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Expression of human Interferon Regulatory Factor 3 (IRF-3) in alveolar macrophages relates to clinical and functional traits in COPD.

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Abstract

Introduction Chronic obstructive pulmonary disease (COPD) is a frequent cause of morbidity and mortality. Dysregulated and enhanced immune-inflammatory responses have been described in COPD. Recent data showed impaired immune responses and, in particular, of interferon (IFNs) signaling pathway in these patients.

Aim To evaluate in peripheral lung of COPD patients, the expression of some of the less investigated key components of the innate immune responses leading to IFN productions including: IFN-receptors (IFNAR1/IFNAR2), IRF-3 and MDA-5. Correlations with clinical traits and with the inflammatory cell profile have been assessed.

Methods Lung specimens were collected from 58 subjects undergoing thoracic surgery: 22 COPD patients, 21 smokers with normal lung function (SC) and 15 non-smoker controls (nSC). The expression of IFNAR1, IFNAR2, IRF-3 and MDA-5, of eosinophils and activated NK cells (NKp46+) were quantified in the peripheral lung by immunohistochemistry.

Results A significant increase of IRF-3 + alveolar macrophages were observed in COPD and SC compared with nSC subjects. However, in COPD patients, the lower the levels of IRF-3 + alveolar macrophages the lower the FEV1 and the higher the exacerbation rate. The presence of chronic bronchitis (CB) was also associated with low levels of IRF-3 + alveolar macrophages. NKp46 + cells, but not eosinophils, were increased in COPD patients compared to nSC patients ($p < 0.0001$).

Conclusions Smoking is associated with higher levels of innate immune response as showed by higher levels of IRF-3 + alveolar macrophages and NKp46 + cells. In COPD, exacerbation rates, severe airflow obstruction and CB were

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associated with lower levels of IRF-3 expression, suggesting that innate immune responses characterize specific clinical traits of the disease.

Keywords Exacerbations, Chronic bronchitis, Airflow limitation, Innate immunity

Introduction

The interferons (IFN) system is an essential arm of the innate immunity against pathogens. IFNs are secreted upon virus recognition by transmembrane (mainly Toll-like receptors, TLRs) or cytosolic receptors (melanoma differentiation-associated protein 5, MDA-5/RIG1). They induce distinct molecular pathways in host cells which interfere with viral replication and are also involved in the recruitment and activation of leukocytes to the site of inflammation. Dysregulated IFN responses may therefore promote increased susceptibility or modulate the severity of the clinical manifestations of infections. Besides their crucial role in response to infections, IFNs orchestrate various distinct homeostatic processes and immune responses to maintain physiological integrity at steady state [1]. Accumulating evidence suggests that dysregulated IFN responses are major players in the pathogenesis of respiratory conditions such as chronic rhinosinusitis, asthma and, more recently, even in the progression toward severe forms of COVID-19 and in COPD [1–6]. COPD is a major cause of morbidity and mortality worldwide and is characterized by an abnormal inflammatory reaction of the lungs to inhaled particles, mainly cigarette smoke [7]. Increasing evidence indicates that chronic inflammatory and immune responses play key roles in the development and progression of COPD [8], with evidence of lymphoid follicles accumulation in the peripheral lung [9]. Both dysregulated and enhanced immune-inflammatory responses have been described in COPD patients. However, whether this enhanced immune response is an effective response or whether it is impaired leading to increase susceptibility to infection and/or is related to specific clinical presentations of the disease is still largely unknown. Experimental data in epithelial cells in vitro, in line with data coming from in vivo models of COPD exacerbations, support the presence of impaired immune responses and, in particular, of interferon (IFNs) signaling pathway in COPD patients [5, 10, 11]. The melanoma differentiation-associated protein 5 (MDA-5 - a cytosolic sensor that recognizes viral double-strand RNA and then triggers the transcription of genes encoding interferons) [12] and the transcription factor interferon responsive factors (IRF) are key elements to the production of type I and III interferons [13]. IRF−3 and IRF-7 have been shown to play a role in the transcriptional activation of virus-inducible cellular genes, primarily in the regulation of viral-induced type I IFNs production [14, 15]. The expression of these elements in vivo in the peripheral lung of COPD patients is scarce. Previous studies, mainly by semiquantitative scoring, have already reported a decreased expression of IRF-7 in the airways of COPD patients [16]. Natural killer (NK) cells are major players of the interplay between innate and adaptive immune responses in COPD [17–19]; however, most of the data relating NK cells to COPD in humans comes from studies on peripheral blood, whereas much less is known about NK in peripheral lung tissues. Finally, previous studies have also highlighted a role for inflammatory milieu, particularly eosinophils, in modulating innate immune responses and increased risk of infectious events and exacerbations in COPD [20–22]. Based on this background, we aimed to assess the expression of key elements of the IFNs signaling pathway in smokers (with or without COPD) and to evaluate whether the findings of reduced innate immune responses previously found in COPD [6, 16] can be extended to other components of the innate immune pathway. Not only, but we aimed to evaluate whether the expression of the innate immune components correlated with specific disease manifestations. To this aim, we quantified by immunohistochemistry the expression of IFN-receptors (IFNAR1/IFNAR2), MDA-5 and IRF-3 in the peripheral lung. Furthermore, we related IFN mediators with lung tissue NK cells and eosinophils and, on the other side, with patients' clinical and functional parameters.

