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Innate immunity but not NLRP3 inflammasome activation correlates with severity of stable COPD

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Key messages:

- IL-27, IL-37 and IL-6 are upregulated in stable COPD
- The NRLP3 inflammasome is not activated in stable COPD

Key words

COPD; bronchial biopsies; airway inflammation; cytokines; neutrophils.

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What is the key question?

- Are innate immunity and/or inflammasome activation in the lower airways involved in the progression of severity of stable COPD?

What is the bottom line?

- In our study the increased expression of the innate immunity inflammatory mediators IL-6, IL-27, IL-37 and NALP7 in the bronchial mucosa and/or bronchoalveolar lavage may be involved in the progression of the severity of stable COPD.

Why read on?

- The lack of evidence for inflammasome activation in the lower airways of stable COPD patients suggest that this inflammatory pathway is not involved in the progression of the severity of stable COPD

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Summary

Background: In models of COPD, environmental stressors induce innate immune responses, inflammasome activation and inflammation. However, the interaction between these responses and their role in driving pulmonary inflammation in stable COPD is unknown.

Objectives: To investigate the activation of innate immunity and inflammasome pathways in the bronchial mucosa and bronchoalveolar lavage (BAL) of stable COPD patients of different severity and control healthy smokers and non-smokers.

Methods: Innate immune mediators (IL-6, IL-7, IL-10, IL-27, IL-37, TSLP, IFNγ and their receptors, STAT1 and pSTAT1) and inflammasome components (NLRP3, NALP7, caspase 1, IL-1β and its receptors, IL-18, IL-33, ST2) were measured in the bronchial mucosa using immunohistochemistry. IL-6, soluble IL-6R, sgp130, IL-7, IL-27, HMGB1, IL-33, IL-37 and soluble ST2 were measured in BAL using ELISA.

Results: In bronchial biopsies IL-27+ and pSTAT1+ cells are increased in severe COPD compared to control healthy smokers. IL-7+ cells are increased in both COPD and control smokers compared to control non-smokers. In severe stable COPD IL-7R+, IL-27R+ and TSLPR+ cells are increased in comparison with both control groups. The NALP3 inflammasome is not activated in stable COPD patients compared with control subjects. The inflammasome inhibitory molecule NALP7 and IL-37 are both increased in COPD compared to control smokers. IL-6 levels are increased in BAL from stable COPD patients compared to control smokers with normal lung function whereas that of IL-1β and IL-18 were similar across all groups.

Conclusion: Increased expression of IL-27, IL-37 and NALP7 in the bronchial mucosa may be involved in progression of stable COPD.

Summary Word count: 250

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Introduction

Inflammation is important in the pathogenesis of stable COPD (1). Environmental stress such as cigarette smoke activates the innate immune response which may drive COPD inflammation (2). Interleukin (IL-6) is a multifunctional pro-inflammatory cytokine (1) that acts via two molecules: the IL-6R (IL-6 receptor) and gp130 (3). Soluble gp130 (sgp130) inhibits both IL-6 trans-signaling via the soluble IL-6R (sIL-6R) and classic signaling via the membrane bound IL-6R (mIL-6R) (4).

IL-27, an IL-12/IL-23 family member, stimulates Th1 lymphocyte differentiation (5). It also stimulates haematopoiesis, increases antigen presentation by antigen-presenting cells and inhibits angiogenesis (6). The IL-27 receptor (IL-27Ra or WSX-1) activates the Janus kinase (JAK) pathway with phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3 (6). IL-10, in contrast, potently inhibits the expression of inflammatory proteins such as IL-1β, TNFα, and MMP-9 (1).

IL-7 is primarily produced by stromal and epithelial cells (7, 8) and promotes human T cell development, naïve T cell homeostasis, T cell proliferation and survival of memory T cells (7, 8). IL-7 binds to the IL-7R, a heterodimer consisting of the IL-7Rα (CD127) and the common gamma chain (γc or CD132), causing STAT1 and STAT3 activation (7). Thymic stromal lymphopoietin (TSLP) is an IL-7 family member involved in the activation, expansion and survival of T lymphocytes and dendritic cells acting through a heterodimeric IL-7Rα and TSLPR complex (9).

High mobility group box-1 (HMGB1) is a nuclear protein that can act as a damage associated molecular pattern (DAMP) to activate immune cells, including Th1 lymphocytes (10).

NLRs are categorized into 5 subfamilies (11, 12). A typical inflammasome is composed of an NLR, an adaptor protein such as apoptosis-associated speck-like protein containing a CARD (ASC) and an effector caspase that activates proinflammatory cytokines. Within the NRLP3 complex, auto-catalytic cleavage of pro-caspase-1 enables removal of IL-1β and IL-18 pro-sequences resulting in biologically active forms and thereby initiating Th1 and Th17 adaptive immune responses (11, 13).

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HMGB1 is also induced by NRLP3 activation (11, 12). The expression of IL-33, another member of the IL-1 family, may also be enhanced through activation of the inflammasome (14). IL-33 acts through its receptor ST2 and may activate immune cells (15). IL-6 is induced by NRLP3 but in an inflammasome-independent manner (16).

The NLRP3 inflammasome is activated by infectious agents, double strand DNA and extracellular ATP (11, 2). NALP7 decreases transcription of pro-IL-1β expression and both IL-10 and IL-27 can modulate NRLP3 activity (2) whereas IL-37 can down-regulate inflammation and innate immunity independently of the inflammasome (17, 18).

The aim of this study was to investigate the presence of innate immune mediators (IL-6, IL-7, IL-10, IL-27, IL-37, TSLP, IFNγ, their receptors, and signaling proteins STAT1 and pSTAT1) and inflammasome components (NLRP3, caspase 1, IL-1β, IL-18, IL-33, NALP7, ST2) in the bronchial mucosa and bronchoalveolar lavage (BAL) of patients with stable COPD of differing severity and age-matched control subjects with normal lung function.

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Methods

Subjects

All subjects were recruited from the Respiratory Medicine Unit of the "Fondazione Salvatore Maugeri" (Veruno, Italy), the Section of Respiratory Diseases of the University Hospital of Ferrara, Italy and the Section of Respiratory Diseases of the University Hospital of Katowice, Poland for immunohistochemistry and ELISA experiments. The severity of the airflow limitation, as determined by spirometry, was graded using GOLD criteria (19). All former smokers had stopped smoking for at least one year. COPD and chronic bronchitis were defined, according to international guidelines: COPD, presence of a post-bronchodilator forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) ratio <70%; chronic bronchitis, presence of cough and sputum production for at least 3 months in each of two consecutive years [www.goldcopd.com]. All COPD patients were stable. The study conformed to the Declaration of Helsinki. We obtained and studied bronchial biopsies from 55 subjects: 32 had a diagnosis of COPD in a stable clinical state (20), 12 were current or ex-smokers with normal lung function, and 11 were non-smokers with normal lung function (Table 1). The smoking history was similar in the three smoker groups: mild/moderate and severe/very severe COPD, and healthy smokers with normal lung function. The clinical details of the patients where has been collected the bronchoalveolar lavage are summarized in Table 2. The results provided below are the data from 26 COPD and 18 control smokers with normal lung function. Due to the necessity to concentrate the BAL supernatants the results provided for each ELISA are the data from 15 COPD and 14 control smokers with normal lung function which are not the same patients for all mediators measured.

A detailed description of subjects, lung function tests, fiberoptic bronchoscopy and processing of bronchial biopsies and bronchoalveolar lavage, immunohistochemistry, scoring system for immunohistochemistry, double staining and confocal microscopy, ELISA tests performed on the

bronchoalveolar lavage fluid and "*in vitro*" experiments performed on normal human bronchial epithelial cells (NHBE) and details of statistical analysis is provided in the online data repository.

Statistical analysis

Differences between groups were analyzed using ANOVA and Kruskal Wallis tests. Correlation coefficients were calculated using the Spearman rank method.

Results

Measurement of inflammatory cells in the bronchial submucosa

These results are reported in full in the online data repository and in Table E1. Briefly, these data confirm elevated numbers of CD8+ T-cells, CD68+ macrophages and neutrophils in COPD (20).

Immunohistochemistry for innate immunity and inflammasome pathways in the bronchial epithelium

The number of IL-27+ (Figure 1 and Table 3), IL-27R+, TSLPR+, NALP7 and STAT1 immunoreactive cells was increased in the epithelium of severe stable COPD patients compared to control non-smokers (p=0.0048, p=0.017, p=0.008, p=0.054, p=0.011, respectively). IL-27+, TSLPR+, NALP7+ and STAT1+ cells also differed significantly in comparison with control healthy smokers (p=0.043, p=0.0019, p=0.0009 and p=0.023, respectively) (Table 3). In contrast, no significant differences in bronchial epithelial expression of IL-1β, IL-1βRI and RII, caspase-1, IL-18, IL-18Rα, IL-18Rβ, IL-18BP, NLRP3, IL-6, IL-6Rα, IL-7, IL-7Rα, IL-10, IL-10Rα, IL-33, ST-2, IFNγ, IFNγRI, pSTAT1, TSLP and IL-37 were observed between groups (Table 3). Due to the fewer number of subjects who were current smokers within the severe stable COPD group we were unable to perform sub-analysis of current versus former smokers across groups.

Immunohistochemistry for innate immunity and inflammasome pathways in the bronchial submucosa

Mononuclear cells (lymphocytes and macrophages) and endothelial cells were the most represented immunostained cells in the submucosa. The number of IFNγ+ cells was significantly higher in mild/moderate (p=0.010) and severe (p=0.008) stable COPD compared to control non-smokers, confirming previously reported data (21). The number of IFNγRI+ cells was increased in severe COPD compared to mild COPD (p=0.031), control smokers (p=0.0035) and control non-smokers

(p=0.006). IL-18Rβ showed a slight increase in severe COPD compared to mild/moderate COPD (p=0.045) and control smokers (p=0.039) but did not differ in comparison with control non-smokers. The number of IL-7+ (Figure E1) cells was higher in severe COPD (p=0.008), mild/moderate COPD (p=0.010) and in control smokers (p=0.012) compared to control non-smokers. In addition the number of IL-7Rα+ cells was significantly higher in severe COPD compared to mild/moderate COPD (p=0.040), control smokers (p=0.009) and control non-smokers (p=0.002). IL-10 was poorly expressed but number of IL-10+ cells was higher in severe stable COPD (p=0.005), mild/moderate COPD (p=0.047) and in control smokers (p=0.054) compared with control non-smokers.

The number of IL-27+ (Figure 1) as well as pSTAT1+ (Figure E2) cells was significantly higher in severe COPD (p=0.032 and p=0.018, respectively) compared to control smokers but did not differ in comparison with the other groups. Interestingly, the number of IL-27R+ cells was significantly higher in severe COPD (p=0.010 and p=0.002) and mild/moderate COPD (p=0.054 and p=0.009) compared to control smokers and non-smokers. Similarly, the number of total STAT1+ cells was significantly higher in severe COPD (p=0.0043 and p=0.015) and mild/moderate COPD (p=0.022 and p=0.029) compared to control smokers and non-smokers.

The number of TSLPR+ cells was higher in severe COPD compared to control smokers (p=0.005) and non-smokers (p=0.044). TSLPR+ cell numbers were also increased in mild/moderate stable COPD compared to control smokers (p=0.013).

The number of IL-37+ (Figure 2) and NALP7+ (Figure E3) cells was higher in severe stable COPD compared to control smokers (p=0.054 and p=0.0015 respectively) and in mild/moderate stable COPD in comparison with control smokers (p=0.008 and p=0.0043 respectively). Furthermore, the number of IL-37+ cells in mild/moderate stable COPD was significantly increased in comparison with control non-smokers (p=0.023) (Table 3).

No significant differences were observed for IL-1β, IL-1βRI, IL-1βRII, caspase-1, IL-18, IL-18Rα, IL-18BP, NLRP3, IL-6, IL-6Rα, IL-10Rα, IL-33, ST-2 and TSLP immunostaining between groups.

Double staining and confocal microscopy

The percentage of CD68+IL-27+ double stained cells was significantly increased in COPD patients (34±8%) compared to control smokers (8±2%, p=0.0209) (Figure E4) (see also online data repository for more details).

ELISA assays in the BAL supernatants

BAL levels of IL-6 were significantly increased in stable COPD patients compared to the control healthy smokers (p=0.0001; Figure 3A), without a significant change in the BAL level of sIL-6R and sgp130 between the two groups (Figure E5A and E6A). In contrast BAL IL-7 (Figure E5B) and IL-27 (Figure E5C) levels were not significantly different between the two subject groups. BAL HMGB1 was significantly decreased in stable COPD patients compared to control healthy smokers (p=0.0174; Figure 3C). However, this difference was lost after removal of the outliers (p=0.0540). The BAL level of soluble ST2 (p=0.0073; Figure 3D) and IL-1RA (p=0.0307; Figure 3B) are both significantly decreased in stable COPD patients compared to control smokers with normal lung function without significant changes in the BAL level of IL-1β (Figure E6B), IL-18 (Figure E6C), IL-18BPa (Figure E6D) and their IL-1β/ILRA (Figure E6E) and IL-18/IL-18BPa (Figure E6F) ratios and IL-37 between the two group of subjects. Finally, BAL levels of IL-33 were under the detection limit of the assay (data not shown) in all subjects. (see also online data repository).

Correlations between inflammatory cell counts, IL-27 related molecules in the bronchi and clinical parameters

Correlations restricted to patients with COPD alone show that the number of IFNγRI+ cells correlated with the number of IL-27+ (R=0.42, p=0.036) and IL-27R+ (R=0.51, p=0.014) cells in the bronchial submucosa (Figure 4, panels a and b). The number of IFNγRI+ cells also correlated with numbers of IL-7Rα (R=0.68, p=0.0009). IL-7+ cell numbers correlated with the number of IL-

27+ (R=0.43, p=0.010) and IL-27R+ (R=0.51, p=0.003) cells in the bronchial submucosa of stable COPD patients alone (Figure 4, panels c and d)(see also online data supplement).

Inflammation and oxidative stress enhance IL-27 mRNA and protein expression in NHBE cells in vitro, but not inflammasome related mRNAs

Stimulation of human bronchial epithelial cells in vitro with a combination of oxidants (H_2O_2) and inflammatory cytokines significantly increased IL-27B mRNA and protein, but not the inflammasome related IL-1β, IL-18 and caspase 1 encoding mRNAs (see online data repository and Figures E7, E8 and E9).

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Discussion

We show significantly increased expression of IL-27 in the bronchial epithelium of severe stable COPD patients compared with both smoking and non-smoking controls. In addition, the number of IL-27+ and pSTAT1+ cells is also increased in the bronchial submucosa of severe stable COPD patients compared to control smokers. This is associated with an increase in the number of IL-27R+ cells in severe stable COPD subjects compared with controls. Finally, in smokers, and in patients with COPD, the number of IFNγRI+ cells correlated with the number of both IL-27+ and IL-27R+ cells. We failed to show evidence for NRLP3 activation in the airways of stable COPD patients. Indeed, the levels of NRLP7 and IL-37, inhibitors of NLRP3 activity, were upregulated in COPD.

We have previously reported increased pSTAT4 expression linked to IFNγ expression (21) but IL-27 can also induce IFNγ transcription through a STAT1- and STAT3-mediated process (6). IL-27 also increases proliferation and IFNγ and granzyme B production from human CD3-activated naïve CD8+ cells. This results in increased CD8+ T cell-mediated cytotoxicity (22). IL-27 also drives inducible regulatory T (Treg) cells to produce IL-10 (23). Although we observed a significant increase in the number of IL-10+ cells in bronchial submucosa of COPD patients and healthy smokers, these numbers were very low in all subjects. Furthermore, there was no difference in IL-10R expression in the bronchial mucosa of COPD patients. This, in conjunction with the previous lack of changes in FoxP3+ regulatory T cells (24) and low FoxP3 expressions in BAL T lymphocytes from stable COPD patients (25), suggests that a predominantly non-regulatory CD25+ helper T-cell population is present in smokers and stable COPD patients. However, IL-10 expression is reduced in the sputum of patients with stable COPD (26) and additional studies are required to clarify this discrepancy.

