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Relation between duration of smoking cessation and bronchial inflammation in COPD

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ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) is associated with airway inflammation. Although smoking cessation improves symptoms and lung function decline in COPD, it is unknown whether bronchial inflammation in patients with established COPD varies with the duration of smoking cessation.

Methods: 114 COPD patients were studied cross-sectionally: 99 males, age 62±8 years, median 42 (IQR: 31-55) pack-years, no inhaled or oral corticosteroids, all current or ex-smokers (n=42, quit >1 month, median cessation duration 3.5 years), postbronchodilator FEV_1 63±9% predicted, FEV_1/IVC 48±9%. Subepithelial T-lymphocyte (CD3, CD4, CD8), neutrophil, macrophage, eosinophil, mast cell, and plasma cell numbers were measured in bronchial biopsies (median [IQR] /0.1 mm^2), using fully automated image analysis.

Results: Ex-smokers with COPD had higher $CD3^+$, $CD4^+$, and plasma cell numbers than current smokers with COPD (149 [88-225] vs. 108 [61-164], p=0.036; 58 [32-90] vs. 40 [25-66], p=0.023; 9.0 [5.5-20] vs. 7.5 [3.1-14], p=0.044, respectively), but no difference in other inflammatory cells. Short-term ex-smokers (<3.5 years) had higher $CD4^+$ and $CD8^+$ cell numbers than current smokers (p=0.017, p=0.023; respectively). Conversely, long-term ex-smokers (quit ≥3.5 years) had lower $CD8^+$ cell numbers than short-term ex-smokers (p=0.009), lower CD8/CD3 ratios than both current smokers and short-term ex-smokers (p=0.012, p=0.003; respectively), and higher plasma cell numbers than current smokers (p=0.003).

Conclusions: With longer duration of smoking cessation CD8 cell numbers decrease and plasma cell numbers increase. This indicates that bronchial T-lymphocyte and plasma cell counts, but not other inflammatory cells, are related to duration of smoking cessation in patients with COPD.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterised by progressive airflow limitation and abnormal inflammatory responses in the airways.[1] Persistent smoking-induced inflammation is thought to play an important role in the pathogenesis of COPD. It is characterised by influx of neutrophils into the airway lumen, and elevated macrophage and T-lymphocyte numbers in the airway wall.[2][3] Particularly, bronchial $CD8^+$ T-lymphocytes appear to be associated with the severity of the disease.[3][4] Moreover, sputum $CD8^+$ cells of COPD patients have elevated cytotoxic activity[5], which may contribute to the tissue damage occurring in these patients. Recently, it was demonstrated that B cell numbers in the small airways of COPD patients are also increased.[3] Plasma cells (terminally differentiated effector B cells) are the cellular source of mucosal immunoglobulin production and consequently play a central role in host defence against infection. However, there is limited data on their role in COPD, and their presence in the lung has not been investigated in relation to smoking status.

Smoking cessation is the only intervention able to reduce COPD progression.[6] Moreover, patients who quit smoking experienced less respiratory symptoms and hyperresponsiveness as compared to those who continued smoking.[7][8] The largest improvements in lung function and symptoms occurred within the first year after cessation. It is yet unclear whether these beneficial effects are accompanied by reversal of smoking-induced pathology.

At present, there is insufficient evidence that smoking cessation reduces inflammation in COPD. There are few cross-sectional studies comparing smokers and ex-smokers regarding bronchial inflammation in heterogeneous and relatively small groups of patients without an established diagnosis of COPD.[9] Most previous studies were performed in patients with chronic bronchitis.[10][11] In patients with symptoms of chronic cough and expectoration, ex-smokers tended to have lower mast cell numbers in the lamina propria than current smokers[10], whereas the number of neutrophils, macrophages, eosinophils, and lymphocytes in bronchial biopsies have been reported to be similar.[11] Most studies did not take duration of smoking cessation into account when comparing current and ex-smokers. However, it has been shown that this may influence the inflammatory response in small airways.[12] These available studies may not be representative for COPD, since relatively low numbers of patients with airflow limitation were included. Therefore, it remains to be investigated whether bronchial inflammation varies with current smoking status and the duration of smoking cessation in patients with an established diagnosis of COPD.

