples, which were then kept on ice until analysed by flow cytometry on a Coulter Epics Altra HyPerSort^{TM} System (Beckman Coulter) with a gate setting for macrophages on forward and side scatter. In total, 10,000 cells were analysed; green fluorescence was determined and mean cellular fluorescence intensities were calculated using LYSIS II software. Cell suspensions served as controls of autofluorescence. The

intensity of the specific fluorescence was calculated and expressed as relative linear mean fluorescence (RLMF), the ratio of specific to nonspecific (autofluorescence) linear fluorescence.

Statistical analysis

Results are shown as mean±SEM. One-way ANOVA (followed by *post hoc* contrast (Scheffe) if appropriate) was used to assess the statistical significance of the differences between groups. To analyse the potential effects of ICS in patients with COPD, the Mann-Whitney U-test was used. Correlations between variables of interest were explored using the Spearman rank test. A p-value <0.05 was considered significant.

RESULTS

Anthropometric and functional data

Table 1 presents the main clinical and lung function data of all particpants. Age was similar in never-smokers and patients with COPD, but smokers with normal lung function were younger. Likewise, the latter had a smaller smoking exposure than patients with COPD (table 1). According to GOLD criteria, patients with COPD showed moderate airflow obstruction. By definition, spirometry was normal in smokers with normal lung function and never-smokers.

BAL cell count

Total cell count was higher in smokers with normal lung function ($3.6\pm0.6\times10^5\cdot mL^{-1}$) than in nonsmokers ($0.9\pm0.5\times10^5\cdot mL^{-1}$; $p<0.05$). Patients with COPD also showed a trend towards higher values ($2.1\pm0.5\times10^5\cdot mL^{-1}$), but differences failed to reach statistical significance. Differential cell counts were similar among groups (table 2). Nevertheless, the number of AM tended to be higher in smokers with normal lung function, and the number of neutrophils appeared to be increased in patients with COPD. Overall, these observations are in keeping with previous studies [16].

LTB_4 release

After 4 h in culture, LTB_4 levels were similar in nonsmokers (222 ± 107 pg·mL^{-1}) and smokers with normal lung function (244 ± 107 pg·mL^{-1}). Patients with COPD showed a trend towards lower values (137 ± 61 pg·mL^{-1}), but differences failed to reach statistical significance. LPS stimulation did not modulate these levels significantly (nonsmokers: 284 ± 144 pg·mL^{-1}; smokers with normal lung function: 342 ± 99 pg·mL^{-1}; patients with COPD: 226 ± 94 pg·mL^{-1}). The current authors did not measure LTB_4 release after 24 h in culture, as previous studies have shown that LTB_4 is almost completely metabolised at this time point [17].

TABLE 1 Clinical and lung function data of all participants

	Nonsmokers	Smokers with normal lung function	COPD
Subjects n	7	16	13
Age yrs	67±7	55±2	67±2#
Pack-yrs	0	40±4**	61±7**,#
Active smokers	0	16	4
Ex-smokers	0	0	9
Inhaled steroids	0	0	4
FEV_1 % pred	94±4	97±4	61±4*,#
FEV_1/FVC %	81±4	81±2	59±2*,#

Data are presented as mean±SEM. COPD: chronic obstructive pulmonary disease; FEV_1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity. *: $p<0.05$; **: $p<0.01$ (*versus* nonsmokers); #: $p<0.05$ (*versus* smokers with normal lung function). Spirometric reference values were those of a Mediterranean population [13].

TABLE 2 Bronchoalveolar lavage fluid total and differential cell counts

	Nonsmokers	Smokers with normal lung function	COPD
Subjects n	7	16	13
Total cell count $\times10^5\cdot mL^{-1}$	0.9±0.5	3.6±0.6*	2.1±0.5
Macrophages %	90±5	94±1	90±2
Lymphocytes %	7±5	5±1	7.5±1
Neutrophils %	0.8±0.5	0.8±0.2	2.5±1

Data are presented as mean±SEM. COPD: chronic obstructive disease. *: $p<0.05$ *versus* nonsmokers.

ROI production

ROI production was similar in nonsmokers (8.32 ± 3.67 RLMF), smokers with normal lung function (5.03 ± 1.47 RLMF) and patients with COPD (6.32 ± 1.68 RLMF). LPS stimulation did not modify these values significantly (nonsmokers: 8.26 ± 4.07 RLMF; smokers with normal lung function: 5.29 ± 1.60 RLMF; patients with COPD: 6.37 ± 1.69 RLMF).