IL-27B mRNA and protein expression was up-regulated in normal primary human bronchial epithelial cells by combined oxidative and pro-inflammatory stimuli. Although elevated levels of

IL-27 have been reported in COPD sputum (27) there was no increase in BAL IL-27 levels in mild to severe stable COPD patients in this study. This may reflect the different compartments sampled by BAL and by sputum (28) or that IL-27 is released from dead or dying sputum macrophages since we show here that CD68+ cells are the main source of IL-27.

IL-7+ cell numbers were increased in the bronchial mucosa of stable COPD and control smokers compared to control non-smokers despite no changes in BAL IL-7 being found. IL-7 is produced by both stromal and epithelial cells whereas its receptor is expressed mainly on T cells and monocytes (7, 8). The increased levels of IL-7, IL-7R and TSLPR observed here indicates that IL-7 may have a local pro-inflammatory function increasing the activation and survival of T cells and monocytes in the bronchial submucosa of severe stable COPD patients (29).

IL-7+ cell numbers correlate with both IL-27+ and IL-27R+ cells suggesting a functional relationship. The correlation between numbers of IL-7Rα+ and INFγRI on one side, and the inverse relationship between IL-7Rα and FEV1 % predicted, also suggests a link between increased expression of this receptor and increased severity in stable COPD. Overexpression of IL-7Rα is linked to increased severity of inflammatory bowel disease (8).

High levels of IL-6 are released by both sputum and BAL macrophages *in vitro* (3) and IL-6 is increased in sputum of COPD patients during exacerbations (30). In our study, BAL levels of IL-6, but not its soluble decoy receptor, were increased in COPD despite no change in the numbers of IL-6+ and IL-6R+ cells. Thus, our BAL data may simply reflect the presence of activated macrophages in the peripheral airways of stable COPD patients Soluble gp130 inhibits both IL-6 trans-signaling via the sIL-6R and classic signaling via the mIL-6R (31). In animal models, sIL-6R-mediated signaling is an important intermediary in the resolution of neutrophilic inflammation (31), but we were unable to observe any significant difference in the expression of sgp130 in BAL from stable COPD patients compared to control smokers with normal lung function.

We report similar expression levels of NLRP3 in COPD and control subjects, an absence of caspase-1 expression in COPD patients and controls and no differences in the expression of IL-1β, its receptors and IL-18 in COPD bronchial biopsies as well as in the BAL level of IL-1β, IL-1RA, IL-18 and IL-18BPa, and their IL-1β/IL-1RA and IL-18/IL-18BPa ratios in stable mild/moderate COPD patients vs control smokers with normal lung function. This suggests that the NRLP3 inflammasome only plays, at most, a minor role in the inflammatory response in the central airways of stable COPD. The increased levels of IL-18Rβ in bronchial biopsies of severe COPD, compared to mild COPD and control smokers, reported here, in the absence of a parallel increase of its own ligand, IL-18, may have a limited biological function (Table 3, Figure 5).

We did not find any difference in either IL-1β or IL-18 expression in the bronchial mucosa of stable COPD patients and control subjects in this study. This is in contrast with increased levels of serum, sputum and BAL IL-1β (32-35) and of plasma and sputum IL-18 (36, 37) reported in COPD compared to control smokers and non-smokers. Furthermore, there is discordance between the levels of IL-18 and IL-1β in COPD suggesting that co-ordinated induction through the NRLP3 inflammasome is not critical for their expression (36, 38). Animal models of COPD also provide discordant data on the role of the NLRP3 inflammasome (35, 38, 39). Original data (38) suggested a role for IL-1β in smoke-induced emphysema and airway remodelling but more recent data in mice favours a role for inflammasome-independent induction of IL-1β in driving smoke-induced inflammation (35, 39). The expression of other inflammasome components in stable COPD has not been reported but the failure to detect increased IL-1β and IL-18 expression both in the bronchial mucosa and the BAL in this study suggests they may also play a limited role in stable COPD. However, NRLP3 inflammasome activation may be important during viral and bacterial infections (11, 13). Previous studies have shown elevated levels of IL-1β in exhaled breath condensate and sputum during COPD exacerbations particularly when associated with bacterial infections (40). We observed a modest, albeit significant, reduction of IL-1RA in the BAL of stable COPD patients as

compared to control smokers with normal lung function, that is in line with previous studies (41), but without significant differences in their IL-1β/IL-1RA ratio, suggesting that the BAL decrease of this endogenous counter-regulatory mechanism of the IL-1β activation pathway may be clinically irrelevant. This is in line with data from unpublished clinical trials showing that monoclonal antibodies neutralizing IL-1β signalling are ineffective in the treatment of stable COPD (http://clinicaltrials.gov/ct2/show/results/NCT00581945).

Plasma and sputum HMGB1 levels have been previously reported to be increased in stable COPD patients compared with control subjects (42) but these studies were limited by not having controls matched for age and smoking-history. BAL levels of HMGB1 have been reported as either increased in stable COPD (41) or no different from controls (43). In steroid-naïve stable COPD patients, we found significantly decreased BAL HMGB1 levels, in comparison with age matched control smokers with normal lung function, although this difference was lost when outliers were removed, again suggesting a minor role of inflammasome activation in the peripheral airways of stable COPD patients.

Overexpression of human IL-37 in mice results in down-regulation of inflammation (17). The increased expression of IL-37 seen in the bronchial submucosa, but not in BAL, of stable COPD patients compared with control smokers, suggests a counteregulatory role of this molecule. NALP7 attenuates caspase 1-dependent IL-1β secretion by inhibiting the processing of pro-IL-1β and pro-caspase 1 (12). The increased expression of NALP7 in the submucosa, but not BAL, of stable COPD patients may act to prevent the activation of the inflammasome pathway (Figure 5).

In summary, our results showing an increased expression of the innate immunity cytokine IL-27 in the bronchial mucosa of stable COPD patients indicate its potential role in the progression of severity of their chronic bronchial inflammation. The absence of NRLP3 inflammasome increase in COPD patients is associated with an increased expression of the inflammatory and inflammasome

inhibitory molecules IL-37 and NALP7. These data suggest a relevant role for innate immunity and a silent state of inflammasome in stable COPD.

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Figure legends

Figure 1

Photomicrographs showing the bronchial mucosa from (a) control non-smoker, (b) control healthy smoker with normal lung function, (c) mild/moderate stable chronic obstructive pulmonary disease (COPD) and (d) severe stable COPD immunostained for identification of IL-27+ cells (arrows) in the epithelium and bronchial submucosa. Results are representative of those from 11 non-smokers, 12 healthy smokers, 14 mild/moderate COPD and 18 with severe COPD. Bar=30 microns.

Figure 2

Photomicrographs showing the bronchial mucosa from (a) control non-smoker, (b) control healthy smoker with normal lung function, (c) mild/moderate stable chronic obstructive pulmonary disease (COPD) and (d) severe stable COPD immunostained for identification of (IL-37)+ cells (arrows) in the bronchial submucosa. Results are representative of those from 11 non-smokers, 12 healthy smokers, 14 mild/moderate COPD and 18 with severe COPD. Bar=30 microns.

Figure 3

BAL levels of (A) IL-6, (B) IL-1RA, (C) HMGB1 and (D) soluble ST2 in stable COPD patients (n=15) compared to the control healthy smokers (n=14). Results are expressed as mean±SEM. Exact p values are shown above each graph.

Figure 4

Regression analysis between numbers of (a) IFNγRI+ and IL-27+, (b) IFNγRI+ and IL-27R+, (c) IL-7+ and (d) IL-27+, IL-7+ and IL-27R+ cells in the bronchial submucosa of all patients with chronic obstructive pulmonary disease (COPD). Correlation coefficients were calculated by using the Spearman rank method.

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Figure 5

Schematic representation of the molecular variations related to innate immunity (panel a) and inflammasome (panel b) in the bronchial biopsy submucosa (values expressed as number of cells/mm2) of healthy non-smokers, healthy smokers, mild/moderate stable COPD and severe/very severe stable COPD patients. *significantly different from healthy non-smokers; # significantly different from healthy smokers. With worsening of the disease, increased levels of IFNγ are accompanied by increased levels of pSTAT1 and molecules inducing innate immunity such as IL-27 and IL-7 (panel a). Molecules inducing activation of the inflammasome are not changed (NLRP3, caspase-1) as well as IL-1β. Concomitantly, molecules inhibiting the inflammasome activation, such as IL-37 and NALP-7 are increased (panel b).

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Tables

Table 1. Clinical characteristics of subjects studied by immunohistochemistry

	Control non-smokers	Control smokers normal lung function	COPD grade I/II (mild/moderate)	COPD grade III/IV (severe/very severe)
Number	11	12	14	18
Age (years)	67± 1	61±7	67±8	66±9
M/F	10/1	9/3	12/2	11/7
Pack years	0	43±26	40±19	54±36
Ex/current smokers	0	2/10	5/9	13/5
FEV_1 pre-β_2 (% predicted)	116±14	104±13	66±14$^{\#}$	35±8$^{\#\&}$
FEV_1 post-β_2 (% predicted)	ND	ND	72±12	38±9
FEV_1/FVC (%)	85±10	81±6	60±8$^{\#}$	44±10$^{\#\&}$
Chronic Bronchitis	0	5	8	6

Patients were classified according to GOLD (http://www-goldcopd.org) grades of severity for COPD into: mild (stage I), moderate (stage II), severe (stage III), and very severe (stage IV).

COPD patients were using short-acting inhaled β_2-agonists (SABA) or short-acting inhaled antimuscarinics (SAMA) prn or regular long-acting inhaled β_2-agonists (LABA) and/or regular inhaled anticholinergics, including SAMA or long-acting inhaled antimuscarinics (LAMA) at the dosage recommended in current COPD guidelines (www.goldcopd.org) at the time of their recruitment.

Data expressed as means ± SEM. For COPD patients FEV_1/FVC (%) are post-bronchodilator values.

Abbreviations: M, male; F, female, FEV_1: forced expiratory volume in one second; FVC, forced vital capacity; ND, not determined; COPD, chronic obstructive pulmonary disease.

Statistics. (ANOVA) #$p<0.0001$, significantly different from control smokers with normal lung function and control never-smokers; $^{\&}p<0.0001$, significantly different from mild/moderate COPD.

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Table 2: Clinical characteristics of the subjects for the BAL study

	Smokers with normal lung function	Mild to moderate stable COPD
number	18	26
Age	64.4 ±2.0	67.9±1.6
Sex (M/F)	15/3	19/F
Ex/current smokers	10/8	12/14
Pack-years	37.8±3.0	39.6±5.5
Chronic bronchitis	10	14
FEV_1 %pred	88.2±4.9	57.9±3.0
FEV_1/FVC%	79.2±1.8	56.8±2.5

Definitions of abbreviations: COPD=chronic obstructive pulmonary disease; M: male; F: female; FEV_1=forced expiratory volume in one second; FVC=forced vital capacity. FEV_1 %predicted and FEV_1/FVC% are post-bronchodilator values. COPD patients were using short-acting inhaled β_2-agonists (SABA) or short-acting inhaled antimuscarinics (SAMA) prn or regular long-acting inhaled β_2-agonists (LABA) and/or regular inhaled anticholinergics, including SAMA or long-acting inhaled antimuscarinics (LAMA) at the dosage recommended in current COPD guidelines (www.goldcopd.org) at the time of their recruitment. Data expressed as means ± SEM.

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Table 3. Immunohistochemical quantification of innate immunity and inflammasome pathways expression in the bronchial mucosa

	Healthy non-smokers	Healthy smokers	Mild/Moderate COPD	Severe COPD	p-value
Epithelium (score 0-3)					
IL-6	0.25 (0-0.5)	0.25 (0-0.5)	0.25 (0-1)	0.25 (0-1)	0.346
IL-6 Rα	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	n.v.
IL-7	0.5 (0.5-1)	0.75 (0.25-2)	0.5 (0.25-1.5)	0.75 (0.5-2)	0.671
IL-7 Rα	0 (0-0)	0 (0-0)	0 (0-0.5)	0 (0-0.5)	0.985
IL-10	0 (0-0.25)	0 (0-0.25)	0 (0-0.5)	0 (0-0.5)	0.513
IL-10 Rα	0 (0-0.25)	0 (0-0.25)	0 (0-0.25)	0 (0-0.25)	0.688
IL-27	0.5 (0.25-1)	0.75 (0.25-1.5)	0.75 (0-1.5)	1 (0.5-2)*&	0.0279
IL-27 R	0 (0-0.5)	0.25 (0-0.5)	0.25 (0-0.75)	0.25 (0-1)*	0.0748
IL-33	0 (0-0.25)	0 (0-0.25)	0 (0-0.25)	0 (0-0.75)	0.962
ST2	0 (0-0)	0 (0-0)	0 (0-0.25)	0 (0-0.25)	0.752
TSLP	0.25 (0-1)	0.25 (0-1)	0.5 (0-1)	0.37 (0-1)	0.545
TSLP-R	0.25 (0.25-0.5)	0.25 (0.25-0.5)	0.5 (0.25-1.5)	1 (0.25-1.25)*&	0.007
IL-18	1 (0.5-2)	1 (0.5-2.5)	1.5 (0.25-2.5)	1 (0.5-2.5)	0.386
IL-18 Rα	2.5 (0.75-2.75)	2.5 (1-3)	2.5 (1-3)	2.5 (1-3)	0.869
IL-18 Rβ	2.25 (1-2.5)	1 (0.25-2.5)	1.5 (0-2.5)	2 (1-2.75)	0.095
IL-18 BP	0 (0-0)	0 (0-0.25)	0 (0-0.5)	0 (0.25)	0.093
NLRP-3	1.62 (1-2)	1.5 (1-2.75)	1.5 (1-3)	2 (1.5-2.5)£	0.220
Caspase-1	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	n.v.
IL-1β	0 (0-0.25)	0 (0-0.25)	0 (0-0.25)	0 (0-0.5)	0.671
IL-1β RI	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	n.v.
IL-1β RII	0 (0-0.25)	0.25 (0-0.5)	0.25 (0-1)	0.25 (0-1)	0.380
IL-37	1.5(0.25-2.25)	1.5(0.25-2.25)	1.12(0.75-2.0)	1.5(0.75-2.25)	0.915
NALP7	1.25(0.5-2.5)	0.62(0.25-1.75)	1.62(0.25-2.5)	1.87(0.5-3.0) *&	0.006
STAT1	0.37(0.25-1.5)	0.5(0.0-2.0)	1.0(0.25-2.25)	1.37(0.25-2.75) *&	0.018
pSTAT1	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	n.v.
IFN-γ	0 (0-0.25)	0 (0-0.25)	0 (0-0)	0 (0-0.25)	0.938
IFN-γ RI	1.5 (0.75-2.25)	1.25 (0.75-2.5)	1.25 (0.75-3)	2.25 (0.75-2.5)	0.282
Submucosa (cells/mm^2) Median (Range)					
IL-6	24 (9-54)	27.5 (8-64)	36.5 (4-126)	44 (6-169)	0.294
IL-6 Rα	0 (0-22)	11 (0-55)	7 (0-18)	10 (0-37)	0.259
IL-7	72 (32-226)	126 (56-269)*	140 (32-484)*	140 (52-323)*	0.023
IL-7 Rα	13 (0-71)	32 (6-108)	44.5 (5-183)	91 (16-302)*&£	0.0053
IL-10	0 (0-12)	7 (0-28)*	6.5 (0-113)*	9 (0-55)*	0.0519
IL-10 Rα	0 (0-4)	0 (0-8)	0 (0-48)	0 (0-23)	0.367
IL-27	54 (8-113)	51 (14-140)	73 (9-400)	77 (36-542)&	0.113
IL-27 R	12 (0-24)	9 (0-51)	35 (0-81)*&	48 (6-155)*&	0.0027
IL-33	5 (0-9)	6 (0-23)	5 (0-54)	6 (0-86)	0.606
ST-2	0 (0-5)	0 (0-8)	0 (0-16)	0 (0-7)	0.722
TSLP	14 (0-29)	11 (0-38)	17 (0-90)	12 (0-81)	0.498
TSLP-R	18 (5-43)	12 (0-86)	55 (0-277) &	49 (7-269)*&	0.011
IL-18	123 (11-203)	59 (16-355)	170 (16-419)	107 (8-488)	0.381
IL-18 Rα	177 (61-203)	152 (81-242)	231 (71-424)	214 (59-525)	0.118
IL-18 Rβ	138 (90-210)	69 (5-236)	104 (5-232)	182 (48-302)£&	0.043

IL-18 BP	5 (0-97)	0 (0-32)	0 (0-18)	0 (0-16)	0.258
NLRP-3	104 (73-183)	111 (41-274)	166 (56-348)	121 (84-403)	0.261
Caspase-1	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	n.v.
IL-1β	0 (0-5)	6 (0-24)	5 (0-56)	4 (0-89)	0.298
IL-1β RI	0 (0-7)	0 (0-7)	0 (0-16)	0 (0-14)	0.362
IL-1β RII	0 (0-11)	0 (0-16)	0 (0-18)	0 (0-26)	0.882
IL-37	145(43-258)	107(58-355)	282(113-441) *&	21364-452) &	0.024
NALP7	167(29-270)	85(11-242)	210(32-462) &	177(45-456) &	0.005
STAT1	35(19-102)	22(13-129)	110(9-210) *&	129(8-274) *&	0.005
pSTAT1	37 (0-156)	8 (0-127)	41 (0-158)	67 (0-173)&	0.0887
IFN-γ	12 (0-46)	14.5 (0-115)	20 (3-90)*	22 (4-82)*	0.051
IFN-γRI	146 (118-298)	183 (113-460)	226 (129-672)	344 (170-631)*&£	0.0046

Abbreviations: COPD, chronic obstructive pulmonary disease. Data expressed as median (range). Statistics: The Kruskal-Wallis test was used for multiple comparisons followed by Mann-Whitney U test for comparison between groups: *$p<0.05$, significantly different from control non-smokers; $^{\&}p<0.05$, significantly different from control smokers with normal lung function; $^{£}p<0.05$, significantly different from mild COPD. The exact "p" values for comparison between groups are given in the Results section.