Methods

Subject characteristics and definitions

Lung specimens were collected from subjects undergoing surgery for appropriate clinical indications [23]: i.e. mainly lung resection for solitary peripheral nodules ($n=53$) or lung volume reduction surgery for severe emphysema ($n=5$). The subjects were categorized into the following three groups: COPD patients (smokers with persistent airflow obstruction; $n=22$); smokers with normal lung function (SC; $n=21$) and non-smokers controls with normal lung function (nSC; $n=15$). Diagnosis of COPD has been done according to the Global Initiative for chronic obstructive lung disease [7]. Patients with current or a history of asthma were excluded from the analysis. Clinical, laboratory and functional data were collected as gold standard clinical practice in preparation to surgical procedure. The presence of chronic bronchitis (CB) was defined as the presence of productive cough of more than 3 months occurring within a span of 2 years [7]. The study conformed to the Declaration of Helsinki. Local ethics committee approval was obtained for sampling collection (Comitato Etico Azienda Ospedaliero

Univeristaria Ferrara, Ferrara, Italy. Ref. number 080399 – Approved in March 2018). Informed written consent was obtained from each participant.

Immunohistochemistry and Morphometric Analysis

Lung tissue preparation and immunohistochemistry were performed as previous described [23, 24]. Briefly, formalin-fixed paraffin embedded lung sections were treated for 60 min with primary antibodies rabbit anti-human IFNAR1 and IFNAR2 (GTX54322 and GTX54289 from GeneTex, Irvine CA, USA), MDA-5 (700360 from Invitrogen, Carlsbad CA, USA) and murine anti-human IRF-3 (sc-376455 from Santa Cruz Dallas TX, USA). Biotinylated secondary antibodies, combined with streptavidin-HRP in the ABC (Avidin Biotin Complex, Vectastain Elite ABC kit; Vector Laboratories, Burlingame, USA) and DAB Chromogen were used to reveal the signal. Sections were counterstained in hematoxylin, dehydrated and mounted. The expression of IFN receptors and signaling mediators was quantified in alveolar macrophages, alveolar walls and bronchiolar epithelium. To quantify expression in alveolar macrophages, 20 nonconsecutive high-power fields and at least 100 macrophages inside the alveolar spaces were evaluated for each subject. Digital images from the stained sections were obtained with a light microscope (Leica DM 2000) connected to a video recorder and a computerized image analysis system (LAS, Leica Application Suite). To standardize the results, cell counts were expressed as percentage of positive macrophages over total macrophages examined. Moreover, the intensity of expression in alveolar macrophages was scored semiquantitatively with a score from 0 to 3 according to increasing intensity (0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining). Expression of the mediators was also evaluated in alveolar walls and bronchiolar epithelium using a semiquantitative score (0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining) [25].

For detection of NK cells Human NKp46/NCR1 MAb from Bio-techne (Minneapolis, MN; USA) was used and signal revealed with streptavidin-HRP Complex and DAB as chromogen. Positively stained cells were quantified in the bronchiolar wall and expressed as number of cells per mm^2 of the examined tissue area. For detection of eosinophils the mouse monoclonal anti-Eosinophil Cationic Protein (Diagnostic Developments, Uppsala, Sweden) was used, upon antigen retrieval (0.1% trypsin in 0.1% calcium chloride (pH 7.8) at 37° C for 15 min). Staining was detected with the EXPOSE Mouse and Rabbit Specific AP (red) Detection IHC Kit (AbCam, Cambridge UK). Positively stained cells were counted in the bronchiolar and alveolar walls and expressed as number of cells per mm^2 of the examined tissue area. Negative controls for nonspecific binding were processed, either omitting the primary antibody or using isotype IgG, and revealed no signal.

Statistical analysis

All cases were coded and the measurements were made without knowledge of clinical data. All analyses were performed using SPSS (v26, IBM Armonk, NY, USA) (level of significance $p<0.05$). For continuous variables, normal distributions were tested using the Shapiro-Wilk test. Group data were expressed as median and interquartile range or frequency (absolute number and percentage) when appropriate. Differences between groups were analyzed using the following tests for multiple comparisons: the analysis of variance and the unpaired Student's t test, the Kruskal-Wallis test and the Mann-Whitney U test, Chi square or Fisher's exact test for nominal variables. Correlation coefficients were calculated using Spearman's rank method.

Results

Clinical characteristics of study subjects

Patients' demographic and clinical characteristics are reported in Table 1. In COPD patients the airflow limitation was very severe in 4 patients (18%); severe in 2 patients (9%), moderate in 8 patients (36%) and mild in 8 patients (36%). Chronic bronchitis was reported in 50% of COPD patients (11 out of 22) and only in one patient among SC ($p<0.001$ vs. COPD). In COPD patients the mean exacerbation rate in the previous year was 1±0.97 events/patient/year. Lung volume measurements (total lung capacity, TLC and residual volume, RV) were available in 10/21 COPD patients. Overall mild increase in RV was found (Median RV 127% predicted [107–143] – TLC 106% predicted [98–116]. No demographic and clinical differences were found between treatment groups (data not shown).

Lung tissue analysis of human interferon system components

Immunohistochemistry analysis was performed in lung specimens including peripheral airways and surrounding lung parenchyma. The expression of IFN receptors (IFNAR1 and 2) and of the components of the human interferon system (IRF-3 and MDA-5) were most prominent in alveolar macrophages.