In the current study, we postulated that bronchial inflammation in patients with established COPD differs between active smokers and patients who stopped smoking, and that such difference is influenced by the duration of smoking cessation. We therefore investigated the number of inflammatory cells (e.g. neutrophils, macrophages, eosinophils, mast cells, T-lymphocytes, plasma cells, and granzyme B^+ cells as a marker of activated cytotoxic cells) in bronchial biopsies of current and ex-smokers with COPD in a large cross-sectional study.

METHODS

Subjects

Hundred fourteen patients with COPD, participating in the GLUCOLD study (Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease), were included in this study. Patient characteristics and methods have been described in detail previously.[13] In short, all patients had irreversible airflow limitation compatible with GOLD stages II and III [postbronchodilator FEV_1 and FEV_1/IVC <90% *confidence interval* (CI) of the predicted value, FEV_1 ≥1.3 liter and >20% predicted] and at least one of the following symptoms: chronic cough, chronic sputum production, or dyspnea on exertion. Patients with a history of asthma, alpha-1 antitrypsin deficiency, or other active lung disease were not permitted to the study. The study subjects did not use a course of oral steroids during the last three months, and did not have maintenance treatment with inhaled or oral steroids during the last six months. Patients were allowed to use short-acting bronchodilators, and were in clinical stable condition. They were current or ex-smokers (quit smoking for at least one month) with at least 10 pack-years of smoking. A validated questionnaire was used to assess the smoking history.[14] Those who had quitted were asked at what age they stopped in order to calculate the duration of smoking cessation (years). All patients gave their written informed consent.

Design and Lung function

The study had a cross-sectional design and consisted of four visits. Spirometry, reversibility to salbutamol, and diffusion capacity were measured according to previously described methods in order to characterise the patients.[13]

Bronchoscopy

Fiberoptic bronchoscopy was performed using a standardised protocol according to recent recommendations.[15] Smokers were requested to refrain from smoking on the day of the bronchoscopy. Patients received pre-medication with 400 µg salbutamol p.i., 20 mg codeine p.o., and 0.5 mg atropine s.c., and local anesthesia with lidocaine (≤3 mg/kg). During the procedure 100% oxygen was delivered through a nasal canula (2 L/min) if required, while transcutaneous oxygen was monitored continuously by oximeter with a finger probe. Bronchoscopies were performed by experienced pulmonary physicians using a fiberoptic bronchoscope (18X, outer diameter 6 mm, Pentax Optical Co., Japan) and pairs of cup forceps (Reda, Tuttlingen, Germany). Six macroscopically adequate bronchial biopsy specimens were randomly taken from (sub) segmental carinae in the right or left lower lobe (left and right was alternated per patient, all biopsies from one lung).

Biopsy processing and staining

Four biopsies were immediately fixed in 4% neutral buffered formalin for 24 hours, then processed and embedded in paraffin, and two were immediately snap frozen and stored at –80°C. Paraffin-embedded biopsies were cut in 4 µm thick sections and haematoxylin/eosin staining was used for evaluation and selection of the two morphological best biopsies per patient for analysis (without crushing artifacts, large blood clots, or only epithelial scrapings). If required, immunohistochemistry included antigen retrieval (Table 1). Specific antibodies against T lymphocytes (CD3, CD4, CD8), macrophages (CD68), neutrophil elastase (NE), mast cell tryptase (AA1), eosinophils (EG2), plasma cells (CD138), and granzyme B were used (Table 1). Besides staining the plasma cells, CD138 (syndecan-1) antibody also stains the bronchial epithelium and submucosal glands[16], but these structures were not present in the areas of subepithelial cell quantification. All stainings, except for CD3 and CD4, were performed using an automatic staining machine (Dako), in two sessions, with one section per patient in each

session. In short, the sections were incubated with an optimal dilution of the primary antibodies in 1% BSA/PBS at room temperature for 60 min. As a secondary antibody, the horseradish peroxidase conjugated anti-mouse or anti-rabbit EnVision system (DAKO, Glostrup, Denmark) was used, with NovaRED (Vector, Burlingame, CA) as the chromagen. The sections were counterstained with Mayer's hematoxylin (Klinipath, Duiven, the Netherlands). For negative controls, the first antibody was omitted from this procedure.