TGF-β_1 release

After 4 h in culture, TGF-β_1 concentration was below the detection limit of the assay in all groups. At 24 h, TGF-β_1 levels were detectable and similar in all of them (fig. 1). The addition

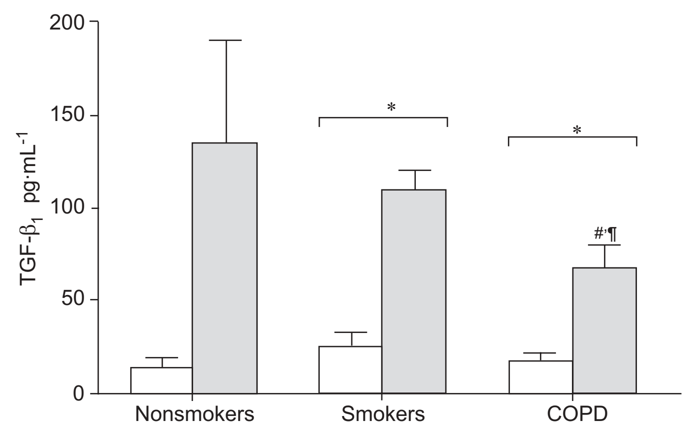

FIGURE 1. Transforming growth factor (TGF)-β_1 release after 24 h in culture in each of the subject groups studied, both with (■) and without (□) *Escherichia coli* lipopolysaccharide (100 ng·mL^{-1}) stimulation. COPD: chronic obstructive pulmonary disease. *: $p<0.05$; **: $p<0.01$; #: $p<0.05$ *versus* nonsmokers; ¶: $p<0.05$ *versus* smokers with normal lung function.

of LPS increased TGF-β_1 release in all groups, but this effect was significantly attenuated in patients with COPD (67 ± 13 ng·mL^{-1}) as compared with smokers with normal lung function (135 ± 55 ng·mL^{-1}; $p<0.01$) or nonsmokers (110 ± 10 ng·mL^{-1}; $p<0.05$).

TIMP-1 release

After 4 h in culture, without LPS stimulation, AM from smokers with normal lung function released less TIMP-1 (6.2 ± 2.1 ng·mL^{-1}) than nonsmokers (11.7 ± 2.4 ng·mL^{-1}; $p<0.05$). This defect was further accentuated in patients with COPD (1.7 ± 0.4 ng·mL^{-1}; $p<0.05$ *versus* smokers with normal lung function; fig. 2). Stimulation with LPS augmented TIMP-1 release in nonsmokers (16.7 ± 4 ng·mL^{-1}; $p<0.05$). Interestingly, this effect was clearly reduced in smokers with normal lung function (6.2 ± 2.1 ng·mL^{-1}), and basically abolished in patients with COPD (2.4 ± 0.8 ng·mL^{-1}). At 24 h, TIMP-1 release increased significantly in all groups (note change of y-axis scale in fig. 2). LPS stimulation did not enhance TIMP-1 release any further in any group, but differences observed at 4 h between groups persisted. Hence, TIMP-1 release was significantly lower in smokers with normal lung function and almost absent in patients with COPD (fig. 2).