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Competing interests

We declare no competing interests in relation to this manuscript

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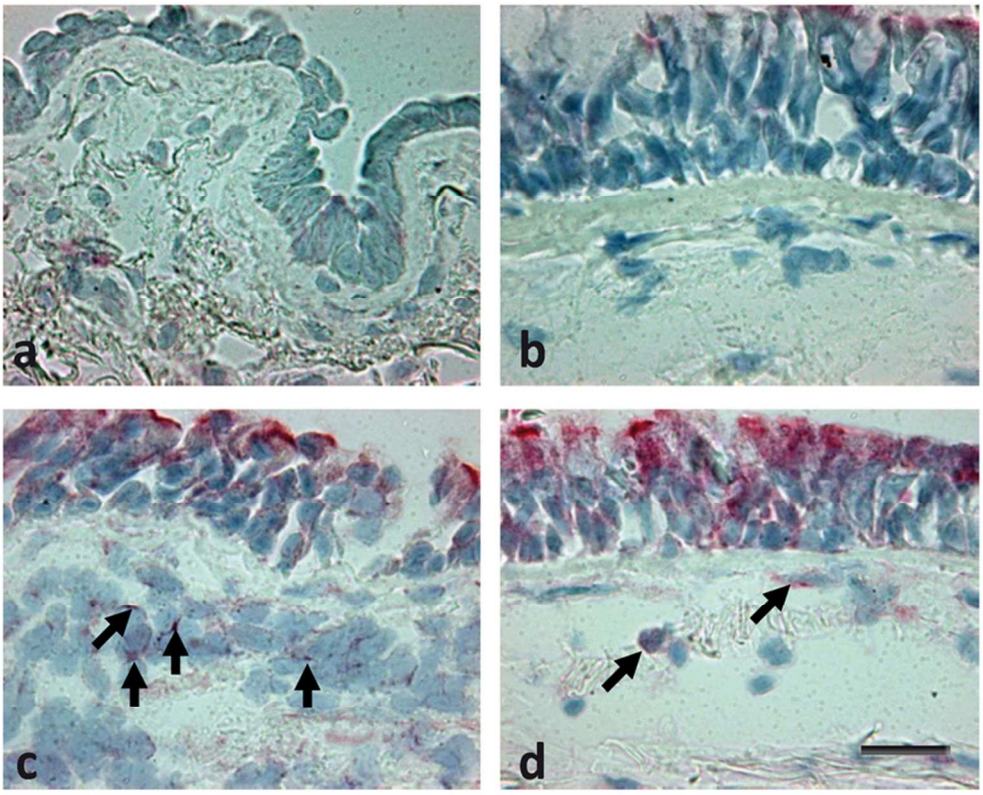

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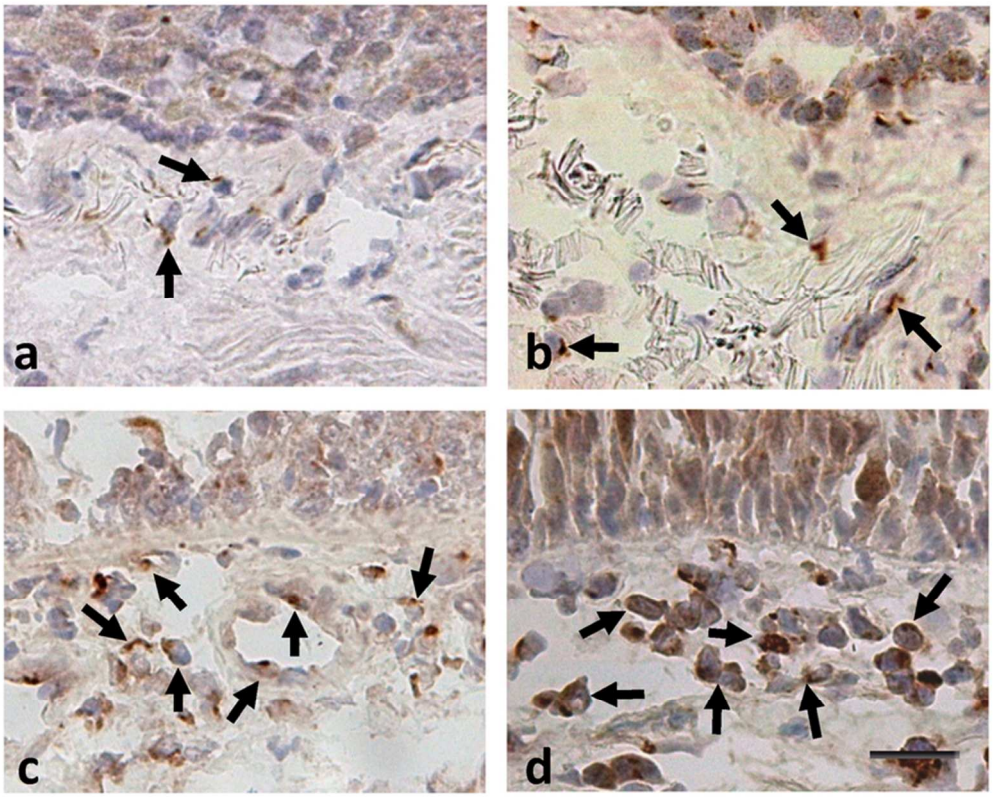

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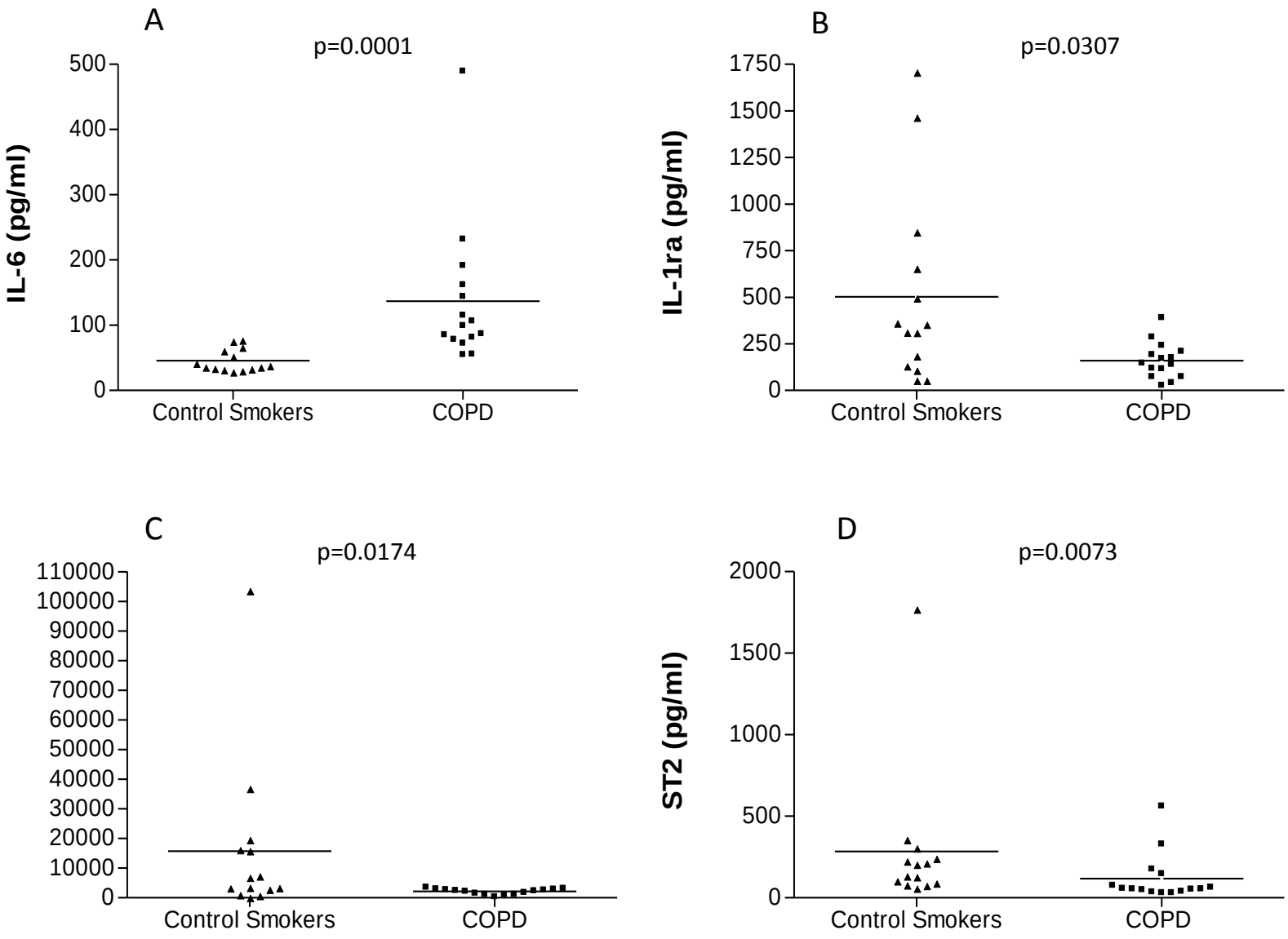
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400
300
200
100
0
Control Smokers
COPD
B
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IL-1ra (pg/ml)
1750
1500
1250
1000
750
500
250
0
Control Smokers
COPD
C
p=0.0174
HMGB1 (pg/ml)
110000
100000
90000
80000
70000
60000
50000
40000
30000
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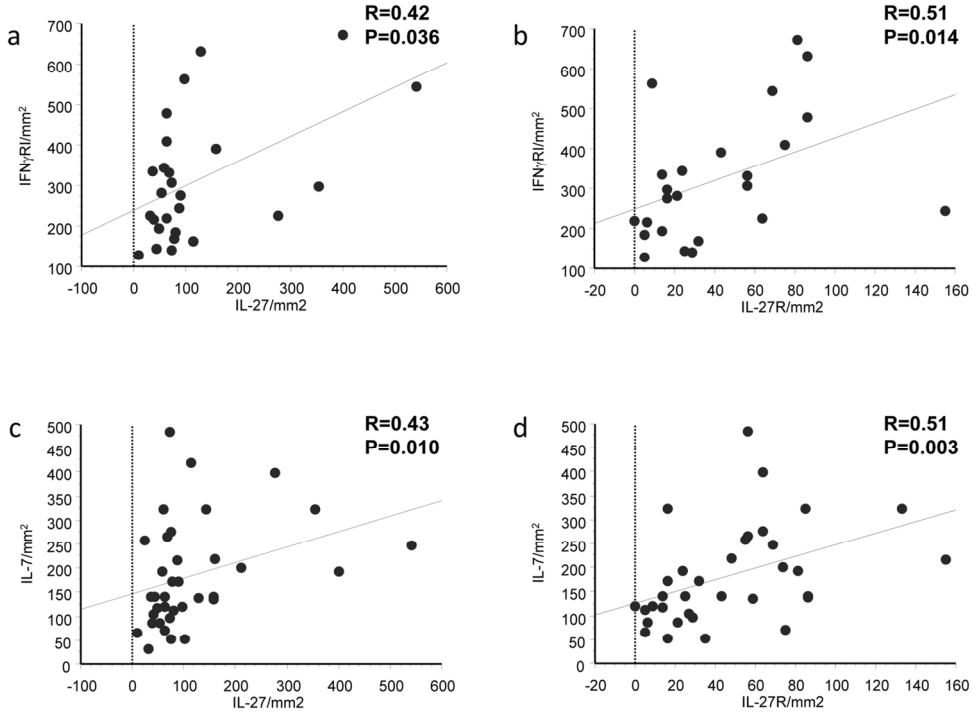
COPD



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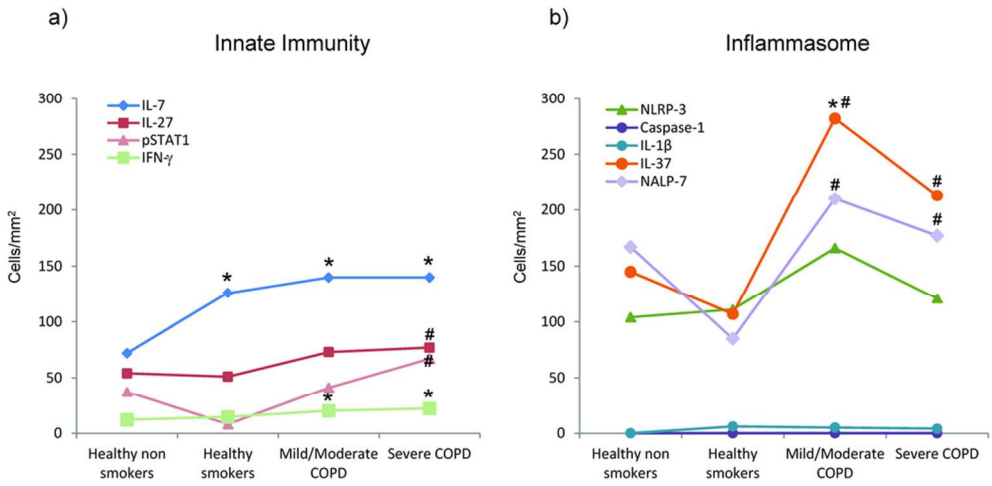

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Online data repository

Innate immunity but not NLRP3 inflammasome activation correlates with severity of stable COPD

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Methods

Subjects

Subjects were recruited from the Respiratory Medicine Unit of the "Fondazione Salvatore Maugeri" (Veruno, Italy), the Section of Respiratory Diseases of the University Hospital of Ferrara, Italy and the Section of Respiratory Diseases of the University Hospital of Katowice, Poland for immunohistochemistry and ELISA experiments. The severity of the airflow obstruction was staged using GOLD criteria [**www.goldcopd.org**]. All former smokers had stopped smoking for at least one year. COPD and chronic bronchitis were defined, according to international guidelines, as follows: COPD, presence of a post-bronchodilator forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) ratio <70%; chronic bronchitis, presence of cough and sputum production for at least 3 months in each of two consecutive years (E1). All COPD patients were stable with no previous exacerbation in the six months before bronchoscopy. None of the subjects was treated with theophylline, antibiotics, antioxidants, mucolytics, and/or glucocorticoids in the month prior bronchoscopy. **COPD patients were using short-acting inhaled β_2-agonists (SABA) or short-acting inhaled antimuscarinics (SAMA) prn or regular long-acting inhaled β_2-agonists (LABA) and/or regular inhaled anticholinergics, including SAMA or long-acting inhaled antimuscarinics (LAMA) at the dosage recommended in current COPD guidelines (www.goldcopd.org) at the time of their recruitment**. The study conformed to the Declaration of Helsinki and was approved by the ethics committees of the Fondazione Salvatore Maugeri [Veruno (Novara), Italy], the University Hospital of Ferrara, Italy and the University Hospital of Katowice, Poland. Written informed consent was obtained from each subject and bronchial biopsies and BAL were performed according to the local ethic committee guidelines.