Analysis of bronchial biopsies

Multiple digital images per coded biopsy section were prepared using a colour camera (Basler A101fc-le) and a dedicated software program (RVC-software, Amersfoort, The Netherlands, http://www.rvc.nl/en/index.html). Next, these different images were united into one large image that consisted of the entire biopsy section (100 μm = 115.7 pixels). Fully automated inflammatory cell counting procedures were performed according to previously described validated methods.[17] The number of subepithelial positively staining inflammatory cells was counted within the largest possible area, of maximal 125 μm deep beneath the basement membrane, per biopsy section, and expressed as the mean number of cells / 0.1 mm^2 of the two biopsies. Because of very low numbers of granzyme B^+ cells, these were analysed using a semi-quantitative score of the entire biopsy section: 0 (absent staining), 1 (1-10 positive cells), or 2 (> 10 positive cells).

Statistical analysis

Mean values and standard deviations (SD) were computed for normally distributed variables. Cell counts, and other variables that did not show a normal distribution, were logarithmically transformed (square root in case of CD8/CD3 ratio) before statistical analysis, and presented as medians with interquartile range (IQR). Normally distributed log-transformed cell counts were analysed using parametric tests. EG2 data remained skewed after log-transformation and were therefore analysed using non-parametric tests. Differences between smokers and ex-smokers were explored using Chi-square tests, 2-tailed unpaired t-tests, or Mann Whitney tests. To study the influence of duration of smoking cessation on cell counts, we compared smokers with ex-smokers who quit <3.5 years and those who quit ≥3.5 years ago, since this was the median duration of smoking cessation, using one-way ANOVA (Kruskal-Wallis tests in case of EG2). If these were statistically significant, 2-tailed unpaired t-tests were applied for further exploration of between-group differences. Multivariate linear regression analysis was applied to adjust for significant differences in patient characteristics between the groups, such as sex, age, pack-years, and FEV_1/IVC. Univariate correlations were evaluated using Spearman and Pearson's correlation coefficient. SPSS 12.0 (SPSS Inc., Chicago, IL) software was used for statistical analysis.

RESULTS

Patient characteristics

Table 2 shows the characteristics of the 72 smoking and 42 ex-smoking patients included in the study. Patients had moderate to severe COPD, based on a postbronchodilator FEV_1 of 63.0 (8.8) % predicted, and a median smoking history of 42 pack-years. Median duration of smoking cessation in ex-smokers was 3.5 (1-10) years. Differences in patient characteristics between current and ex-smokers, and between current smokers, short-term ex-smokers (quit <3.5 years ago), and long-term ex-smokers (quit ≥3.5 years ago) are demonstrated in table 2.

Bronchial inflammatory cell counts in smokers versus ex-smokers

All 114 patients underwent bronchoscopy; from one patient (ex-smoker) none of the biopsies taken were adequate for analysis. Figure 1 shows examples of biopsy sections stained with haematoxylin/eosin, CD138, CD8, and CD4. The median analysed surface area of biopsy sections (mucosal area per patient, average of all antibodies, in which cells were counted, not corrected for shrinkage) was 0.35 (0.26-0.42) mm^2. Ex-smokers had higher $CD3^+$, $CD4^+$, and $CD138^+$ cell numbers than current smokers (p=0.036, p=0.023, p=0.044; respectively), but no significant difference in other inflammatory cell counts (Table 3). When differences in sex, age, and FEV_1/IVC were taken into account in multivariate linear regression analyses, differences in CD3, CD4, and CD138 remained significant. There were very few granzyme B^+ cells in most patients [median score: 0.5 (IQR: 0-1)], and the score was not different between current and ex-smokers with COPD.