We then performed a quantitative analysis (expressing the results as % of positive over total alveolar macrophages) and we observed that the percentage of IRF-3^+ alveolar macrophages was increased in COPD and in control smokers, compared with non-smokers ($p=0.013$ and $p=0.032$, respectively, Table 2; Fig. 1A). Moreover, the intensity of IRF-3 staining was also graded as a semiquantitative score ranging from 1 to 3. Even in this analysis we confirmed that IRF3 intensity score was increased

Table 1 Clinical and demographic characteristics of the study groups

	COPD *n* = 22	Smoker controls (SC) *n* = 21	Non-smoker controls (nSC) *n* = 15	*p*-value
Age (yrs)	70 [65–76]	68 [60–64]	69 [65–72]	0.592
Male/Females, n (%)	20 (90) / 2 (10)	17 (81) / 4 (19)	5 (33)/10 (67)	0.005
Current/Former smokers, n (%)	11 (50) / 11 (50)	7 (33) / 14 (67)	---	0.278
Smoking exposure (pack-yrs)	42 [35–50]	40 [23–50]	---	0.451
Dyspnoea (mMRC)	1 [1–2]°	0 [0–1]	0 [0–0]	0.025
Acute exacerbations (n)	1 [0–2] [£]	n.a.	n.a.	---
FEV1/FVC (%)	61 [47–65]*	75 [73–81][#]	81 [79–83]	< 0.001
FEV1 (%)	67 [49–84][$]	102 [91–109]	104 [93–118]	< 0.001
deltaFEV1 postBD (L)	0.06 [-0.02–0.1]	n.a.	n.a.	---
Chronic bronchitis, n (%)	11 (50) /11 (50)	1 (4) / 20 (96)	—	< 0.001
Inhaled treatments	6 (27%)	NA	NA	
ICS/LABA, n (%)	1 (5%)			
LABA + LAMA, n (%)	15 (68%)			
LAMA, n (%)				

Data are expressed as median [interquartile range] or absolute number (percentage). P-value is referred to the comparison between the 3 groups performed by Kruskal-Wallis (or by Mann-Whitney/Chi-square in the case of 2 groups), as appropriate

*$p<0.001$ vs. SC and nSC; #$p=0.044$ vs. nSC; $$p<0.001$ vs. SC and nSC. °data available in 14/22 patients; [£] data available in 20/22 patients (ICS/LABA: inhaled corticosteroids + long-acting beta-2 agonists fixed combinations; LABA + LAMA: long-acting beta-2 agonists + long acting antimuscarinic agents; LAMA: long acting antimuscarinic agents)

Table 2 Main immunohistochemical findings in the study groups

	COPD *n* = 22	Smoker controls (SC) *n* = 21	Non-smoker controls (nSC) *n* = 15	*p*-value
IRF3$^+$macrophages (%)	6.5 [1–35]*	5 [0–17] *	0 [0-1.7]	0.023
IRF3- intensity score	1 [1–2] °	1 [0-1.25] *	0 [0–1]	0.031
MDA5$^+$macrophages (%)	56 [37–79]	74 [34–86]	53 [34–71]	0.365
MDA5- intensity score	1 [1–2]	1 [1–2]	2 [1–2]	0.745
IFNAR1$^+$macrophages (%)	10 [0–32]	6 [0–13]	2 [0–12]	0.499
IFNAR1- intensity score	1 [0–1]	1 [0–1]	1 [0–1]	0.811
IFNAR2$^+$macrophages (%)	1.5 [0–18]	6 [0–15]	4 [0–14]	0.794
IFNAR2- intensity score	1 [1–1]	1 [0–1]	1 [0–1]	0.623
Bronchiolar eosinophils (cell/mm^2)	10 [1–20]	3 [0–13]	0 [0–5]	0.125
Parenchymal eosinophils (cell/mm^2)	0.3 [0-1.3]	0.7 [0.3–3.7]	0.2 [0-0.9]	0.094
Bronchiolar NKp46 + cells (cell/mm^2)	10.5 [9–17][$]	5 [3–9][£]	4 [1–4]	< 0.001

in smokers with COPD and in control smokers compared to nonsmokers (Table 2). Representative images of the immunohistochemical staining are shown in Fig. 2.

Conversely, no differences in MDA-5 were found among the 3 groups (either expressed as % of positive cells or intensity score). Similarly, no difference in the expression of IFNAR1 and IFNAR2 in alveolar macrophage was found among groups (Table 2).

A positive correlation was found between the expression of IRF3 and MDA-5 in alveolar macrophages when the analysis was performed in the entire cohort of the study population ($p=0.01$; $r=0.34$) but not when restricted to COPD patients ($p=0.13$; $r=0.35$).

Distinct immunoreactivity was also observed in the peripheral airways (epithelial cells) and in the alveolar walls (either on pneumocytes and interstitial infiltrating cells). Expression of IFNAR1 and IFNAR2, IRF-3 and MDA-5 in the alveolar walls and bronchiolar epithelium was quantified by a semiquantitative score and revealed no statistical difference among the three groups (Table 3).