We obtained and studied bronchial biopsies from 55 subjects: 32 had a diagnosis of COPD in a stable clinical state (E2), 12 were current or ex-smokers with normal lung function, and 11 were non-smokers with normal lung function (Table 1 **of the main manuscript**). **Two out of 12 current or ex-smokers were pure volunteers. All the other control subjects, smokers, ex-smokers and non-smokers, attended our respiratory clinic mostly for of unexplained persistent cough. At their first visit they were screened and after exclusion of the inappropriate subjects determined by the clinical history, physical examination, lung function and imaging studies and laboratory examinations for lung, upper airways and/or cardiac or systemic disease, subjects were recruited.** COPD patients were divided in two groups, according to the grade of severity of their airflow limitation (mild/moderate, grade I-II; or severe/very severe, grade III-IV; n=14 and 18, respectively) (E1). Subjects in all four groups were age-matched. The smoking history was similar in the three smoker groups: mild/moderate and severe/very severe COPD, and healthy

smokers with normal lung function. 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). Forty-**three** percent (n=1**4**) of the total COPD patients and **41**% (n=**5**) 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.

The clinical details of the subjects undergoing bronchoalveolar lavage (BAL) are summarized in Table 2 **of the main manuscript**. **We analyzed the BAL fluid obtained from 26 COPD and 18 control smokers with normal lung function but due to** the necessity to concentrate the BAL supernatants for many ELISA assays **we were unable to perform all these assays in all subjects**. **The results provided for each ELISA are the data from 15 COPD and 14 control smokers with normal lung function.**

Of the 44 subjects included for the bronchoalveolar lavage (BAL) analysis, 21 patients were recruited in the Veruno's Hospital and 23 subjects in Poland and Ferrara. All the BAL procedures were well standardized in accordance with standard guidelines. All the bronchoscopists in the three centers followed the same SOP for BAL collection and processing and the ELISAs on the BAL supernatants were run in a single center (Ferrara).

Lung function tests and volumes

Pulmonary function tests were performed as previously described (E2) according to published guidelines (E3). 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, CA). In order to assess the reversibility of airflow obstruction and post bronchodilator functional values the FEV_1 and FEV_1/FVC% measurements in the groups of subjects with FEV_1/FVC%≤70% pre-bronchodilator was repeated 20 min after the inhalation of 0.4 mg of albuterol.

Fiberoptic bronchoscopy, collection and processing of bronchial biopsies

Subjects were at the bronchoscopy suite at 8.30 AM after having fasted from midnight and were pre-treated with atropine (0.6 mg IV) and midazolam (5-10 mg IV). 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

fiberoptic 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 four 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 extracted from the forceps and processed for light microscopy as previously described (E2). Two samples were embedded in Tissue Tek II OCT (Miles Scientific, Naperville, IL), frozen within 15 min in isopentane pre-cooled in liquid nitrogen, and stored at –80°C. The best frozen sample was then oriented and 6μm thick cryostat sections were cut for immunohistochemical light microscopy analysis and processed as described below.

Immunohistochemistry

Two sections from each sample were stained applying immunohistochemical methods with a panel of antibodies specific for inflammatory cells, innate immune mediators and inflammasome components (Table E1). 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 (0.15M saline containing 0.05 M TRIS-hydrochloric acid at pH 7.6) and incubated for 1hr at room temperature in a humid chamber. Antibody binding was demonstrated with secondary antibodies anti-mouse (Vector, BA 2000), anti-rabbit (Vector, BA 1000) or anti-goat (Vector, BA 5000) followed by ABC kit AP AK5000, Vectastain and fast-red substrate (red color) or ABC kit HRP Elite, PK6100, Vectastain and diaminobenzidine substrate (brown color). Slides were included in each staining run using human tonsil, nasal polyp or breast cancer, as a positive control. For the negative control slides, normal non-specific goat, mouse or rabbit immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at the same protein concentration as the primary antibody.

Scoring system for immunohistochemistry

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 630x.

The immunostaining for all the antigens studied was scored (range: 0 = absence of immunostaining to 3 = extensive intense immunostaining) in the intact (columnar and basal epithelial cells) bronchial epithelium, as previously described (E2). The final result was expressed as the average of

all scored fields performed in each biopsy. A mean±SD of 0.70±0.26 millimeters of epithelium was analyzed in COPD patients and control subjects.

Immunostained cells in bronchial submucosa (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 millimeter, was calculated as the average of all the cellular counts performed in each biopsy.

Double staining and confocal microscopy

Four patients with COPD (FEV_1, 64±15%; FEV_1/FVC, 61±8%) and 4 control smokers (FEV_1, 104±14%; FEV_1/FVC, 81±3%) were used for double staining immunofluorescence and confocal microscopy. Double staining was performed as previously reported (E2). For confocal microscopy sections were fixed with 4% paraformaldehyde, washed with phosphate buffered saline (PBS) and incubated (1 hour) with PBS containing 5% bovine serum albumin and 5% donkey serum. After blocking, sections were incubated 1 hour with the primary antibodies diluted as indicated in Table E1 in PBS containing 5% bovine serum albumin. The following antibodies were used: rabbit anti-human IL-27 (LS B2565, R&D); and mouse anti human CD4, CD8 and CD68, M716, M7103 and M814, respectively, Dako). After washing with PBS, the preparations were incubated for a further 30 min with the appropriate secondary Alexa Fluor 488- or Alexa Fluor 647 conjugated antibodies diluted 1:200 in PBS. Negative controls included non-specific mouse and rabbit immunoglobulins revealed as for primary antibodies. Slides were mounted using a specific mounting medium (Vector, H-1400, Vectashield Hard Set). The slides for confocal microscopy were analysed using a three-channel Leica TCS SP5 laser scanning confocal microscope. The Leica LCS software package was used for acquisition, storage, and visualization. The quantitative estimation of co-localized proteins was performed calculating the "co-localization coefficients" (E4, E5).

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed from the right middle lobe using four successive aliquots of 50ml of 0.9% NaCl. BAL cells were spun (500×*g*; 10min) and washed twice with Hanks' buffered salt solution (HBSS). Cytospins were prepared and stained with May-Grünwald stain for differential cell counts. Cell viability was assessed using the trypan blue exclusion method. BAL supernatants were aliquoted and left at -80°C before its use for the ELISA assays summarised in Table E2. These assays have been performed according to the manufacturers' instructions.

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Cell culture and treatments

We used normal human bronchial epithelial cells (NHBE), of **non-smoking subjects** obtained from Lonza (Cologne, Germany) grown in BEGM media (Lonza) with Singlequot supplement (Lonza) following the suppliers instructions. Cells were passaged using the ReagentPack ™ Subculture Reagents (Lonza) following the manufacturer's instructions. Passage number of cells used in this study ranged from 3 to 6. Prior to all the experiments, 70-80% cell monolayers were incubated in supplement–free medium for the 18hrs. The cells were treated with hydrogen peroxide (H_2O_2, 100μM), IL-1β (1 ng/ml), combined IL-1β and H_2O_2, cytomix (50ng/ml of TNFα, IL-1β and IFNγ) or combined cytomix and H_2O_2 for 2hrs **to analyse the messenger RNAs or 24 hours to measure protein expression**. All experiments were performed at least three times.

Extraction and quantification of RNA and qRT-PCR from NHBE

Total RNA was isolated from cells using the RNeasy RNA extraction kit following manufacturer's instructions (Qiagen, UK). cDNA was made from quantified RNA by reverse transcription using the high capacity cDNA kit following manufacturer's instructions (Applied Biosystems, UK). The expression of genes of interest was measured using Syber green (Qiagen, UK) for qPCR in a Corbett Rotorgene 6 (Corbett, Cambridge, UK). We detected the expression of IL-27p28 and EBI3 (IL-27B) using the following primers (E6): IL-27p28 forward, agc tgc atc ctc tcc atg tt; reverse, gag cag ctc cct gat gtt tc; EBI3 (IL-27B) forward, tgt tct cca tgg ctc cct ac; reverse, gct ccc tga cgc ttg taa c. mRNA was normalized using a housekeeping gene 18S, using the following primers 18S forward ctt aga ggg aca agt ggc g; reverse acg ctg agc cag tca gtg ta, for each experimental condition.

Extraction and quantification of IL-27 protein from NHBE

IL-27 levels in the cell culture supernatant following stimulation with H_2O_2 (100μM), IL-1β (1 ng/ml), combined IL-1β and H_2O_2, cytomix (50ng/ml of TNFα, IL-1β and IFNγ) or combined cytomix and H_2O_2 for 24 hours was quantified by sandwich ELISA (R&D Systems Europe, Abingdon, UK) exactly according to the manufacturer`s instructions.

Statistical analysis

Group data were expressed as mean (standard deviation) for functional data or median (range) or interquartile range (IQR) for morphologic data. **We tested for a normal distribution for functional data (i.e. FEV1%, FVC, age etc.) and for a non normal distribution for morphological parameters. Normality tests were performed on all group data. The Grubb's**

outlier test was used to determine whether individual values were outside the rest of the group. Then we applied the analysis of variance (ANOVA) for functional data in comparing subgroups of patients and control subjects for functional data. The non parametric Kruskal Wallis test was applied for multiple comparisons, without application of Bonferroni correction, when morphologic data were analysed followed by the Mann-Whitney U test for comparison between groups. The statistical guide to GraphPad Prism recommends that the Bonferroni correction should not be used when comparing more than 5 variables due to the conservative nature of the test and the subsequent likeliness of missing real differences. We believe that this comparative analysis is of value and represents part of our informative findings. For this reason we applied specific non parametric statistical tests to our data of Table 3 without including the Bonferroni correction. To verify the degree of association between functional or morphological parameters, in all smokers with and without COPD or in smokers with COPD alone the correlation coefficients between functional and morphological and morphological-morphological data 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, CA-USA) **and GraphPad Prism software (www.graphpad.com/scientific-software/prism/).**

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Results

Measurement of inflammatory cells in the bronchial submucosa

The results are summarized in Table E3. The number of CD8 positive lymphocytes was significantly increased in severe/very severe (p=0.021) and mild/moderate (p=0.027) stable COPD compared to control non-smokers. The number of CD4 positive lymphocytes did not differ significantly between the four groups of subjects. Compared with control non-smokers, the number of CD68 positive macrophages was significantly higher in severe/very severe (p=0.033) and mild/moderate (p=0.036) stable COPD. The number of neutrophils was also significantly higher in severe/very severe stable COPD patients compared with control smokers (p=0.011) and non-smokers (p=0.010). Stable COPD patients with chronic bronchitis had a similar number of neutrophils when compared with stable COPD patients without chronic bronchitis (data not shown).

Immunohistochemistry for innate immunity and inflammasome pathways in the bronchial submucosa

For all the proteins studied mononuclear cells (lymphocytes and macrophages) and endothelial cells were the most represented immunostained cells in the submucosa. The number of IFNγ+ cells was significantly higher in mild/moderate (p=0.010) and severe (p=0.008) COPD compared to control non-smokers, confirming previously reported data (E2). The number of IFNγRI+ cells was also increased in severe COPD compared to mild COPD (p=0.031), control smokers (p=0.0035) and control non-smokers (p=0.006). IL-18Rβ showed a slight increase in severe COPD compared to mild/moderate COPD (p=0.045) and control smokers (p=0.039) but did not differ in comparison with control non-smokers. The number of IL-7+ (Figure E1) cells was higher in severe (p=0.008), mild/moderate COPD (p=0.010) and in control smokers (p=0.012) compared to control non-smokers. In addition the number of IL-7Rα+ cells was significantly higher in severe compared to mild/moderate COPD (p=0.040), control smokers (p=0.009) and control non-smokers (p=0.002). IL-10 was poorly expressed but number of IL-10+ cells was higher in severe (p=0.005), mild/moderate COPD (p=0.047) and in control smokers (p=0.054) in comparison with control non-smokers.

The number of IL-27+ (Figure 1) as well as pSTAT1+ (Figure E2) cells was significantly higher in severe COPD (p=0.032 and p=0.018, respectively) compared to control smokers but did not differ in comparison with the other groups. Interestingly, compared to control smokers and non-smokers, the number of IL-27R+ cells was higher in severe (p=0.010 and p=0.002, respectively) and mild/moderate COPD (p=0.054 and p=0.009, respectively). Similarly, the number of total STAT1+

cells was significantly higher in severe (p=0.0043 and p=0.015, respectively) and mild/moderate COPD (p=0.022 and p=0.029, respectively).

No significant differences were observed for IL-1β, IL-1βRI, IL-1βRII, caspase-1, IL-18, IL-18Rα, IL-18BP, NLRP3, IL-6, IL-6Rα, IL-10Rα, IL-33, ST-2 and TSLP immunostaining in the four groups of subjects examined. However, caspase-1 staining was observed in the positive control represented by human breast tumour tissue (Table E1).

Double staining and confocal microscopy

Double staining for identification of CD4+, CD8+ lymphocytes and macrophages (CD68+) co-expressing IL-27 was performed in four representative healthy smokers with normal lung function and in four patients with COPD. We found no difference in the percentage (mean±SD) of CD4+IL-27+ double stained cells between control smokers (12±2%) and COPD patients (11±6%). Similarly, there was no difference in the percentage of CD8+IL-27+ double stained cells between control smokers (14±11%) and COPD patients (9±2%). Interestingly, the percentage of CD68+IL-27+ double stained cells was significantly increased in COPD patients (34±8%) compared to control smokers (8±2%, p=0.0209) (Figure E4).

ELISA assays in the BAL supernatants

The BAL levels of IL-6 were significantly increased in stable COPD patients compared to the control smokers **with normal lung function [median (range) 99.4(54.8-489.6) pg/ml vs 36.6 (28.2-76-8) pg/ml, respectively; p=0.0001; Figure 3A),** without a significant change in sIL-6R expression between the two groups **[127.2(28-435-7) pg/ml vs 197.1(21.7-843-2) pg/ml, respectively; p=0.2658; Figure E5A].**

The BAL levels of IL-7 **[9.4(5-35.9) pg/ml vs 9.1(2.5-99.7) pg/ml, respectively; p=0.8273; Figure E5B], IL-27 [4.3(0.3-64.6) pg/ml vs 15.1(0.8-248.1) pg/ml, respectively; p=0.112; Figure E5C]** and **sgp130 [3213(278.4-9350) pg/ml vs 5663(453.2-11660) pg/ml, respectively; p=0.2850; Figure E6A]** were not significantly different between the two group of subjects. However, the BAL level of HMGB1 was significantly decreased in stable COPD patients compared to control smokers **with normal lung function [2340 (314-3550) pg/ml vs 5105 (0-103521) pg/ml, COPD vs control smokers respectively; p=0.0174; Figure 3C]. However, this difference was lost after removal of the outliers.**

The BAL level of soluble ST2 **[55(30.3-563) pg/ml vs 168.5(57-1770) pg/ml, respectively; p=0.0073; Figure 3D] and IL-1RA [147(26.7-390) pg/ml vs 333(53.4-1707) pg/ml, respectively; p=0.0307; Figure 3B] are both significantly decreased in stable COPD patients compared to**

control smokers with normal lung function without significant changes **in the BAL level of IL-1β [0.22(0.11-1.2) pg/ml vs 0.4(0.13-6.7) pg/ml, respectively; p=0.2214; Figure E6B], IL-18 [13.1(4.7-82.2) pg/ml vs 18(8.4-73.2) pg/ml, COPD vs control smokers respectively; p=0.1063; Figure E6C] and IL-18BPa [78.9(25.2-102.8) pg/ml vs 57.9(35.2-558.4) pg/ml, COPD vs control smokers respectively; p=0.3947; Figure E6D]. In addition, the IL-1β/ILRA [0.002(0.0007-0.007) vs 0.001 (0.0002-0.02), COPD vs control smokers respectively; p=0.6625; Figure E6E] and IL-18/IL-18BPa [0.2(0.06-1) vs 0.3 (0.09-2), COPD vs control smokers respectively; p=0.3710; Figure E6F] ratios and** IL-37 **levels [60(53.4-74.1) pg/ml vs 57.8(44.4-68.1) pg/ml, COPD vs control smokers respectively; p=0.1980; Figure E5D] were not different** between the two group of subjects. The BAL level of IL-33 was under the detection limit of the assay (data not shown) in all subjects.