Bronchial inflammatory cell counts and duration of smoking cessation

There were significant differences for $CD4^+$, $CD8^+$, $CD8^+/CD3^+$, and $CD138^+$ cells between current smokers, short-term ex-smokers, and long-term ex-smokers with COPD (Table 3). Short-term ex-smokers had higher $CD4^+$ and $CD8^+$ cell numbers than current smokers (p=0.017, p=0.023, respectively; Table 3, Figure 2). These differences persisted after adjustment for differences in postbronchodilator FEV_1/IVC between the groups. In contrast, long-term ex-smokers had lower $CD8^+$ cell numbers than short-term ex-smokers (p=0.009), lower $CD8^+/CD3^+$ ratios than both current smokers (p=0.012) and short-term ex-smokers (p=0.003), and higher plasma cell numbers than current smokers (p=0.003; Table 3, Figure 2) and a trend towards significance versus short-term ex-smokers (p=0.069). When adjusting for differences in patient characteristics between the groups, all differences remained significant, except for the difference in $CD8^+$ cells between short-term and long-term quitters. On the other hand, the difference in plasma cells between short-term and long-term quitters became significant when adjusting for age.

Correlations between bronchial inflammation and smoking behavior

When defining current smoking as 0 years stopped, longer duration of smoking cessation was associated with higher numbers of $CD3^+$ cells (r_s=0.221, p=0.019), $CD4^+$ cells (r_s=0.194, p=0.040), $CD138^+$ cells (r_s=0.217, p=0.021), and a trend with CD4/CD8 ratios (r_s=0.181, p=0.056). Excluding current smokers, longer duration of smoking cessation was associated with lower CD8/CD3 ratios (r_s=-0.395, p=0.011), and a trend with higher numbers of $CD138^+$ cells (r_s=0.307, p=0.051). The number of pack-years smoked was inversely correlated with $CD138^+$ cells (R=-0.295, p=0.002).

DISCUSSION

The present study aimed to determine whether the inflammatory cell profile in the bronchial mucosa is different between current smokers and ex-smokers with COPD, and whether this profile is influenced by duration of smoking cessation. Ex-smokers had higher numbers of $CD3^+$, $CD4^+$, and plasma cells, whereas numbers of neutrophils, macrophages, eosinophils, mast cells, and $CD8^+$ cells were not different from current smokers. Interestingly, short-term smoking cessation (below the median value of our cohort, i.e. <3.5 years) was associated with higher $CD4^+$ and $CD8^+$ T-lymphocytes, whereas long-term smoking cessation (≥3.5 years) was associated with higher plasma cell numbers and lower CD8/CD3 ratios. These results indicate that the number of bronchial T-lymphocytes and plasma cells in patients with COPD is related to current smoking status and the duration of smoking cessation.

To our knowledge, this is the first study comparing bronchial inflammation in current and ex-smokers within a group of COPD patients, and examining the association with duration of smoking cessation. The observed higher numbers of $CD3^+$ and $CD4^+$ lymphocytes, and plasma cells in ex-smokers with COPD compared to current smokers with COPD is novel. Consistent with our results, one previous study also reported increased inflammation in peripheral airways of ex-smokers with mild COPD and mucus hypersecretion compared to current smokers, though the type of cells was not specified.[18] Our observation that large airway inflammation in COPD persists after smoking cessation is in line with preceding results in smaller numbers of patients with chronic bronchitis.[11] Rutgers *et al.*, also reported ongoing airway inflammation in ex-smokers with COPD when compared to healthy ex-smokers, however, in contrast to the present study, they did not include current smokers with COPD in the analysis.[19] Our results of T-lymphocytes and plasma cells being associated with duration of smoking cessation in COPD are not in line with a previous study, showing no difference in general peripheral airway inflammation between patients with COPD who quit >2 years, <2 years, and current smokers.[20] However, Lams et al. reported that CD8/CD3 ratios in peripheral airways are inversely associated with duration of smoking cessation in patients with and without airflow limitation.[12] Taken together, it can now be inferred that within a group of COPD patients, T-lymphocytes and plasma cell numbers are related to current smoking status and duration of smoking cessation, whereas other inflammatory cells are not.