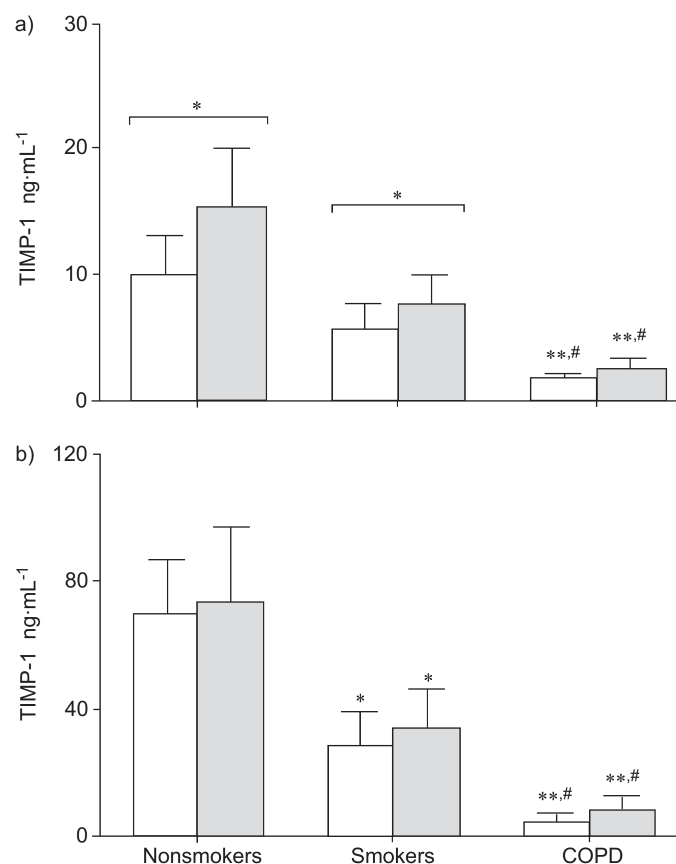

FIGURE 2. Tissue inhibitor of metalloproteinase (TIMP)-1 release after a) 4 h, and b) 24 h in culture in each of the subject groups studied, both with (■) and without (□) *Escherichia coli* lipopolysaccharide (100 ng·mL^{-1}) stimulation. COPD: chronic obstructive pulmonary disease. *: $p<0.05$; **: $p<0.01$ *versus* nonsmokers; #: $p<0.05$ *versus* smokers with normal lung function.

Effect of ICS

LTB_4, TGF-β_1, TIMP-1 and ROI production were not different at any time point in patients treated with (n=4) or without ICS (n=9).

Physiological correlates

As smokers with normal lung function were younger and had smoked less than patients with COPD (table 1), in order to investigate a potential confounding effect of these two variables upon the different mediators studied, the biological relationships between the former and the latter were explored. However, no significant relationship could be found.

DISCUSSION

Tobacco smoking is the major risk factor for COPD [1]. However, only a small percentage of smokers develop the disease [2]. An excessive and persistent inflammatory response appears to be the hallmark of these latter individuals [5]. In theory, this can be due either to an enhanced inflammatory response to tobacco smoking and/or a failure to switch off the inflammatory machinery when the stimuli cease [5]. AM are the major immunocompetent cell population in the lower human respiratory tract and, as such, they are likely to play a significant role in the initiation, modulation and eventual termination of the inflammation that follows cigarette smoking [7]. In this study, the capacity of AM to secrete both pro- (LTB_4, ROI) and anti-inflammatory molecules (TGF-β_1 and TIMP-1) in patients with COPD was investigated. It was found that AM from these patients released similar amounts of LTB_4 and ROI than those from smokers with preserved lung function or nonsmokers. In contrast, the released TGF-β_1 and TIMP-1 by AM were markedly impaired in COPD.

LTB_4 is increased in breath condensate [18], induced sputum [18] and serum [19] of patients with COPD. However, AM release of LTB_4 has not been previously investigated in these patients. An abnormal release of LTB_4 by AM in COPD was not observed. This is similar to that reported by SPENCER *et al.* [20] in AM from patients with α-antitrypsin deficiency, and suggests that the main cellular source of LTB_4 in COPD is a different cell type, perhaps the neutrophil [21]. This is not to say that AM are not important sources of other inflammatory mediators in COPD, such as IL-8 [9].

Oxidative stress is an important factor in the pathogenesis of COPD [22], and AM can produce ROI [6]. A previous study by MCLEOD *et al.* [23] could not detect significant differences in superoxide anion release by AM harvested from smokers with and without COPD. The results of the present study support this previous observation and, hence, also suggest a marginal role for AM in the pathogenesis of the oxidative stress that characterises COPD.