Correlations between inflammatory cell counts, IL-27 related molecules in the bronchi and clinical parameters

In all smokers there was a positive correlation between the number of IFNγRI+ cells and the number of IL-27+ (R=0.43, p=0.009) and IL-27R+ (R=0.56, p=0.001) cells in the bronchial submucosa. The number of IFNγRI+ cells also correlated with numbers of IL-7Rα+ cells (R=0.62, p=0.0003). Numbers of IL-7Rα+ cells correlated inversely with pre- and post-bronchodilator FEV_1% predicted values (R=-041, p=0.005 and R=-0.42, p=0.013, respectively). Furthermore, the number of IL-7+ cells correlates significantly with the number of IL-27+ (R=0.49, p=0.0007) and IL-27R+ (R=0.43, p=0.003) cells in the bronchial submucosa of all smokers.

When correlations were restricted to patients with COPD alone all reported correlations were maintained. In fact, the number of IFNγRI+ cells correlated again with the number of IL-27+ (R=0.42, p=0.036) and IL-27R+ (R=0.51, p=0.014) cells in the bronchial submucosa (Figure 4, panels a and b). The number of IFNγRI+ cells also correlated with numbers of IL-7Rα (R=0.68, p=0.0009).

Numbers of IL-7Rα correlated inversely with pre- and post-bronchodilator FEV1% predicted values (R=-045, p=0.009 and R=-0.36, p=0.049, respectively). Furthermore, the number of IL-7+ cells correlated significantly with the number of IL-27+ (R=0.43, p=0.010) and IL-27R+ (R=0.51, p=0.003) cells in the bronchial submucosa of COPD patients alone (Figure 4, panels c and d).

No other significant correlations were found between inflammatory cells, innate immunity and inflammasome pathways expression in bronchial mucosa and/or BAL and any clinical parameters.

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IL-27 and inflammasome mRNAs *expression in NHBE cells induced by oxidative and inflammatory stimuli "In vitro"*

Normal human bronchial epithelial (NHBE) cells were stimulated with H_2O_2 (100μM), IL-1β (1ng/ml) and H_2O_2 + IL-1β at the same concentrations as for single treatments, cytomix alone (TNFα, IL-1β and IFNγ each at 50ng/ml) and combined cytomix + H_2O_2 at the same concentrations as for single treatments and quantified the expression of IL-27p28 (**Figure E7A**) and IL-27B (**Figure E7B**) mRNA by qRT-PCR. IL-27B mRNA was significantly increased (Figure **E7B** and **E7C**) after combined treatment with cytomix plus H_2O_2 (n=3, paired T test, $p<0.05$). **The same stimulation did not significantly stimulate the expression of inflammasome-related IL-1β, IL-18 and caspase 1 encoding mRNAs (Figures E8A, E8B and E8C).** The 2hr time point was selected after an initial time-course study was performed (data not shown).

IL-27 protein expression in NHBE cells induced by oxidative and inflammatory stimuli "In vitro"

The levels of IL-27 protein was measured in the supernatant and whole cell extract of NHBE cells, treated with H_2O_2 (100μM), IL-1β (1ng/ml), cytomix (50ng/ml TNFα, IL-1β and IFNγ) and combined IL-1β+H_2O_2 or cytomix+H_2O_2. No IL-27 protein was detected in the supernatant of any of the samples (data not shown). Protein from the whole cell extract showed a small but significant increase in the levels of IL-27 when treated with the combined treatment of cytomix+H_2O_2 s (n=3, paired T test, $p<0.05$) (Figure E9).

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Table E1. Primary antibodies and immunohistochemical conditions used for identification of cytokines, proteins and inflammatory cells

Target	Supplier	[a]Cat#	Source	Dilution	Positive control
CD4	Dako	M716	Mouse	1:100	Human tonsil
CD8	Dako	M7103	Mouse	1:200	Human tonsil
CD68	Dako	M814	Mouse	1:200	Human tonsil
Neutrophil elastase	Dako	M752	Mouse	1:100	Nasal polyp
IL-6	R&D	AF206NA	Goat	1:80	Human tonsil
IL-6 Rα	Diaclone	852.033.020	Mouse	1:20	Human tonsil
IL-7	R&D	MAB207	Mouse	1:40	Nasal polyp
IL-7 Rα	R&D	AF306PB	Goat	1:80	Nasal polyp
IL-10	R&D	AF217NA	Goat	1:100	Nasal polyp
IL-10 Rα	R&D	AF274NA	Goat	1:50	Nasal polyp
IL-27p28	R&D	LS B2565	Rabbit	1:250	Human tonsil
IL-27 R	R&D	ANP1619	Rabbit	1:300	Human tonsil
IL-33	S.Cruz	Sc-98659	Rabbit	1:150	Nasal polyp
ST-2	S.Cruz	Sc-18687	Goat	1:100	Nasal polyp
TSLP	Peprotech	500-P258	Rabbit	1:40	Nasal polyp
TSLP-R	R&D	AF981	Goat	1:25	Nasal polyp
IL-18	S.Cruz	Sc-133127	Mouse	1:50	Nasal polyp
IL-18 Rα	R&D	AF840	Goat	1:15	Nasal polyp
IL-18 Rβ	S.Cruz	Sc-107635	Goat	1:75	Nasal polyp
IL-18 BP	Epitomics	1893-1	Rabbit	1:100	Nasal polyp
NLRP-3	Sigma	C-33680	Rabbit	1:75	Nasal polyp
Caspase-1	Epitomics	3345-1	Rabbit	1:200	Breast Tumor
IL-1β	R&D	AB201NA	Goat	1:150	Human tonsil
IL-1βRI	S.Cruz	Sc-66054	Mouse	1:50	Human tonsil
IL-1βRII	R&D	AF263NA	Goat	1:50	Human tonsil
IL-37	R&D	AF1975	Goat	1:40	Nasal polyp
NALP7 (NLRP7)	Thermo Scientific	PA5-21023	Rabbit	1:400	Nasal polyp
STAT1	Atlas	HPA000931	Rabbit	1:200	Human tonsil
pSTAT1	BD	612133	Mouse	1:80	Human tonsil
IFN-γ	R&D	MAB2851	Mouse	1:100	Human tonsil
IFN-γ RI	R&D	AF673	Goat	1:30	Human tonsil

[a]Cat#, catalogue number

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Table E2. Summary of the ELISA assays performed on BAL supernatants

Specificity	Molecular weight (kDa)	Manufacturer website	Catalogue code	Standard curve sensibility range	
IL-1β	**17.5**	**www.rndsystems.com**	**HSLB00C**	**0-8 pg/ml**	**Not concentrated**
IL-1RA	**22-25**	**www.rndsystems.com**	**DRA00B**	**0-2000 pg/ml**	**Not concentrated**
IL-6	**20-30**	**www.raybiotech.com**	**ELH-IL6-001**	**0-1000 pg/ml**	**Concentrated x30 with Vivaspin**
IL-6sR	50-55	www.biosupply.co.uk	EL10034	0-2000 pg/ml	Concentrated x30 with Vivaspin
sgp130	130	www.rndsystems.com	DGP00	0-8 ng/ml	Concentrated x30 with Vivaspin
IL-7	20-25	www.raybiotech.com	ELH-IL7-001	0-1000 pg/ml	Concentrated x30 with Vivaspin
IL-18	**18-24**	**www.mblintl.com**	**7620**	**0-1000 pg/ml**	**Not concentrated**
IL-18BPa	**22-40**	**www.raybiotech.com**	**ELH-IL18BPA-001**	**0-18.000 pg/ml**	**Not concentrated**
IL-27	**69**	**www.uscnk.us**	**E90385Hu**	**0-1000 pg/ml**	**Concentrated x30 with Vivaspin**
IL-33	**30**	**www.biolegend.com**	**435907**	**0-1000 pg/ml**	**Concentrated x30 with Vivaspin**
IL-37	**30**	**www.adipogen.com**	**AG-45A-0041EK-KI01**	**0-1000 pg/ml**	**Not concentrated**
ST2/IL-1R4	**60**	**www.rndsystems.com**	**DST200**	**0-2000 pg/ml**	**Concentrated x30 with Vivaspin**
HMGB1	**30**	**www.ibl-international.com**	**ST51011**	**0-80 ng/ml**	**Not concentrated**

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Table E3. Immunohistochemical quantification of inflammatory cells in the bronchial mucosa

Target Group					
	Control non-smokers	**Control smokers normal lung function**	**COPD stage I/II (mild/moderate)**	**COPD stage III/IV (severe/very severe)**	***p* value**
Submucosa (cells/mm^2)					
CD4	168 (88-378)	218 (37-500)	245 (86-731)	252 (42-671)	0.360
CD8	120 (15-301)	187 (78-657)	208 (86-523) #	215 (59-355) #	0.125
CD68	284 (110-516)	369 (97-945)	566 (158-833) #	428 (204-1054) #	0.130
Neutrophil Elastase	89 (58-179)	101 (17-308)	125 (28-512)	173 (47-500) #ε	0.030

Abbreviations: COPD, chronic obstructive pulmonary disease; Data expressed as median (range). Statistics: Kruskal-Wallis test for multiple comparisons **were performed and the "p" values across groups shown**. For comparison between groups the Mann-Whitney U test was applied: $^{\#}p<0.05$, significantly different from control non-smokers; $^{\varepsilon}p<0.05$, significantly different from control smokers with normal lung function.

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Data supplement E-Figures

Figure E1

Photomicrographs showing the bronchial mucosa from (a) control non-smoker, (b) control healthy smoker with normal lung function, (c) mild/moderate stable chronic obstructive pulmonary disease (COPD) and (d) severe stable COPD immunostained for identification of IL-7+ cells (arrows) in the bronchial submucosa. Results are representative of those from 11 non-smokers, 12 healthy smokers, 14 mild/moderate COPD and 18 with severe COPD. Bar=30 microns.

Figure E2

Photomicrographs showing the bronchial mucosa from (a) control non-smoker, (b) control healthy smoker with normal lung function, (c) mild/moderate stable chronic obstructive pulmonary disease (COPD) and (d) severe stable COPD immunostained for identification of pSTAT1+ cells (arrows) in the bronchial submucosa. Results are representative of those from 11 non-smokers, 12 healthy smokers, 14 mild/moderate COPD and 18 with severe COPD. Bar=30 microns.

Figure E3

Photomicrographs showing the bronchial mucosa from (a) control non-smoker, (b) control healthy smoker with normal lung function, (c) mild/moderate stable chronic obstructive pulmonary disease (COPD) and (d) severe stable COPD immunostained for identification of NALP7+ cells (arrows) in the bronchial epithelium and submucosa. Results are representative of those from 11 non-smokers, 12 healthy smokers, 14 mild/moderate COPD and 18 with severe COPD. Bar=30 microns.

Figure E4

Representative double-labelled confocal fluorescence images showing double staining for CD68+ macrophages and IL-27 in the bronchial mucosa from four healthy smokers with normal lung function (A-B) and four patients with stable chronic obstructive pulmonary disease (COPD) (C-D). Images A and C were obtained from one healthy control smoker and one patient with severe COPD, respectively. Arrows in panel C indicate double stained cells. Images B and D show the co-expression levels of IL-27 (Alexa Fluor 488-green) and CD68 (Alexa Fluor 647-red) and represent the correlation cytofluorogram of the images in A and C, respectively.

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Figure E5

BAL supernatant levels of (A) IL-6R, (B) IL-7, (C) IL-27 and (D) IL-37 in stable COPD patients **(n=15)** compared to the control **smokers with normal lung function (n=14).** Exact p values are shown above each graph.

Figure E6

BAL supernatant levels of (A) sgp130, (B) IL-1β, (C) IL-18, (D) IL-18BPa and (respectively E and F) their IL-1β/IL.1RA and IL-18/IL-18BPa ratios in stable COPD patients (n=15) compared to the control smokers with normal lung function (n=14). Exact p values are shown above each graph.

Figure E7.

In vitro expression of IL-27p28 (A) and IL-27B (B and C) in normal primary human bronchial epithelial cells treated with H_2O_2 (100μM), IL-1β (1ng/ml), cytomix (50ng/ml TNFα, IL-1β and IFNγ) and combined IL-1β + H_2O_2 or cytomix + H_2O_2. Combined treatment with cytomix + H_2O_2 significantly up-regulated the mRNA expression of IL-27B (B and C) (n=3, paired T test, $p<0.05$). Panel C shows a direct comparison between results from 3 subjects to indicate level of variability.

Figure E8

***In vitro* expression of expression of the inflammasone-related genes (*IL1B*, *IL18* and *CASP1*) in normal human bronchial epithelial cells were measured in cells treated with H_2O_2 (100μM), IL-1β (1ng/ml), cytomix (50ng/ml TNFα, IL-1β and IFNγ) and combined IL-1β + H_2O_2 or cytomix + H_2O_2. Whilst treatment with cytomix alone did significantly increase *IL1B* gene expression (n=3, paired T test, $p<0.05$) no other gene or treatment showed significant increase in expression relative to the untreated controls.**

Figure E9

***In vitro* expression of IL-27 protein in normal primary human bronchial epithelial cells treated with H_2O_2 (100μM), IL-1β (1ng/ml), cytomix (50ng/ml TNFα, IL-1β and IFNγ) and combined IL-1β+H_2O_2 or cytomix+H_2O_2. Combined treatment with cytomix+H_2O_2 significantly up-regulated the protein expression of IL-27 in the whole cell extract (n=3, paired T test, $p<0.05$). Panel B shows a direct comparison between results from 3 subjects to indicate level of variability.**

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Innate immunity but not NLRP3 inflammasome activation correlates with severity of stable COPD

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Methods

Subjects

Subjects were recruited from the Respiratory Medicine Unit of the "Fondazione Salvatore Maugeri" (Veruno, Italy), the Section of Respiratory Diseases of the University Hospital of Ferrara, Italy and the Section of Respiratory Diseases of the University Hospital of Katowice, Poland for immunohistochemistry and ELISA experiments. The severity of the airflow obstruction was staged using GOLD criteria [**www.goldcopd.org**]. All former smokers had stopped smoking for at least one year. COPD and chronic bronchitis were defined, according to international guidelines, as follows: COPD, presence of a post-bronchodilator forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) ratio <70%; chronic bronchitis, presence of cough and sputum production for at least 3 months in each of two consecutive years (E1). All COPD patients were stable with no previous exacerbation in the six months before bronchoscopy. None of the subjects was treated with theophylline, antibiotics, antioxidants, mucolytics, and/or glucocorticoids in the month prior bronchoscopy. **COPD patients were using short-acting inhaled β_2-agonists (SABA) or short-acting inhaled antimuscarinics (SAMA) prn or regular long-acting inhaled β_2-agonists (LABA) and/or regular inhaled anticholinergics, including SAMA or long-acting inhaled antimuscarinics (LAMA) at the dosage recommended in current COPD guidelines (www.goldcopd.org) at the time of their recruitment**. The study conformed to the Declaration of Helsinki and was approved by the ethics committees of the Fondazione Salvatore Maugeri [Veruno (Novara), Italy], the University Hospital of Ferrara, Italy and the University Hospital of Katowice, Poland. Written informed consent was obtained from each subject and bronchial biopsies and BAL were performed according to the local ethic committee guidelines.

We obtained and studied bronchial biopsies from 55 subjects: 32 had a diagnosis of COPD in a stable clinical state (E2), 12 were current or ex-smokers with normal lung function, and 11 were non-smokers with normal lung function (Table 1 **of the main manuscript**). **Two out of 12 current or ex-smokers were pure volunteers. All the other control subjects, smokers, ex-smokers and non-smokers, attended our respiratory clinic mostly for of unexplained persistent cough. At their first visit they were screened and after exclusion of the inappropriate subjects determined by the clinical history, physical examination, lung function and imaging studies and laboratory examinations for lung, upper airways and/or cardiac or systemic disease, subjects were recruited.** COPD patients were divided in two groups, according to the grade of severity of their airflow limitation (mild/moderate, grade I-II; or severe/very severe, grade III-IV; n=14 and 18, respectively) (E1). Subjects in all four groups were age-matched. The smoking history was similar in the three smoker groups: mild/moderate and severe/very severe COPD, and healthy

smokers with normal lung function. 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). Forty-**three** percent (n=1**4**) of the total COPD patients and **41**% (n=**5**) 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.