It seems unlikely that our results are affected by methodological errors. To our knowledge, this is the largest study to date including bronchial biopsies of 114 well-characterised patients with stable COPD of GOLD stages II and III, not using inhaled or oral steroids, and without a clinical diagnosis of asthma. It needs to be emphasised that this was a cross-sectional study, and it cannot be ruled out that our ex-smoking group is a selected group of patients who quit smoking because they suffered more from smoking related symptoms, and may already have had different cell numbers before quitting. Nevertheless, in the present study ex-smokers had significantly less respiratory symptoms than current smokers, whilst having similar pack-years and duration of smoking. In addition, in our analysis we did adjust for clinical differences between the groups. We did not confirm smoking status by laboratory tests, and therefore cannot exclude that some ex-smokers were still smoking. However, this problem is comparable to those in other cross-sectional studies in this area.[10][11][19] A fully automated image analysis system was applied for cell counting in airway area sections.[17] We are aware that counting cells in a 2d manner has limitations, since it does not take into account the volume of the cell in a given sample – the smallest cells have the least chance to be counted in a single biopsy. Nevertheless, we were able to demonstrate differences in the smallest cells (LY) between the groups. There is still debate in the literature whether the theoretic basis of stereology fits well with the limitations of endobronchial biopsies.[21] Still,

most of the present data in the literature is based on counting profiles/area, what allows, although somehow limited by other methodological factors, comparison among studies. Because we observed no granzyme B^+ cells in the majority of biopsy sections analysed (56% of all sections), and no differences in a semi-quantitative score between the groups, we did not use digital image analysis for granzyme B^+ cell quantification, which is a more time-consuming procedure. Finally, we choose 3.5 years as a cut-off value for short-term versus long-term smoking cessation groups because this was the median duration of smoking cessation, providing equal sample sizes in both groups.

How can we interpret these data? The ongoing inflammation in ex-smokers with COPD suggests the presence of a persistent stimulus that may act independently of cigarette smoking. There are several potential mechanisms. For instance, chronic colonization of the airways with viral and/or bacterial pathogens in smokers with COPD[22], which may be responsible for the inflammatory response[23][24] and persist after smoking cessation. In agreement with this, it was observed previously that latent adenovirus could persist in ex-smokers with COPD, which is associated with amplification of inflammation.[25][26] Smoking may also trigger self-perpetuating inflammatory mechanisms by altering the balance between endogenous pro- and anti-inflammatory mechanisms, or – as recently suggested – may induce autoimmune-like phenomena.[27][28] In addition, it was recently observed that apoptosis of airway epithelial cells persists after smoking cessation in patients with COPD[29], which may induce persistence of inflammation. It has been suggested that in smokers without COPD or chronic bronchitis who stopped smoking, lung inflammation is at least partially reversible[9], whereas it persists in patients with COPD. Therefore, it is possible that the before mentioned stimuli may not persist in ex-smokers without COPD or chronic bronchitis.

The initial increase in $CD4^+$ and $CD8^+$ cells, and later increase in plasma cells, after smoking cessation might be explained by reversal of immunosuppression. Since both CD4 and CD8 cell numbers returned to similar levels as current smokers in long-term quitters, this reversal of immunosuppression seems transient for lymphocytes, but persistent for plasma cells. Smoking cessation may result in reversal of smoking-induced harmful effects on airway epithelial cells[30], such as a reduction in metaplastic secretory cell numbers in small airways[20][31], leading to improved lung defence mechanisms. Hence, the effector mechanisms of immunity to environmental antigens could be stimulated more efficiently in the absence of smoking, leading to a higher number of immunocompetent cells. Whereas the pro-inflammatory effects of active tobacco smoking have been extensively documented, it may also have selective anti-inflammatory effects, as has been described for acute effects of smoking.[32] Consistent with this and with our results, it was recently observed that cigarette smoke exposure reduced CD4 cell expansion following virus infection.[33] In addition, tobacco smokers have decreased serum levels of immunoglobulins IgG and IgA[33][34], suggesting that cigarette smoke modulates the humoral arm of adaptive immunity. Indeed, Soutar *et al.* described decreased numbers of IgA^+ cells in patients with fatal chronic bronchitis, and suggested that these patients were deficient in plasma cells.[35] These effects of decreased serum immunoglobulin levels appear to be reversible after smoking cessation.[36] It is possible that smoking cessation leads to an improved capacity of producing immunoglobulins in the airway mucosa of patients with COPD, by causing an increase in plasma cell numbers. Loss of these suppressive effects with smoking cessation may also explain our findings of increased numbers of inflammatory cells in ex-smokers, and thereby ameliorating lung defence mechanisms.