To deeper evaluate the role of active smoking a further analysis was performed to compare the expression of the innate immune components in active vs. former smokers. Firstly, no difference was found in the prevalence of active vs. former smokers in COPD patients and smoker controls (40% of active smokers in the COPD group vs. 33% in smoker control group, $p>0.05$). Secondly, we found higher levels of IRF3+alveolar macrophages in active smokers compared to former smokers ($p=0.02$, Figure S1 Online Data Supplement). No other difference was found between active vs. former smokers for the evaluated molecules.A detailed analysis of the immunohistochemical findings according to smoking status in

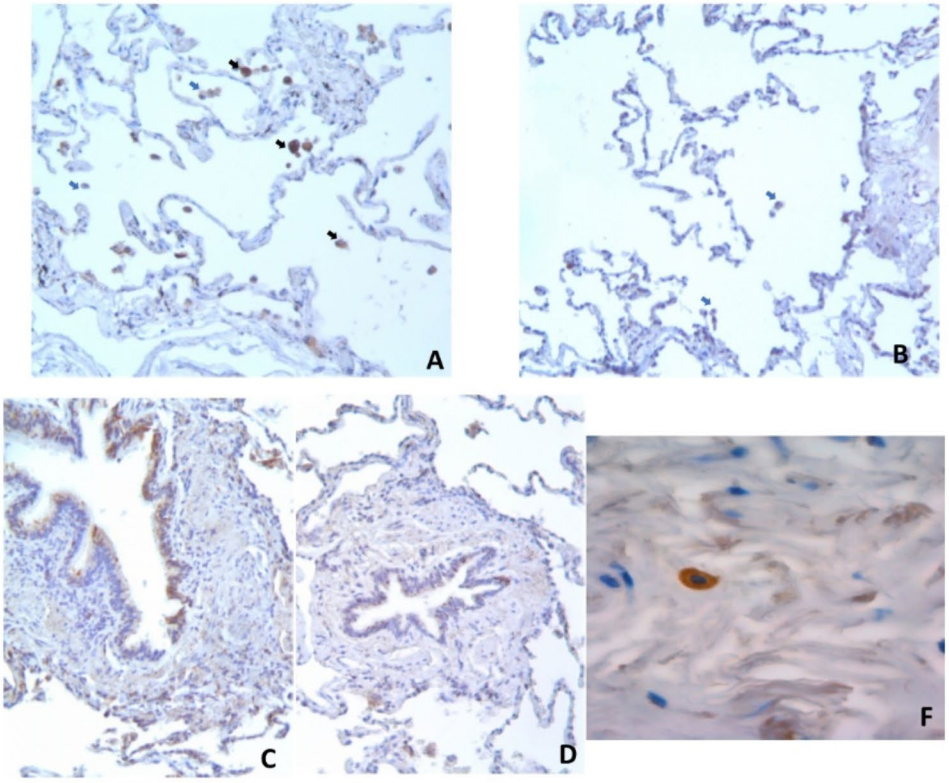

Fig. 1 Representative photomicrographs showing the immunoistochemical staining for detection of IRF-3 on alveolar macrophages from a smoker with mild COPD (Panel **A**) and a nonsmoker control (panel **B**). Blue arrows indicate negative alveolar macrophages, black arrows indicate positive cells (stained in brown). Representative examples of IRF3 staining in bronchiolar epithelial cells are shown in panel **C** (COPD) and **D** (NS control). Finally, Panel E illustrates staining for NK-P46 (in the bronchiolar wall). Original magnification X200 in all panels, except Panel E (X600)

COPD subjects and smoker controls is reported in the Supplementary Table S1).

No difference was found between the mean internal perimeter of the bronchioles examined of COPD patients (1.880 μm, range 1.200–2.700), control smokers (1.885 μm, 1.500–2.350) and nonsmokers (1.670 μm, 1.150–2.550). This data show that the immunostaining analyses were performed on lung samples originating from comparable airway generations.

Quantification of NK cells and eosinophils and relation with interferon system components.

Higher levels of bronchiolar NKp46+cells were found in COPD patients compared to smokers with normal lung function and non-smoker controls ($p<0.0001$ for both comparisons); a numerically increased level of NK cells was found also in smokers compared to non-smokers controls ($p=0.062$) (Table 2; Fig. 1B). Conversely, no difference between groups was found in the levels of eosinophils either in peripheral airways or alveolar walls (Table 2).

Of importance, macrophages expressing IRF-3 were positively related to the numbers of NKp46+cells in peripheral airways ($p=0.016$; $r=0.31$) in all subjects. Moreover, the number of macrophages expressing IRF-3 and MDA-5 were both positively related to the numbers of eosinophils in alveolar walls ($p=0.011$; $r=0.34$ and $p=0.004$; $r=0.38$). All these correlations kept their statistical significance when analysis was limited to the combined smokers' group (COPD and smokers with normal lung function) but not when we restricted the analysis to COPD group alone ($p=0.15$; $r=0.31$ and $p=0.42$; $r=0.37$).