TGF-β_1 is a potent anti-inflammatory cytokine produced by several cell lineages, including AM [24–29]. It also has important anti-elastolytic properties because it reduces the production of MMP [30], and stimulates that of TIMP-1 and matrix proteins [31]. The current authors observed that AM from patients with COPD released significantly lower concentrations of TGF-β_1 in response to LPS than smokers with preserved lung function and never-smokers (fig. 1). To the present authors' knowledge, this has not been previously

reported. Previous studies have reported increased expression of TGF-β_1 in the airway epithelium of patients with chronic bronchitis or COPD [32, 33]. None, however, analysed its release by AM. Differences between the current study and those previously can be related to the different cell lineages analysed and techniques used. Studies in bronchial epithelium tissue used *in situ* hybridisation and/or immunochemistry to ascertain cell-associated proteins, which may give different results [32, 33], whereas, in the current study, ELISA was used to determine TGF-β_1 levels in the supernatant. Furthermore, it is also possible that the airway epithelium and AM behave differently in terms of TGF-β_1 release in COPD. The increased expression of TGF-β_1 in the epithelium may reflect the relevance of this cytokine in airway remodelling in COPD [34]. In contrast, the decreased release of TGF-β_1 from AM may reflect a deficient anti-inflammatory/repair capacity of these cells in patients with COPD. The different behaviour of airway epithelium and AM in patients with COPD has also been reported for other cytokines [35]. Interestingly, a polymorphism of the TGF-β_1 gene that is associated with low TGF-β_1 levels is common in patients with COPD [36]. This observation would be in keeping with the current findings of reduced TGF-β_1 release by AM in COPD patients.

Likewise, it was also observed that AM from patients with COPD release less TIMP-1 than those from smokers with normal lung function or nonsmokers (fig. 2). As TGF-β_1 stimulates TIMP-1 release [31], it is tempting to speculate that the lower TIMP-1 release seen in COPD may be mechanistically linked to the reduced TGF-β_1 release discussed previously. In any case, given that TIMP-1 is an important anti-elastolytic molecule [10], its reduced release in COPD may contribute to the development of pulmonary emphysema. However, the present results contrast with a previous study reporting no difference in TIMP-1 release by AM between COPD patients and smokers with normal lung function [10]. Several methodological aspects may contribute towards explaining this difference because, at variance with this former study [10], the current authors were very careful at controlling the acute effects of smoking known to cause oxidative stress and to decrease TIMP-1 activity [37]. An uneven distribution of active smokers in the individuals studied [10] may have blurred potential differences between groups. Likewise, at variance with previous studies [10, 11], bronchial infection was systemically excluded because it enhances lung inflammation [38].

Other methodological aspects of the current study also deserve comment. First, patients with COPD were not perfectly matched to smokers with normal lung function in terms of age and smoking history. However, the absence of any significant relationship between these two variables and any of the biological mediators studied here suggests that the relatively small differences (albeit statistically significant) of both age and smoking history did not exert any relevant confounding effects. Secondly, no significant differences were found in BAL cell counts between patients with COPD and smokers with normal lung function. This is in keeping with some previous studies [16], and in contrast to others [10], suggesting a high variability of the inflammatory cell counts in these subjects. Thirdly, previous studies isolated AM attending to their adherent properties [10, 11], whereas the present authors used flow-sorted AM. This strategy avoids the loss of nonadhering AM [39], although it does not prevent the priming of the adherent ones, because, for the duration of the exposition period, the cells were cultured in plastic well plates and may have presumably also adhered to the plastic surface. Fourthly, previous reports incubated AM 24 h before stimulation [9, 10, 40]. As mentioned earlier, this can alter AM response [39]. Accordingly, the present authors decided to stimulate AM immediately after sorting and to quantify the concentration of the different inflammatory mediators both early (4 h) and late (24 h). Fifthly, the concentration of LPS used and the time points chosen were similar to those of previous studies [10, 41, 42]. However, different results in LTB_4 and/or ROI release may have been found if different LPS concentrations and/or different time points had been used [17, 41]. Sixthly, the current authors did not analyse other inflammatory markers that might be relevant in COPD, such as IL-8 and MMP-9. This was because previous studies have already done so [9, 43], and the present authors wanted to focus on other less well-characterised molecules. Finally, the current authors excluded a potential confounding effect of ICS because there were no significant differences in the levels of the mediators studied between patients with (n=4) and without ICS (n=9). This is in keeping with many studies suggesting a relative steroid resistance in COPD [44–46].

In conclusion, the present study shows that alveolar macrophages in chronic obstructive pulmonary disease release significantly less transforming growth factor-β_1 and tissue inhibitor of metalloproteinase-1 than smokers with preserved lung function and never-smokers. This observation suggests a lower anti-inflammatory and anti-proteolytic activity in these patients that may, eventually, contribute to the pathogenesis of the disease.

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