The clinical details of the subjects undergoing bronchoalveolar lavage (BAL) are summarized in Table 2 **of the main manuscript**. **We analyzed the BAL fluid obtained from 26 COPD and 18 control smokers with normal lung function but due to** the necessity to concentrate the BAL supernatants for many ELISA assays **we were unable to perform all these assays in all subjects**. **The results provided for each ELISA are the data from 15 COPD and 14 control smokers with normal lung function.**

Of the 44 subjects included for the bronchoalveolar lavage (BAL) analysis, 21 patients were recruited in the Veruno's Hospital and 23 subjects in Poland and Ferrara. All the BAL procedures were well standardized in accordance with standard guidelines. All the bronchoscopists in the three centers followed the same SOP for BAL collection and processing and the ELISAs on the BAL supernatants were run in a single center (Ferrara).

Lung function tests and volumes

Pulmonary function tests were performed as previously described (E2) according to published guidelines (E3). 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, CA). In order to assess the reversibility of airflow obstruction and post bronchodilator functional values the FEV_1 and FEV_1/FVC% measurements in the groups of subjects with FEV_1/FVC%≤70% pre-bronchodilator was repeated 20 min after the inhalation of 0.4 mg of albuterol.

Fiberoptic bronchoscopy, collection and processing of bronchial biopsies

Subjects were at the bronchoscopy suite at 8.30 AM after having fasted from midnight and were pre-treated with atropine (0.6 mg IV) and midazolam (5-10 mg IV). 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

fiberoptic 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 four 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 extracted from the forceps and processed for light microscopy as previously described (E2). Two samples were embedded in Tissue Tek II OCT (Miles Scientific, Naperville, IL), frozen within 15 min in isopentane pre-cooled in liquid nitrogen, and stored at –80°C. The best frozen sample was then oriented and 6μm thick cryostat sections were cut for immunohistochemical light microscopy analysis and processed as described below.

Immunohistochemistry

Two sections from each sample were stained applying immunohistochemical methods with a panel of antibodies specific for inflammatory cells, innate immune mediators and inflammasome components (Table E1). 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 (0.15M saline containing 0.05 M TRIS-hydrochloric acid at pH 7.6) and incubated for 1hr at room temperature in a humid chamber. Antibody binding was demonstrated with secondary antibodies anti-mouse (Vector, BA 2000), anti-rabbit (Vector, BA 1000) or anti-goat (Vector, BA 5000) followed by ABC kit AP AK5000, Vectastain and fast-red substrate (red color) or ABC kit HRP Elite, PK6100, Vectastain and diaminobenzidine substrate (brown color). Slides were included in each staining run using human tonsil, nasal polyp or breast cancer, as a positive control. For the negative control slides, normal non-specific goat, mouse or rabbit immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at the same protein concentration as the primary antibody.

Scoring system for immunohistochemistry

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 630x.

The immunostaining for all the antigens studied was scored (range: 0 = absence of immunostaining to 3 = extensive intense immunostaining) in the intact (columnar and basal epithelial cells) bronchial epithelium, as previously described (E2). The final result was expressed as the average of

all scored fields performed in each biopsy. A mean±SD of 0.70±0.26 millimeters of epithelium was analyzed in COPD patients and control subjects.

Immunostained cells in bronchial submucosa (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 millimeter, was calculated as the average of all the cellular counts performed in each biopsy.

Double staining and confocal microscopy

Four patients with COPD (FEV_1, 64±15%; FEV_1/FVC, 61±8%) and 4 control smokers (FEV_1, 104±14%; FEV_1/FVC, 81±3%) were used for double staining immunofluorescence and confocal microscopy. Double staining was performed as previously reported (E2). For confocal microscopy sections were fixed with 4% paraformaldehyde, washed with phosphate buffered saline (PBS) and incubated (1 hour) with PBS containing 5% bovine serum albumin and 5% donkey serum. After blocking, sections were incubated 1 hour with the primary antibodies diluted as indicated in Table E1 in PBS containing 5% bovine serum albumin. The following antibodies were used: rabbit anti-human IL-27 (LS B2565, R&D); and mouse anti human CD4, CD8 and CD68, M716, M7103 and M814, respectively, Dako). After washing with PBS, the preparations were incubated for a further 30 min with the appropriate secondary Alexa Fluor 488- or Alexa Fluor 647 conjugated antibodies diluted 1:200 in PBS. Negative controls included non-specific mouse and rabbit immunoglobulins revealed as for primary antibodies. Slides were mounted using a specific mounting medium (Vector, H-1400, Vectashield Hard Set). The slides for confocal microscopy were analysed using a three-channel Leica TCS SP5 laser scanning confocal microscope. The Leica LCS software package was used for acquisition, storage, and visualization. The quantitative estimation of co-localized proteins was performed calculating the "co-localization coefficients" (E4, E5).

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed from the right middle lobe using four successive aliquots of 50ml of 0.9% NaCl. BAL cells were spun (500×*g*; 10min) and washed twice with Hanks' buffered salt solution (HBSS). Cytospins were prepared and stained with May-Grünwald stain for differential cell counts. Cell viability was assessed using the trypan blue exclusion method. BAL supernatants were aliquoted and left at -80°C before its use for the ELISA assays summarised in Table E2. These assays have been performed according to the manufacturers' instructions.

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Cell culture and treatments

We used normal human bronchial epithelial cells (NHBE), of **non-smoking subjects** obtained from Lonza (Cologne, Germany) grown in BEGM media (Lonza) with Singlequot supplement (Lonza) following the suppliers instructions. Cells were passaged using the ReagentPack ™ Subculture Reagents (Lonza) following the manufacturer's instructions. Passage number of cells used in this study ranged from 3 to 6. Prior to all the experiments, 70-80% cell monolayers were incubated in supplement–free medium for the 18hrs. The cells were treated with hydrogen peroxide (H_2O_2, 100μM), IL-1β (1 ng/ml), combined IL-1β and H_2O_2, cytomix (50ng/ml of TNFα, IL-1β and IFNγ) or combined cytomix and H_2O_2 for 2hrs **to analyse the messenger RNAs or 24 hours to measure protein expression**. All experiments were performed at least three times.

Extraction and quantification of RNA and qRT-PCR from NHBE

Total RNA was isolated from cells using the RNeasy RNA extraction kit following manufacturer's instructions (Qiagen, UK). cDNA was made from quantified RNA by reverse transcription using the high capacity cDNA kit following manufacturer's instructions (Applied Biosystems, UK). The expression of genes of interest was measured using Syber green (Qiagen, UK) for qPCR in a Corbett Rotorgene 6 (Corbett, Cambridge, UK). We detected the expression of IL-27p28 and EBI3 (IL-27B) using the following primers (E6): IL-27p28 forward, agc tgc atc ctc tcc atg tt; reverse, gag cag ctc cct gat gtt tc; EBI3 (IL-27B) forward, tgt tct cca tgg ctc cct ac; reverse, gct ccc tga cgc ttg taa c. mRNA was normalized using a housekeeping gene 18S, using the following primers 18S forward ctt aga ggg aca agt ggc g; reverse acg ctg agc cag tca gtg ta, for each experimental condition.

Extraction and quantification of IL-27 protein from NHBE

IL-27 levels in the cell culture supernatant following stimulation with H_2O_2 (100μM), IL-1β (1 ng/ml), combined IL-1β and H_2O_2, cytomix (50ng/ml of TNFα, IL-1β and IFNγ) or combined cytomix and H_2O_2 for 24 hours was quantified by sandwich ELISA (R&D Systems Europe, Abingdon, UK) exactly according to the manufacturer`s instructions.

Statistical analysis

Group data were expressed as mean (standard deviation) for functional data or median (range) or interquartile range (IQR) for morphologic data. **We tested for a normal distribution for functional data (i.e. FEV1%, FVC, age etc.) and for a non normal distribution for morphological parameters. Normality tests were performed on all group data. The Grubb's**

outlier test was used to determine whether individual values were outside the rest of the group. Then we applied the analysis of variance (ANOVA) for functional data in comparing subgroups of patients and control subjects for functional data. The non parametric Kruskal Wallis test was applied for multiple comparisons, without application of Bonferroni correction, when morphologic data were analysed followed by the Mann-Whitney U test for comparison between groups. The statistical guide to GraphPad Prism recommends that the Bonferroni correction should not be used when comparing more than 5 variables due to the conservative nature of the test and the subsequent likeliness of missing real differences. We believe that this comparative analysis is of value and represents part of our informative findings. For this reason we applied specific non parametric statistical tests to our data of Table 3 without including the Bonferroni correction. To verify the degree of association between functional or morphological parameters, in all smokers with and without COPD or in smokers with COPD alone the correlation coefficients between functional and morphological and morphological-morphological data 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, CA-USA) **and GraphPad Prism software (www.graphpad.com/scientific-software/prism/).**

Results

Measurement of inflammatory cells in the bronchial submucosa

The results are summarized in Table E3. The number of CD8 positive lymphocytes was significantly increased in severe/very severe (p=0.021) and mild/moderate (p=0.027) stable COPD compared to control non-smokers. The number of CD4 positive lymphocytes did not differ significantly between the four groups of subjects. Compared with control non-smokers, the number of CD68 positive macrophages was significantly higher in severe/very severe (p=0.033) and mild/moderate (p=0.036) stable COPD. The number of neutrophils was also significantly higher in severe/very severe stable COPD patients compared with control smokers (p=0.011) and non-smokers (p=0.010). Stable COPD patients with chronic bronchitis had a similar number of neutrophils when compared with stable COPD patients without chronic bronchitis (data not shown).

Immunohistochemistry for innate immunity and inflammasome pathways in the bronchial submucosa

For all the proteins studied mononuclear cells (lymphocytes and macrophages) and endothelial cells were the most represented immunostained cells in the submucosa. The number of IFNγ+ cells was significantly higher in mild/moderate (p=0.010) and severe (p=0.008) COPD compared to control non-smokers, confirming previously reported data (E2). The number of IFNγRI+ cells was also increased in severe COPD compared to mild COPD (p=0.031), control smokers (p=0.0035) and control non-smokers (p=0.006). IL-18Rβ showed a slight increase in severe COPD compared to mild/moderate COPD (p=0.045) and control smokers (p=0.039) but did not differ in comparison with control non-smokers. The number of IL-7+ (Figure E1) cells was higher in severe (p=0.008), mild/moderate COPD (p=0.010) and in control smokers (p=0.012) compared to control non-smokers. In addition the number of IL-7Rα+ cells was significantly higher in severe compared to mild/moderate COPD (p=0.040), control smokers (p=0.009) and control non-smokers (p=0.002). IL-10 was poorly expressed but number of IL-10+ cells was higher in severe (p=0.005), mild/moderate COPD (p=0.047) and in control smokers (p=0.054) in comparison with control non-smokers.

The number of IL-27+ (Figure 1) as well as pSTAT1+ (Figure E2) cells was significantly higher in severe COPD (p=0.032 and p=0.018, respectively) compared to control smokers but did not differ in comparison with the other groups. Interestingly, compared to control smokers and non-smokers, the number of IL-27R+ cells was higher in severe (p=0.010 and p=0.002, respectively) and mild/moderate COPD (p=0.054 and p=0.009, respectively). Similarly, the number of total STAT1+

cells was significantly higher in severe (p=0.0043 and p=0.015, respectively) and mild/moderate COPD (p=0.022 and p=0.029, respectively).

No significant differences were observed for IL-1β, IL-1βRI, IL-1βRII, caspase-1, IL-18, IL-18Rα, IL-18BP, NLRP3, IL-6, IL-6Rα, IL-10Rα, IL-33, ST-2 and TSLP immunostaining in the four groups of subjects examined. However, caspase-1 staining was observed in the positive control represented by human breast tumour tissue (Table E1).

Double staining and confocal microscopy

Double staining for identification of CD4+, CD8+ lymphocytes and macrophages (CD68+) co-expressing IL-27 was performed in four representative healthy smokers with normal lung function and in four patients with COPD. We found no difference in the percentage (mean±SD) of CD4+IL-27+ double stained cells between control smokers (12±2%) and COPD patients (11±6%). Similarly, there was no difference in the percentage of CD8+IL-27+ double stained cells between control smokers (14±11%) and COPD patients (9±2%). Interestingly, the percentage of CD68+IL-27+ double stained cells was significantly increased in COPD patients (34±8%) compared to control smokers (8±2%, p=0.0209) (Figure E4).

ELISA assays in the BAL supernatants

The BAL levels of IL-6 were significantly increased in stable COPD patients compared to the control smokers **with normal lung function [median (range) 99.4(54.8-489.6) pg/ml vs 36.6 (28.2-76-8) pg/ml, respectively; p=0.0001; Figure 3A),** without a significant change in sIL-6R expression between the two groups **[127.2(28-435-7) pg/ml vs 197.1(21.7-843-2) pg/ml, respectively; p=0.2658; Figure E5A].**

The BAL levels of IL-7 **[9.4(5-35.9) pg/ml vs 9.1(2.5-99.7) pg/ml, respectively; p=0.8273; Figure E5B], IL-27 [4.3(0.3-64.6) pg/ml vs 15.1(0.8-248.1) pg/ml, respectively; p=0.112; Figure E5C]** and **sgp130 [3213(278.4-9350) pg/ml vs 5663(453.2-11660) pg/ml, respectively; p=0.2850; Figure E6A]** were not significantly different between the two group of subjects. However, the BAL level of HMGB1 was significantly decreased in stable COPD patients compared to control smokers **with normal lung function [2340 (314-3550) pg/ml vs 5105 (0-103521) pg/ml, COPD vs control smokers respectively; p=0.0174; Figure 3C]. However, this difference was lost after removal of the outliers.**

The BAL level of soluble ST2 **[55(30.3-563) pg/ml vs 168.5(57-1770) pg/ml, respectively; p=0.0073; Figure 3D] and IL-1RA [147(26.7-390) pg/ml vs 333(53.4-1707) pg/ml, respectively; p=0.0307; Figure 3B] are both significantly decreased in stable COPD patients compared to**

control smokers with normal lung function without significant changes **in the BAL level of IL-1β [0.22(0.11-1.2) pg/ml vs 0.4(0.13-6.7) pg/ml, respectively; p=0.2214; Figure E6B], IL-18 [13.1(4.7-82.2) pg/ml vs 18(8.4-73.2) pg/ml, COPD vs control smokers respectively; p=0.1063; Figure E6C] and IL-18BPa [78.9(25.2-102.8) pg/ml vs 57.9(35.2-558.4) pg/ml, COPD vs control smokers respectively; p=0.3947; Figure E6D]. In addition, the IL-1β/ILRA [0.002(0.0007-0.007) vs 0.001 (0.0002-0.02), COPD vs control smokers respectively; p=0.6625; Figure E6E] and IL-18/IL-18BPa [0.2(0.06-1) vs 0.3 (0.09-2), COPD vs control smokers respectively; p=0.3710; Figure E6F] ratios and** IL-37 **levels [60(53.4-74.1) pg/ml vs 57.8(44.4-68.1) pg/ml, COPD vs control smokers respectively; p=0.1980; Figure E5D] were not different** between the two group of subjects. The BAL level of IL-33 was under the detection limit of the assay (data not shown) in all subjects.

Correlations between inflammatory cell counts, IL-27 related molecules in the bronchi and clinical parameters

In all smokers there was a positive correlation between the number of IFNγRI+ cells and the number of IL-27+ (R=0.43, p=0.009) and IL-27R+ (R=0.56, p=0.001) cells in the bronchial submucosa. The number of IFNγRI+ cells also correlated with numbers of IL-7Rα+ cells (R=0.62, p=0.0003). Numbers of IL-7Rα+ cells correlated inversely with pre- and post-bronchodilator FEV_1% predicted values (R=-041, p=0.005 and R=-0.42, p=0.013, respectively). Furthermore, the number of IL-7+ cells correlates significantly with the number of IL-27+ (R=0.49, p=0.0007) and IL-27R+ (R=0.43, p=0.003) cells in the bronchial submucosa of all smokers.

When correlations were restricted to patients with COPD alone all reported correlations were maintained. In fact, the number of IFNγRI+ cells correlated again with the number of IL-27+ (R=0.42, p=0.036) and IL-27R+ (R=0.51, p=0.014) cells in the bronchial submucosa (Figure 4, panels a and b). The number of IFNγRI+ cells also correlated with numbers of IL-7Rα (R=0.68, p=0.0009).