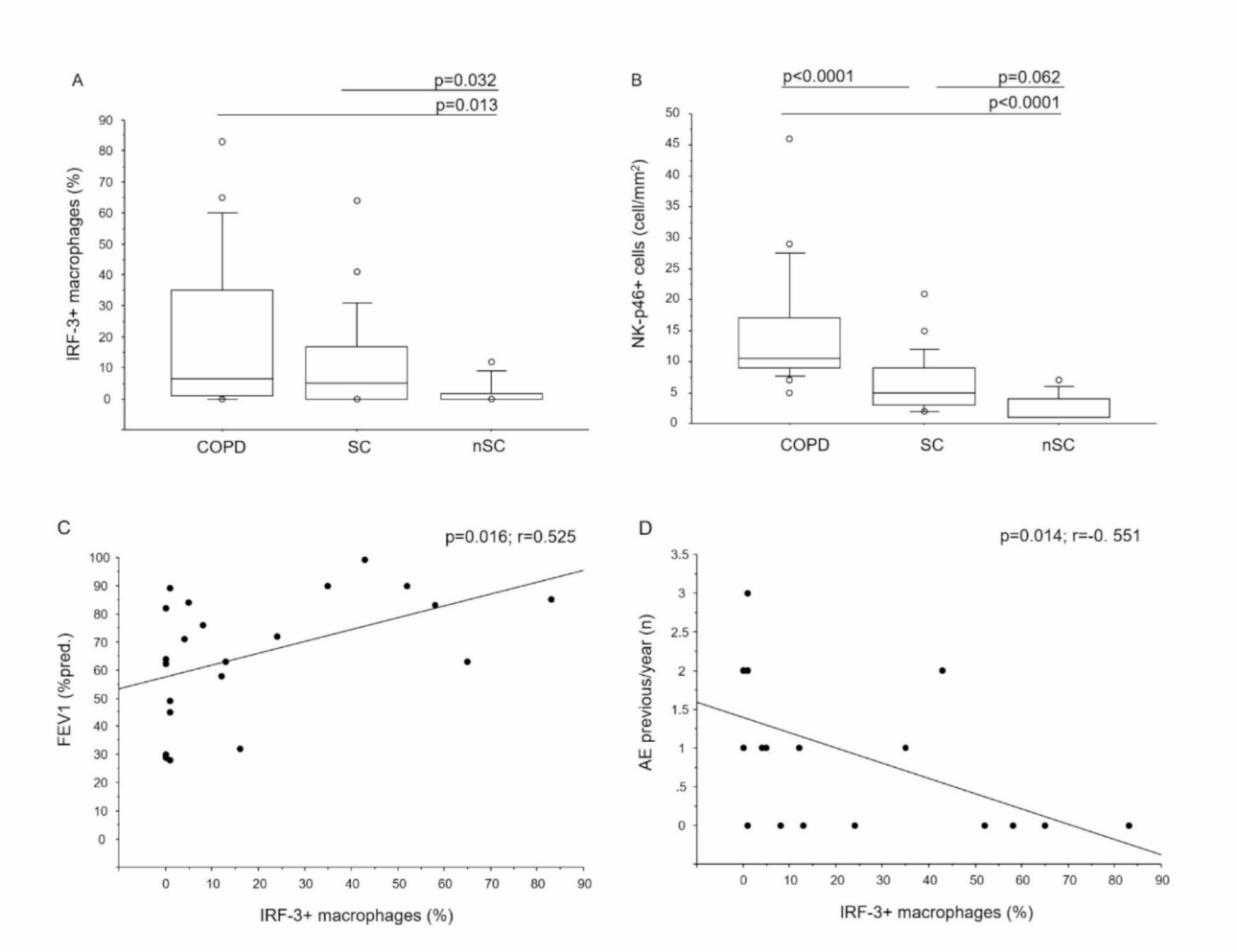

Fig. 2 Boxplots illustrating the percentage of IRF3$^+$ macrophages **(A)** and NKp46 + cells **(B)** in patients with COPD, smoker controls (SC) and non-smoker controls (nSC). Solid line represents the median; bottom and top of the boxes are the 25th and 75th percentiles; brackets correspond to the 10th and the 90th percentiles. Bivariate scatterplots reporting in the COPD group the relationship between the percentage of IRF3$^+$ macrophages (x axes) and FEV1 (%pred.) **(C)** and the number of acute exacerbations in the previous year **(D)**

Table 3 Additional immunohistochemical findings (intensity score in alveolar walls and bronchiolar epithelium)

	COPD *n* = 22	Smoker controls (SC) *n* = 21	Non-smoker controls (nSC) *n* = 15	*p*-value
IRF3 score alveolar wall	0 [0–1]	0 [0–1]	0 [0–0]	0.577
IRF3 score epithelium	0 [0–2]	0 [0–1]	0 [0–0]	0.965
MDA5 score alveolar wall	1 [0–2]	1 [1–2]	1 [0–1]	0.211
MDA5 score epithelium	0 [0–2]	0 [0–2]	0 [0–2]	0.444
IFNAR1 score alveolar wall	0 [0–1]	0 [0–1]	0 [0–1]	0.987
IFNAR1 score epithelium	1 [0–2]	1 [0–1]	1 [0–1]	0.998
IFNAR2 score alveolar wall	0 [0–0]	0 [0–1]	0 [0–0]	0.905
IFNAR2 score epithelium	1 [0–2]	1 [0–2]	1 [0–2]	0.542

Correlations between immune-inflammatory mediators and disease severity

To assess the clinical relevance of our immunohistochemical findings we correlated the expression of innate immunity mediators to functional parameters and clinical presentation including mMRC scale values, exacerbation rate in the previous year and presence of chronic bronchitis. In COPD patients, the percentage of IRF-3 + alveolar macrophages was directly related to FEV1 values ($p = 0.016$; $r = 0.525$; Fig. 1C).

Moreover, a trend for lower levels ($p=0.063$) of IRF-3$^+$ alveolar macrophages was found in COPD patients with chronic bronchitis compared to COPD patients not reporting chronic bronchitis. No relation was found between IRF-3+alveolar macrophages and mMRC values. Of importance, IRF3$^+$ alveolar macrophages were significantly lower in COPD patients experiencing at least one exacerbation in the previous year compared to patients without exacerbations (1 [0–12] vs. 20 [8–58], $p=0.023$). In line with this observation, IRF3 expression was inversely related to the number of acute exacerbations during the previous year ($p=0.01$; r=-0.55 Fig. 1D). These results were confirmed even when immunostaining was expressed by the semiquantitative intensity score. Indeed, IRF 3 intensity score was negatively related to the number of exacerbations ($p=0.043$; r=-0.39) and positively correlated with FEV1 values ($p=0.028$; $r=0.479$).

We then performed a combined analysis on exacerbations in the previous year, chronic bronchitis, pulmonary function and IRF3 expression in COPD patients with or without chronic bronchitis. As shown in Fig. 3A, we observed that patients with lower lung function (FEV1%pred. < 25th percentile) and concomitant lower expression of IRF3 (<75th percentile) had a higher prevalence of CB ($p=0.001$). Similarly, the COPD patients with chronic bronchitis and at least one exacerbation in the previous year ($N=6$) showed the lowest levels of IRF3$^+$ alveolar macrophages ($p=0.004$; Fig. 3B). As expected [26], a lower but not significant FEV1% pedicted (66±24% predicted vs. 71±12% predicted, $p>0.05$) was found in patients experiencing at least one exacerbation

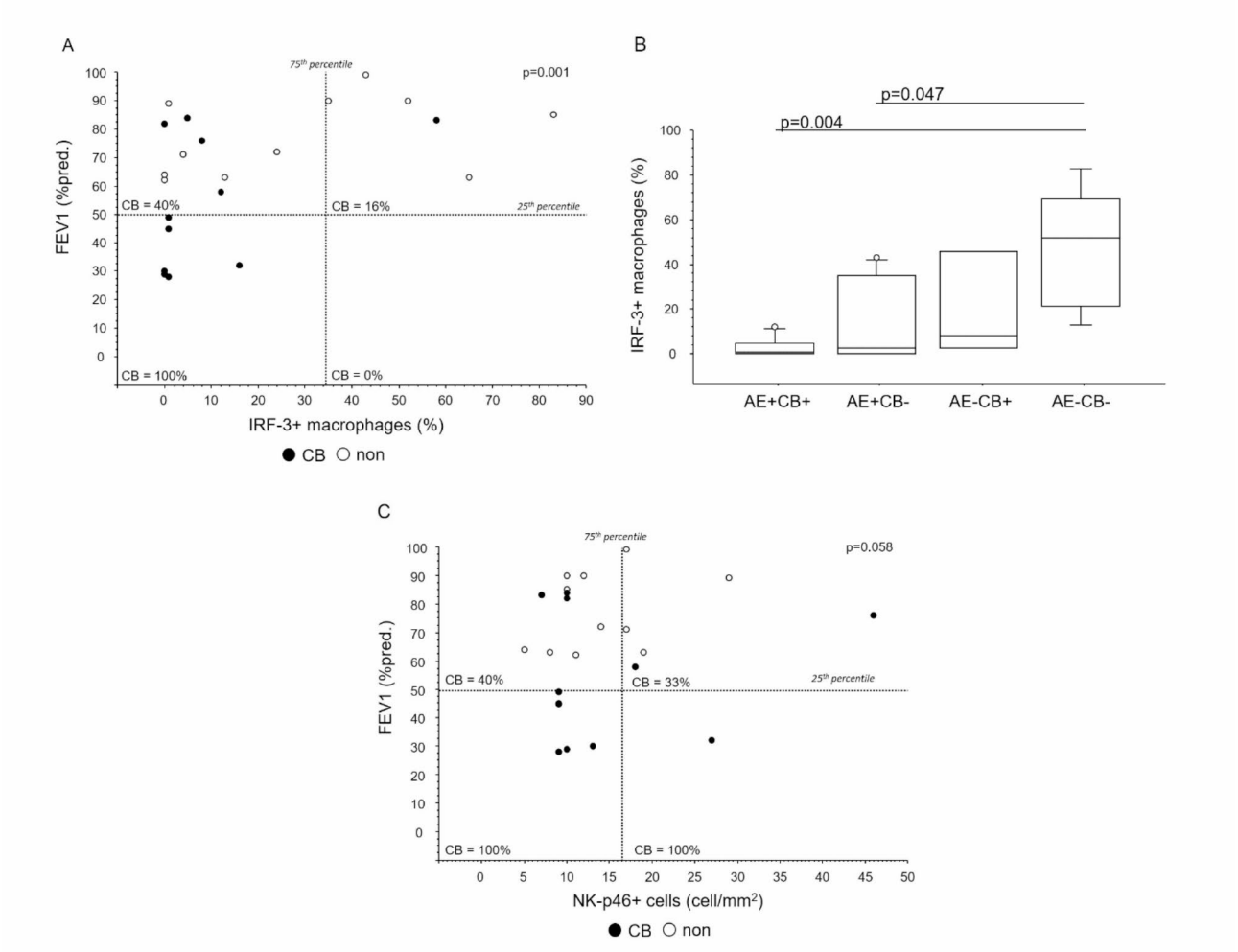

Fig. 3 Bivariate scattergram reporting the relationship between FEV1 (%pred.) and the percentage of IRF3$^+$ macrophages **(A)** or lymphocytes NK **(C)** in COPD patients stratified for the presence (black dots) or the absence (white dots) of chronic bronchitis (CB). Fisher analysis showed a significant association between CB presence, airflow impairment and low IRF3 expression. P-value have been calculated by Fisher's exact test 4×2 contingency table. **(B)** Boxplot illustrating the percentage of IRF3$^+$ macrophages in patients with COPD stratified on the presence/absence of chronic bronchitis (CB+/-) and at least one acute exacerbation during the previous year (AE +/-) (AE + CB + $n=6$; AE + CB- $n=6$; AE-CB + $n=3$; AE-CB- $n=5$). Solid line represents the median; bottom and top of the boxes are the 25th and 75th percentiles; brackets correspond to the 10th and the 90th percentiles

in the previous year compared to patients without exacerbation. Similar results were obtained were IRF3 immunostaining was expressed as intensity score (data not shown).