Numbers of IL-7Rα correlated inversely with pre- and post-bronchodilator FEV1% predicted values (R=-045, p=0.009 and R=-0.36, p=0.049, respectively). Furthermore, the number of IL-7+ cells correlated significantly with the number of IL-27+ (R=0.43, p=0.010) and IL-27R+ (R=0.51, p=0.003) cells in the bronchial submucosa of COPD patients alone (Figure 4, panels c and d).

No other significant correlations were found between inflammatory cells, innate immunity and inflammasome pathways expression in bronchial mucosa and/or BAL and any clinical parameters.

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IL-27 and inflammasome mRNAs *expression in NHBE cells induced by oxidative and inflammatory stimuli "In vitro"*

Normal human bronchial epithelial (NHBE) cells were stimulated with H_2O_2 (100μM), IL-1β (1ng/ml) and H_2O_2 + IL-1β at the same concentrations as for single treatments, cytomix alone (TNFα, IL-1β and IFNγ each at 50ng/ml) and combined cytomix + H_2O_2 at the same concentrations as for single treatments and quantified the expression of IL-27p28 (**Figure E7A**) and IL-27B (**Figure E7B**) mRNA by qRT-PCR. IL-27B mRNA was significantly increased (Figure **E7B** and **E7C**) after combined treatment with cytomix plus H_2O_2 (n=3, paired T test, $p<0.05$). **The same stimulation did not significantly stimulate the expression of inflammasome-related IL-1β, IL-18 and caspase 1 encoding mRNAs (Figures E8A, E8B and E8C).** The 2hr time point was selected after an initial time-course study was performed (data not shown).

IL-27 protein expression in NHBE cells induced by oxidative and inflammatory stimuli "In vitro"

The levels of IL-27 protein was measured in the supernatant and whole cell extract of NHBE cells, treated with H_2O_2 (100μM), IL-1β (1ng/ml), cytomix (50ng/ml TNFα, IL-1β and IFNγ) and combined IL-1β+H_2O_2 or cytomix+H_2O_2. No IL-27 protein was detected in the supernatant of any of the samples (data not shown). Protein from the whole cell extract showed a small but significant increase in the levels of IL-27 when treated with the combined treatment of cytomix+H_2O_2 s (n=3, paired T test, $p<0.05$) (Figure E9).

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Table E1. Primary antibodies and immunohistochemical conditions used for identification of cytokines, proteins and inflammatory cells

Target	Supplier	[a]Cat#	Source	Dilution	Positive control
CD4	Dako	M716	Mouse	1:100	Human tonsil
CD8	Dako	M7103	Mouse	1:200	Human tonsil
CD68	Dako	M814	Mouse	1:200	Human tonsil
Neutrophil elastase	Dako	M752	Mouse	1:100	Nasal polyp
IL-6	R&D	AF206NA	Goat	1:80	Human tonsil
IL-6 Rα	Diaclone	852.033.020	Mouse	1:20	Human tonsil
IL-7	R&D	MAB207	Mouse	1:40	Nasal polyp
IL-7 Rα	R&D	AF306PB	Goat	1:80	Nasal polyp
IL-10	R&D	AF217NA	Goat	1:100	Nasal polyp
IL-10 Rα	R&D	AF274NA	Goat	1:50	Nasal polyp
IL-27p28	R&D	LS B2565	Rabbit	1:250	Human tonsil
IL-27 R	R&D	ANP1619	Rabbit	1:300	Human tonsil
IL-33	S.Cruz	Sc-98659	Rabbit	1:150	Nasal polyp
ST-2	S.Cruz	Sc-18687	Goat	1:100	Nasal polyp
TSLP	Peprotech	500-P258	Rabbit	1:40	Nasal polyp
TSLP-R	R&D	AF981	Goat	1:25	Nasal polyp
IL-18	S.Cruz	Sc-133127	Mouse	1:50	Nasal polyp
IL-18 Rα	R&D	AF840	Goat	1:15	Nasal polyp
IL-18 Rβ	S.Cruz	Sc-107635	Goat	1:75	Nasal polyp
IL-18 BP	Epitomics	1893-1	Rabbit	1:100	Nasal polyp
NLRP-3	Sigma	C-33680	Rabbit	1:75	Nasal polyp
Caspase-1	Epitomics	3345-1	Rabbit	1:200	Breast Tumor
IL-1β	R&D	AB201NA	Goat	1:150	Human tonsil
IL-1βRI	S.Cruz	Sc-66054	Mouse	1:50	Human tonsil
IL-1βRII	R&D	AF263NA	Goat	1:50	Human tonsil
IL-37	R&D	AF1975	Goat	1:40	Nasal polyp
NALP7 (NLRP7)	Thermo Scientific	PA5-21023	Rabbit	1:400	Nasal polyp
STAT1	Atlas	HPA000931	Rabbit	1:200	Human tonsil
pSTAT1	BD	612133	Mouse	1:80	Human tonsil
IFN-γ	R&D	MAB2851	Mouse	1:100	Human tonsil
IFN-γ RI	R&D	AF673	Goat	1:30	Human tonsil

[a]Cat#, catalogue number

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Table E2. Summary of the ELISA assays performed on BAL supernatants

Specificity	Molecular weight (kDa)	Manufacturer website	Catalogue code	Standard curve sensibility range	
IL-1β	**17.5**	**www.rndsystems.com**	**HSLB00C**	**0-8 pg/ml**	**Not concentrated**
IL-1RA	**22-25**	**www.rndsystems.com**	**DRA00B**	**0-2000 pg/ml**	**Not concentrated**
IL-6	**20-30**	**www.raybiotech.com**	**ELH-IL6-001**	**0-1000 pg/ml**	**Concentrated x30 with Vivaspin**
IL-6sR	50-55	www.biosupply.co.uk	EL10034	0-2000 pg/ml	Concentrated x30 with Vivaspin
sgp130	130	www.rndsystems.com	DGP00	0-8 ng/ml	Concentrated x30 with Vivaspin
IL-7	20-25	www.raybiotech.com	ELH-IL7-001	0-1000 pg/ml	Concentrated x30 with Vivaspin
IL-18	**18-24**	**www.mblintl.com**	**7620**	**0-1000 pg/ml**	**Not concentrated**
IL-18BPa	**22-40**	**www.raybiotech.com**	**ELH-IL18BPA-001**	**0-18.000 pg/ml**	**Not concentrated**
IL-27	**69**	**www.uscnk.us**	**E90385Hu**	**0-1000 pg/ml**	**Concentrated x30 with Vivaspin**
IL-33	**30**	**www.biolegend.com**	**435907**	**0-1000 pg/ml**	**Concentrated x30 with Vivaspin**
IL-37	**30**	**www.adipogen.com**	**AG-45A-0041EK-KI01**	**0-1000 pg/ml**	**Not concentrated**
ST2/IL-1R4	**60**	**www.rndsystems.com**	**DST200**	**0-2000 pg/ml**	**Concentrated x30 with Vivaspin**
HMGB1	**30**	**www.ibl-international.com**	**ST51011**	**0-80 ng/ml**	**Not concentrated**

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Table E3. Immunohistochemical quantification of inflammatory cells in the bronchial mucosa

Target Group					
	Control non-smokers	**Control smokers normal lung function**	**COPD stage I/II (mild/moderate)**	**COPD stage III/IV (severe/very severe)**	***p* value**
Submucosa (cells/mm^2)					
CD4	168 (88-378)	218 (37-500)	245 (86-731)	252 (42-671)	0.360
CD8	120 (15-301)	187 (78-657)	208 (86-523) #	215 (59-355) #	0.125
CD68	284 (110-516)	369 (97-945)	566 (158-833) #	428 (204-1054) #	0.130
Neutrophil Elastase	89 (58-179)	101 (17-308)	125 (28-512)	173 (47-500) #ε	0.030

Abbreviations: COPD, chronic obstructive pulmonary disease; Data expressed as median (range). Statistics: Kruskal-Wallis test for multiple comparisons **were performed and the "p" values across groups shown**. For comparison between groups the Mann-Whitney U test was applied: $^{\#}p<0.05$, significantly different from control non-smokers; $^{\varepsilon}p<0.05$, significantly different from control smokers with normal lung function.

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Data supplement E-Figures

Figure E1

Photomicrographs showing the bronchial mucosa from (a) control non-smoker, (b) control healthy smoker with normal lung function, (c) mild/moderate stable chronic obstructive pulmonary disease (COPD) and (d) severe stable COPD immunostained for identification of IL-7+ cells (arrows) in the bronchial submucosa. Results are representative of those from 11 non-smokers, 12 healthy smokers, 14 mild/moderate COPD and 18 with severe COPD. Bar=30 microns.

Figure E2

Photomicrographs showing the bronchial mucosa from (a) control non-smoker, (b) control healthy smoker with normal lung function, (c) mild/moderate stable chronic obstructive pulmonary disease (COPD) and (d) severe stable COPD immunostained for identification of pSTAT1+ cells (arrows) in the bronchial submucosa. Results are representative of those from 11 non-smokers, 12 healthy smokers, 14 mild/moderate COPD and 18 with severe COPD. Bar=30 microns.

Figure E3

Photomicrographs showing the bronchial mucosa from (a) control non-smoker, (b) control healthy smoker with normal lung function, (c) mild/moderate stable chronic obstructive pulmonary disease (COPD) and (d) severe stable COPD immunostained for identification of NALP7+ cells (arrows) in the bronchial epithelium and submucosa. Results are representative of those from 11 non-smokers, 12 healthy smokers, 14 mild/moderate COPD and 18 with severe COPD. Bar=30 microns.

Figure E4

Representative double-labelled confocal fluorescence images showing double staining for CD68+ macrophages and IL-27 in the bronchial mucosa from four healthy smokers with normal lung function (A-B) and four patients with stable chronic obstructive pulmonary disease (COPD) (C-D). Images A and C were obtained from one healthy control smoker and one patient with severe COPD, respectively. Arrows in panel C indicate double stained cells. Images B and D show the co-expression levels of IL-27 (Alexa Fluor 488-green) and CD68 (Alexa Fluor 647-red) and represent the correlation cytofluorogram of the images in A and C, respectively.

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Figure E5

BAL supernatant levels of (A) IL-6R, (B) IL-7, (C) IL-27 and (D) IL-37 in stable COPD patients **(n=15)** compared to the control **smokers with normal lung function (n=14).** Exact p values are shown above each graph.

Figure E6

BAL supernatant levels of (A) sgp130, (B) IL-1β, (C) IL-18, (D) IL-18BPa and (respectively E and F) their IL-1β/IL.1RA and IL-18/IL-18BPa ratios in stable COPD patients (n=15) compared to the control smokers with normal lung function (n=14). Exact p values are shown above each graph.

Figure E7.

In vitro expression of IL-27p28 (A) and IL-27B (B and C) in normal primary human bronchial epithelial cells treated with H_2O_2 (100μM), IL-1β (1ng/ml), cytomix (50ng/ml TNFα, IL-1β and IFNγ) and combined IL-1β + H_2O_2 or cytomix + H_2O_2. Combined treatment with cytomix + H_2O_2 significantly up-regulated the mRNA expression of IL-27B (B and C) (n=3, paired T test, $p<0.05$). Panel C shows a direct comparison between results from 3 subjects to indicate level of variability.

Figure E8

***In vitro* expression of expression of the inflammasone-related genes (*IL1B*, *IL18* and *CASP1*) in normal human bronchial epithelial cells were measured in cells treated with H_2O_2 (100μM), IL-1β (1ng/ml), cytomix (50ng/ml TNFα, IL-1β and IFNγ) and combined IL-1β + H_2O_2 or cytomix + H_2O_2. Whilst treatment with cytomix alone did significantly increase *IL1B* gene expression (n=3, paired T test, $p<0.05$) no other gene or treatment showed significant increase in expression relative to the untreated controls.**

Figure E9

***In vitro* expression of IL-27 protein in normal primary human bronchial epithelial cells treated with H_2O_2 (100μM), IL-1β (1ng/ml), cytomix (50ng/ml TNFα, IL-1β and IFNγ) and combined IL-1β+H_2O_2 or cytomix+H_2O_2. Combined treatment with cytomix+H_2O_2 significantly up-regulated the protein expression of IL-27 in the whole cell extract (n=3, paired T test, $p<0.05$). Panel B shows a direct comparison between results from 3 subjects to indicate level of variability.**

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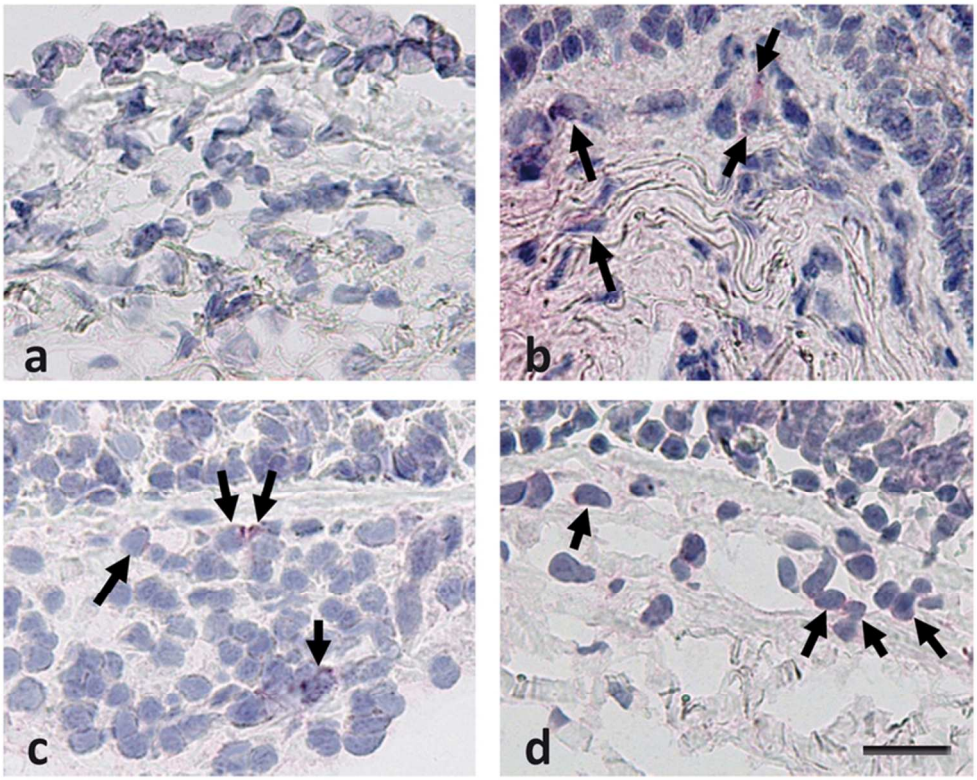

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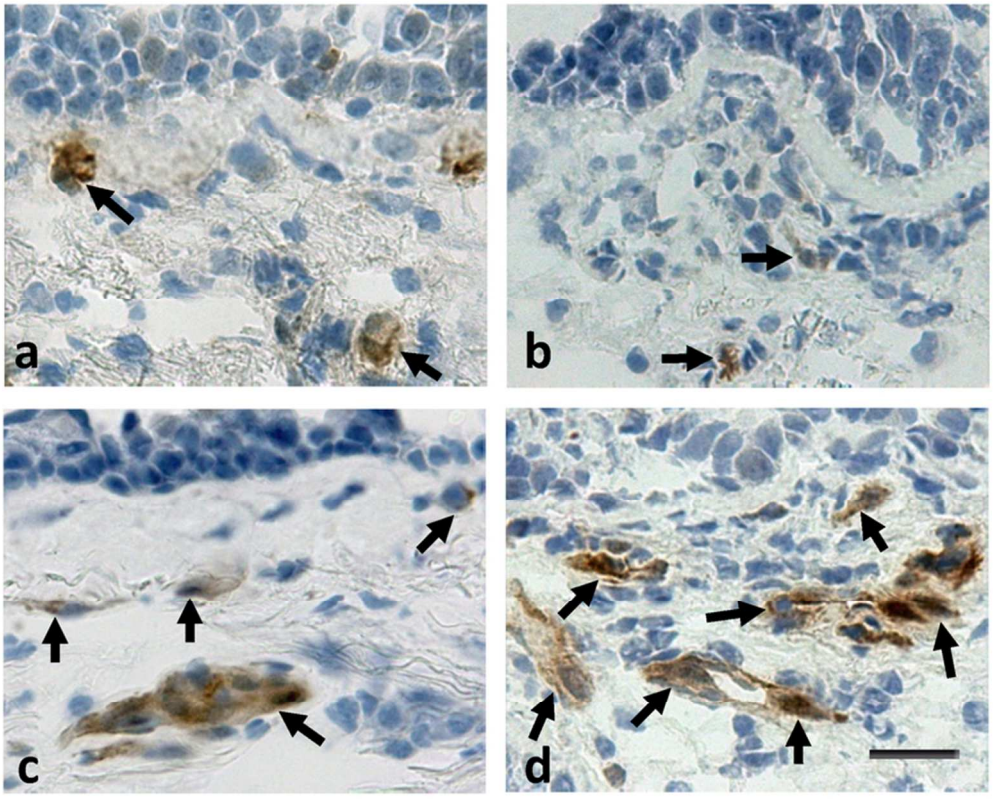