In COPD patients, no correlations were found between airway NKp46+cells and clinical variables. However, in COPD patients with combined lower lung function (FEV1%pred. < 25th percentile) and lower levels of NK (<75th percentile) a higher prevalence of CB was found ($p=0.058$).

No difference was found in the levels of innate immune components, and of NK cells between ICS-treated and not ICS-treated COPD patients (data not shown).

Discussion

Small airways and lung parenchyma are the elective site of pathological abnormalities in COPD. Several studies have shown that an intense immune-inflammatory reaction does occur in the peripheral lung of COPD patients and correlate with the extent of lung remodeling and with the progression of airflow obstruction [27]. Within the microenvironment of the lung tissue, several pathways can be activated to induce the release of damage-associated molecular patterns (DAMPs) and sustain the prolonged activation of immune mediators [28]. Cigarette smoke is known to affect many cellular pathways, disrupting the complex chain of processes that ensure adequate immune control.

We hypothesized that disturbance of the immune host response may occur in vivo in peripheral lungs of COPD and that such innate immune response could affect important clinical outcomes of the disease particularly those linked with infectious events such as exacerbations and chronic bronchitis.

In this study we found increased IRF-3$^+$ expression in alveolar macrophages of smokers (COPD and controls with normal lung function) compared to non-smoker subjects. However, among subjects with COPD, decreased numbers of IRF-3$^+$ alveolar macrophages and NK cells were found in COPD patients with concomitant chronic bronchitis and severe lung function impairment. Also, IRF-3$^+$ alveolar macrophages were significantly reduced in COPD patients experiencing at least one exacerbation in the previous year compared to patients without exacerbations. Our data extend previous studies showing reduced expression of innate immune components in the airways of COPD patients compared to controls [6]. However, with a similar methodologic approach, a recent study found reduced expression of IRF-7, but not IRF-3, in epithelial cells of bronchial biopsies of COPD patients compared to non-smoker controls but not compared to control smokers [16]. Differences in findings can be related to different lung function severity between study populations (milder patients in the previous study compared to the present study) but also to the site of assessment (alveolar macrophages compared to central airways).

The role of smoking on modulating immune responses has been highly investigated. Smoking impacts both innate and adaptive immunity and plays dual roles in regulating immunity by either exacerbation of immune responses or attenuation of defensive immunity [29]. Interestingly, and in line with this concept, we found increased IRF-3 expression and NK levels in smokers compared to non-smokers but reduced in severe COPD patients compared to smokers with normal lung function. This trend toward enhanced smoking induced immune- responses followed by deranged/impaired immune responses in patients with advanced disease has been already showed for several pathways and biomarkers [29–31].

IRF-3 is an important transcription factor that controls multiple IFN-inducing pathways that lead to type I and III production [32]. Alveolar macrophages are also important elements of the innate immune responses and a source of IFN production [33, 34]. Dysregulated alveolar macrophage responses have been shown in COPD [35]Our study provides the evidence that reduced components of the innate immune response can be associated with more severe clinical traits of the disease.

Previous studies, mainly by semiquantitative scoring, have already reported a decreased expression of IFN pathway elements in the airways of smokers with COPD, particularly MDA-5 and IRF-7 [6]. Here we report the novel association between the degree of impairment of the innate immune response component IRF3 and the clinical manifestation of COPD. Indeed, we showed that among COPD subjects, those with concomitant chronic bronchitis and FEV1<50% had the lowest levels of IRF-3+alveolar macrophages. This observation adds an important piece of information by pointing out at chronic bronchitis as a condition related to severe impairment of innate responses in the lung. Such a condition may indeed represent to a colonization-prone environment. The natural history of COPD is punctuated by episodes of worsening in respiratory symptoms that require medical and pharmacological treatment, named exacerbations. Exacerbations are key clinical manifestation of the disease. Indeed, exacerbations are directly related to disease progression, mortality, and disease-related costs [36, 37]. Infections of the tracheobronchial tree are the most common causes of COPD exacerbations [26, 38]. A significant proportion of COPD patients continue to experience frequent exacerbations despite being regularly treated. The comprehension of the molecular and inflammatory mechanisms associated with COPD exacerbation can identify novel targets for intervention. In line with a previous study showing reduced mRNA expression of

the anti-viral immune mediators in sputum of COPD patients with frequent exacerbations [39], we were able to correlate IRF-3 expression in alveolar macrophages to the number of exacerbation events during the previous year. The lower the IRF3 expression the higher the number of exacerbations, an observation that was particularly prominent in those COPD patients with concomitant chronic bronchitis. These data implicate deficient airway innate immunity in the increased propensity to exacerbate observed in some patients with COPD.

The innate immune system is an interplay of many pathways to detect and eliminate pathogens. In this context, a particular role is that of NK cells, which are considered the first line of defense against lung infections [17, 18]. Previous studies that examined the involvement of NK in COPD produced diverging results [19], possibly due to the different markers used and the heterogeneity of the populations. Some studies reported activated NKT cells in patients with COPD, marked by increased expression of granzyme B and perforin, and are involved in epithelial and parenchymal destruction [40, 41]. In line with this data, in our study we observed an increased infiltration of cells expressing the activating natural cytotoxicity receptor NKp46 in COPD subjects. However, we should also remark that, as with the previous analyses with IRF3, subjects with most severe COPD and CB had the lowest numbers of NK cells. This would support the conclusions that while smokers with normal lung function and mild COPD are able to mount a proper innate immune response, this capacity is lost in most severe forms of COPD. Our study has some limitations. Firstly, we did not perform a comprehensive assessment of the IFN system, nor mechanistic studies to describe the activation of IFN components. As so, we cannot exclude that other regulatory factors may be involved. Nevertheless, we believe that our results are important because they relate the reduction of IRF 3 expression directly in the lung tissue to the detailed clinical presentation of COPD patients, something that is usually not explored in mechanistical studies. Secondly, our cohort was not homogeneous for gender distribution, with a higher proportion of females in nonsmokers. Females are known to have enhanced innate immune responses (including IFNs) compared to males [42] and our data show an increase in COPD and smoker groups (those with higher prevalence of males) compared to nonsmokers controls (with more female). As for NK, previous studies showed that oestrogen can inhibit NK cell cytotoxicity during the ovulatory phase [43]. However, this does not apply to the context of our study where the median age of the subjects included is well beyond postmenopausal age. Indeed, if anything, women over the age of 70 have more vigorous NK cell cytotoxic activity relative to men [44]. Thirdly, our cohort is also lacking a group of COPD patients unrelated to smoking, which may have added important information on dissecting the role of smoking from that of COPD. Due to the design of our study, which was performed in lung samples obtained at lobectomy, it is extremely rare to have non-smoking subjects with both COPD and lung cancer. Finally, we have also to acknowledge that: (i) in the study we evaluated clinical characteristics (lung function and chronic bronchitis) mainly reflecting more proximal airway pathology and compared them with observations of small airway histopathology, and (ii) our analysis was not performed on consecutive serial sections for each marker, bur rather from nearby sections from the same paraffin embedded lung samples, reflecting the same area of the lung.

To summarize, these data indicate that cigarette smoke per se can heighten immune-inflammatory responses. However, a reduced expression of immune response components can occur in those patients with more severe clinical manifestation of the disease and can be linked to clinical traits of the diseased where respiratory infections can play a relevant role (exacerbations and chronic bronchitis). The mechanisms leading to decreased innate immune responses in COPD are still debated.

Data are expressed as median [interquartile range]. P-values in table reported the comparison among groups performed by Kruskal-Wallis. *$p<0.05$ vs. nSC; ° $p<0.001$ vs. nSC; $ $p<0.001$ vs. SC and nSC; £ $p=0.062$ vs. nSC calculated by Mann-Whitney-U test.

Data are expressed as median [interquartile range]. P-value reported the comparison among groups performed by Kruskal-Wallis.

Abbreviations

CB	Chronic bronchitis
COPD	Chronic obstructive pulmonary disease
IFN	Interferon
IFNAR	Interferon-α/β receptor
IRF	Interferon regulatory factors
MDA5	Melanoma differentiation-associated gene-5
NK	Natural killer

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-024-02952-6.

Supplementary Material 1

Author contributions

MC, and SB conceived, designed, and supervised the study, co-wrote the first draft of the manuscript. MB, FuBr, MS, and AP contributed to data analysis, data interpretation and manuscript finalization. SC, PC, LDF, MT, FeBa, TB, and AMR performed laboratory assays and contributed to data analy-sis.

Funding

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethical approval

The study conformed to the Declaration of Helsinki. Local ethics committee approval was obtained for sampling collection (Comitato Etico Azienda Ospedaliero Univeristaria Ferrara, Ferrara, Italy. Ref. number 080399 – Approved in March 2018). Informed written consent was obtained from each participant.

Consent for

Not Applicable.

Conflict of interest disclosure statement

SB has nothing to declare. **MB** has nothing to declare. **SC** has nothing to declare. **PC** has nothing to declare. **LDF** has nothing to declare. **MT** has nothing to declare. **FeBa** has nothing to declare. **TB** has nothing to declare. **AMR** has nothing to declare. **FuBr** declares personal payments for participation on Advisory Board from ASTRAZENECA, BOEHRINGER INGELHEIM, CHIESI, GSK, MENARINI GROUP, SANOFI, P&G; personal payments or honoraria for lectures, presentations from ASTRAZENECA, BOEHRINGER INGELHEIM, CHIESI, GSK, MENARINI GROUP, SANOFI. **MS** has nothing to declare. **A.P.** declares: research grants from CHIESI, ASTRAZENECA, GSK, SANOFI, Agenzia Payments Italiana del farmaco (AIFA); personal payments for consultation from Chiesi, ASTRAZENECA, GSK, NOVARTIS, SANOFI, AVILLION, ELPEN PHARMACEUTICA; Personal payments for lectures, presentations, or educational events from CHIESI, ASTRAZENECA, GSK, MENARINI, NOVARTIS, ZAMBON, MUNDIPHARMA, SANOFI, EDMOND PHARMA, IQVIA, AVILLION, ELPEN PHARMACEUTICALS; Personal payments for Advisory boards: CHIESI, ASTRAZENECA, GSK, MSD, NOVARTIS, SANOFI, IQVIA, AVILLION, ELPEN PHARMACEUTICALS; nothing to declare related to the present manuscript. **M.C** declares: research grants from Chiesi and GSK; personal payments for lectures, presentations or educational events from CHIESI, ASTRAZENECA, GSK, NOVARTIS, ZAMBON, SANOFI, ALK ABELLO; personal payments for participation on Advisory Board from CHIESI, ASTRAZENECA, GSK, ALK ABELLO, SANOFI; nothing to declare related to the present manuscript. All of the authors contributed to the study implementation, as well as to the development of this report.

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