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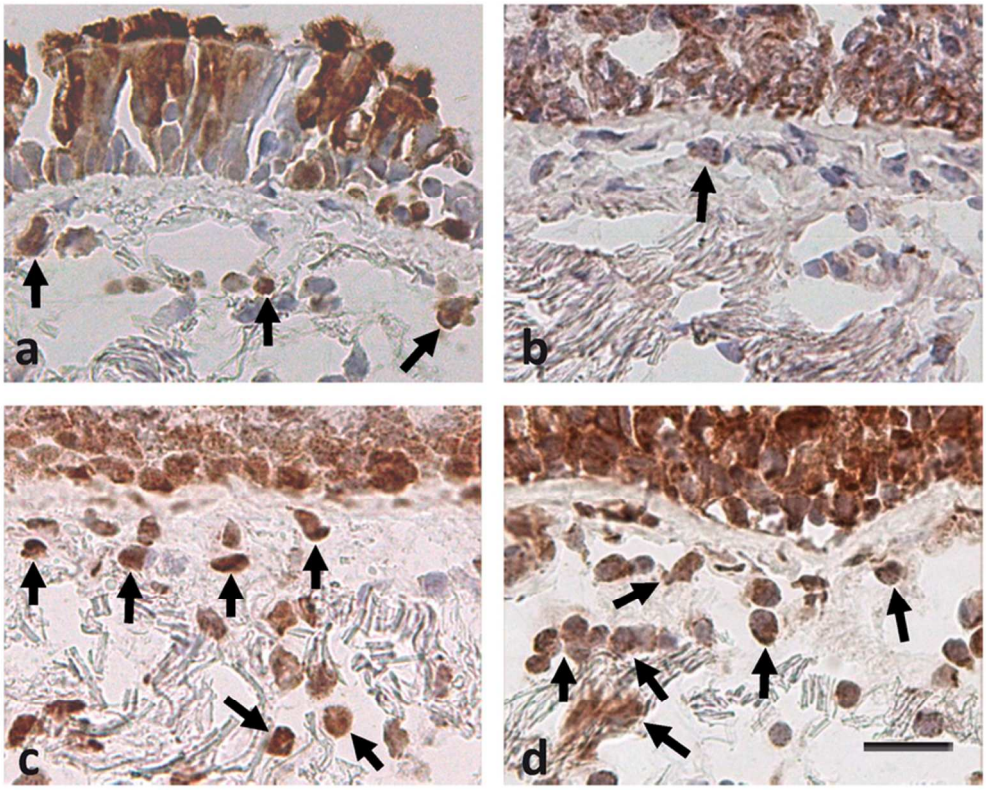

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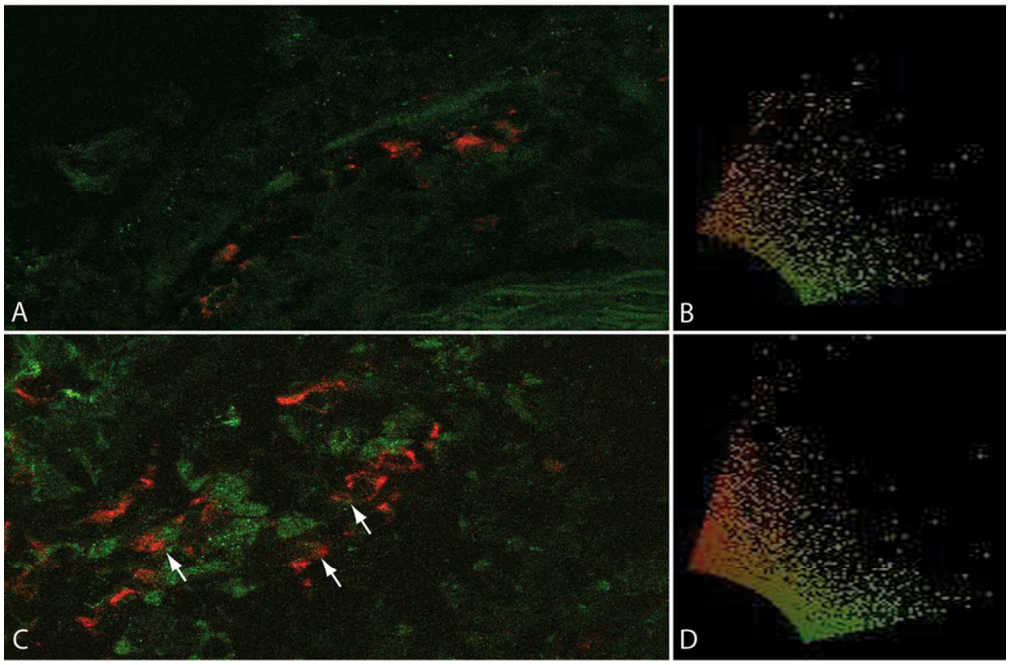

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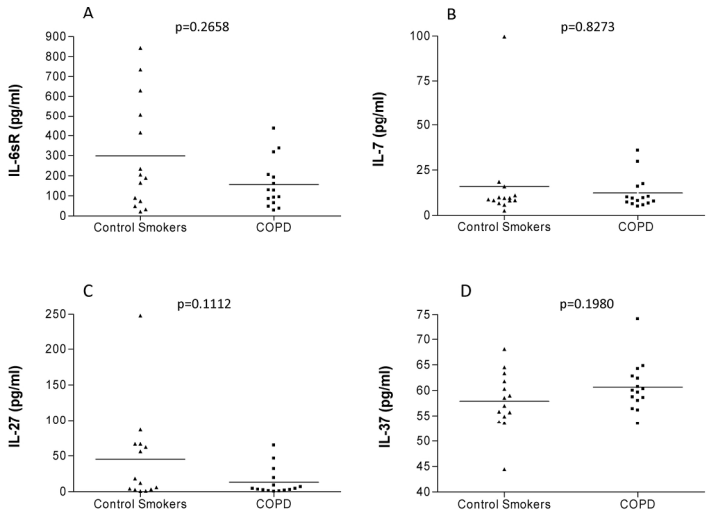

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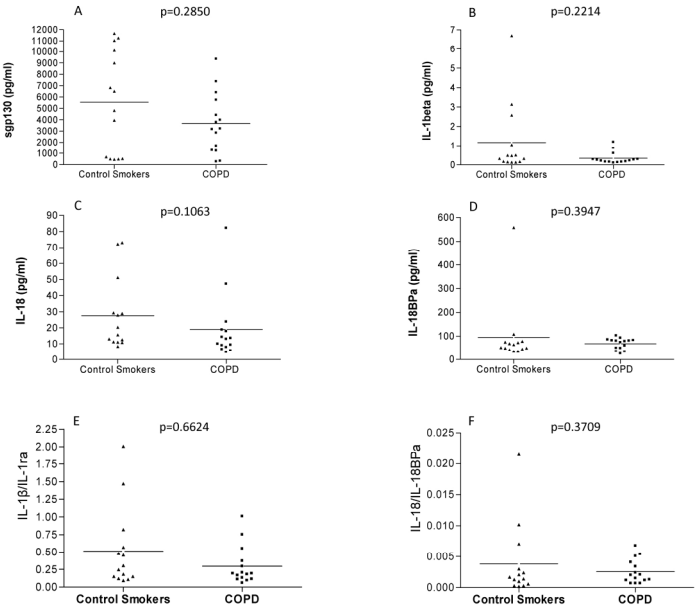

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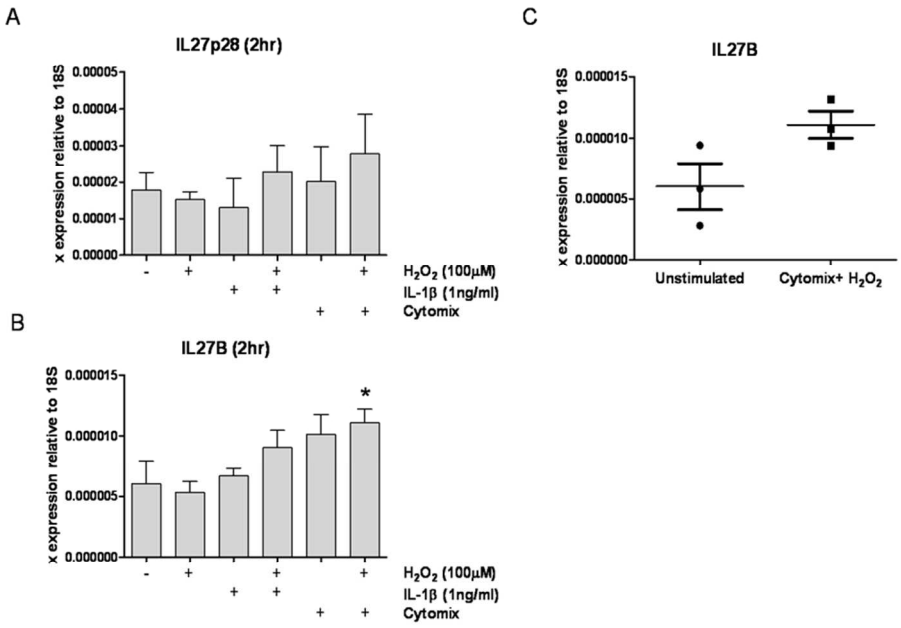

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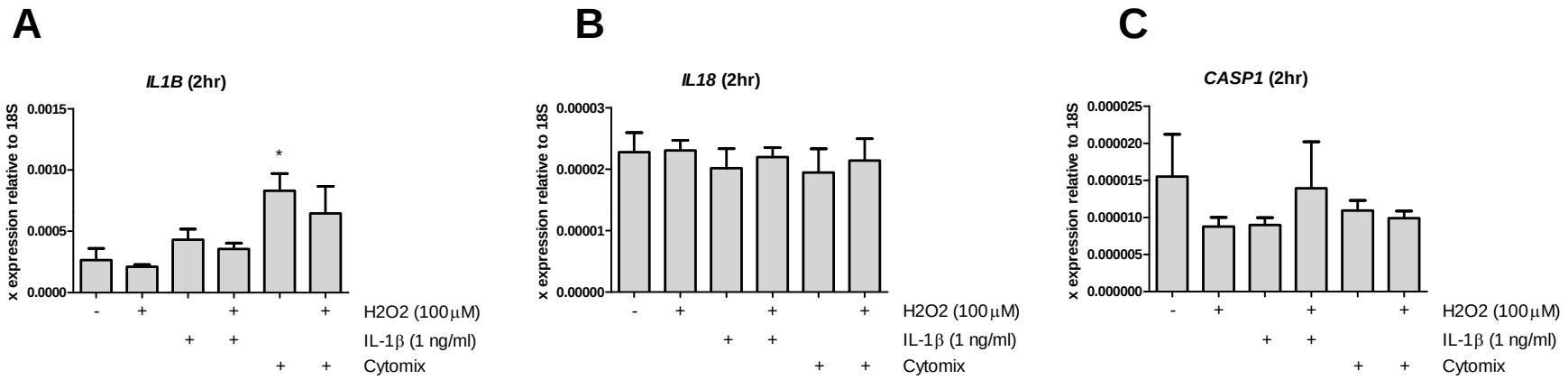
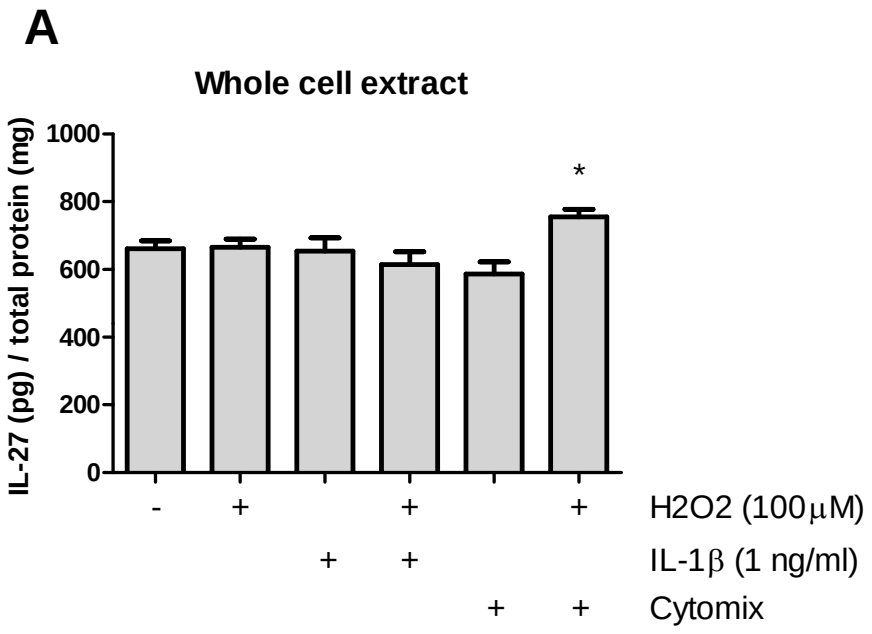

A
IL1B (2hr)
x expression relative to 18S
0.0015
0.0010
0.0005
0.0000
*
- + + + + H2O2 (100μM)
+ + IL-1β (1 ng/ml)
+ + Cytomix
B
IL18 (2hr)
x expression relative to 18S
0.00003
0.00002
0.00001
0.00000
- + + + + H2O2 (100μM)
+ + IL-1β (1 ng/ml)
+ + Cytomix
C
CASP1 (2hr)
x expression relative to 18S
0.000025
0.000020
0.000015
0.000010
0.000005
0.000000
- + + + + H2O2 (100μM)
+ + IL-1β (1 ng/ml)
+ + Cytomix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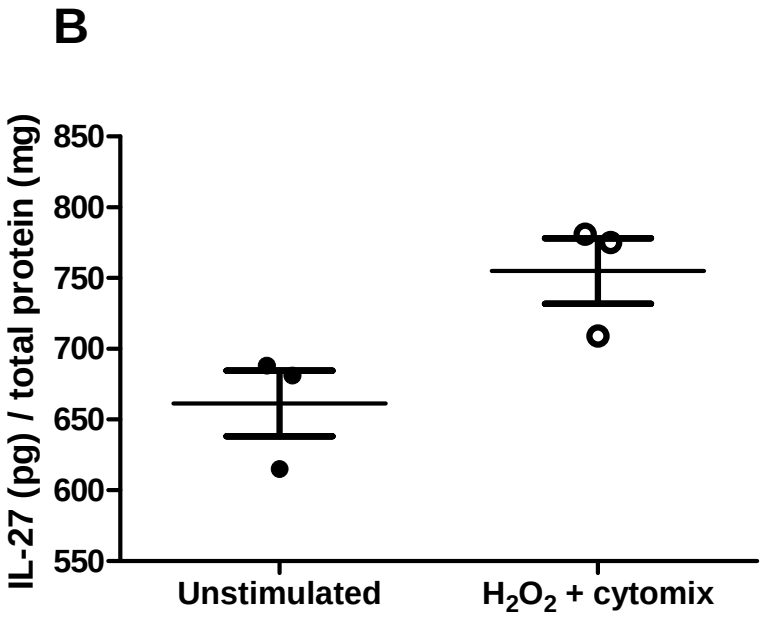
Whole cell extract
IL-27 (pg) / total protein (mg)
1000
800
600
400
200
0
*
-
+
+
+
+
+
+
+
+
H2O2 (100μM)
IL-1β (1 ng/ml)
Cytomix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IL-27 (pg) / total protein (mg)
850
800
750
700
650
600
550
Unstimulated
H2O2 + cytomix