The decreased CD8/CD3 ratio after quitting for ≥3.5 years compared to current smokers and short-term quitters, and the lower $CD8^+$ cell numbers in long-term compared to short-term quitters, suggest that smoking cessation eventually may result in decreased $CD8^+$ cell

numbers. Since $CD8^+$ cells have been implicated in the pathogenesis of COPD, a decrease in number of these cells or their cytotoxic activity may have beneficial effects. However, our findings of very low numbers of cells that stain positive for the CD8 effector molecule granzyme B in the airways of patients with COPD, in the absence of differences between current and ex-smokers, suggest that these cells may not be cytotoxic. This may be because granzymes are involved in the pathogenesis of emphysema rather than in the conducting airways.[37] In this respect, it is interesting to note that antigen-specific $CD8^+$ T-lymphocytes can persist in the lung long after clearance of a respiratory virus, and that these cells are highly activated and can be stimulated to proliferate, but do not express constitutive effector functions.[38] In line with these findings, we did observe high numbers of granzyme B positive cells in lung tissue from children with childhood bronchiolitis obliterans, a disease that is thought to result from acute viral bronchiolitis.[39] In vitro studies may provide additional information by examining more extensively the possible lack of cytotoxicity of $CD8^+$ T-lymphocytes in COPD.

What could be the clinical implications of our findings? There is good evidence that smoking cessation results in a decrease in respiratory symptoms[7], a lower decline in FEV_1[6], and a reduction of airway hyperresponsiveness.[8][40] Our data suggest that T-lymphocytes and plasma cell numbers change after smoking cessation in COPD, while other inflammatory cells persist. Whether these, relatively small, changes in T-lymphocytes and plasma cell numbers contribute to the clinical benefits of smoking cessation in patients with COPD, remains to be established in longitudinal studies. The results propose that smoking cessation may result in improvement of the local humoral immunity, which may result in less respiratory infections and exacerbations, and thereby a reduced progression of COPD. However, the data also suggest that the clinical benefits of smoking cessation do not simply result form a reduction in inflammatory cells. The mechanisms causing this sustained inflammatory pattern after smoking cessation in COPD remain to be clarified.

In conclusion, the present study has shown that ex-smokers with COPD have higher numbers of bronchial $CD4^+$ and plasma cells than current smokers, whereas numbers of neutrophils, macrophages, and $CD8^+$ cells are not different between both groups. T-lymphocytes are higher in short-term quitters, whereas longer duration of smoking cessation is associated with lower CD8/CD3 ratios, and higher numbers of plasma cells. This suggests that changes in T-lymphocyte and plasma cell numbers may contribute to the clinical benefits of smoking cessation in COPD.

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ETHICS APPROVAL
The medical ethics committees of the Leiden University Medical Center and the Groningen University Medical Center approved the study.

COMPETING INTEREST STATEMENT
PS and PH have received, as members of the department of Pulmonology of the LUMC, a grant from GSK (UK and NL) as part of the sponsorship of this GLUCOLD study. The other authors declare no competing interests.

FIGURE LEGENDS

Figure 1.
(A) Bronchial biopsy section of a patient with COPD stained with haematoxylin/eosin, showing goblet cell hyperplasia of the bronchial epithelium (*arrow*), fibrosis (*FI*), and scattered inflammatory cells in the submucosa. (B) Plasma cell staining (CD138). The epithelial layer also stained positive, however for the analysis only the subepithelial layer was taken into account. (C) $CD8^+$ T-lymphocyte staining. (D) $CD4^+$ T-lymphocytes staining, also showing squamous cell metaplasia of the epithelium (*arrow*). Scale bars: (A) 100 μm, (B, C and D) 20 μm.

Figure 2.
Difference in CD4, CD8, CD8/CD3, and CD138 cell counts in the lamina propria of smokers (S), ex-smokers who quit <3.5 years ago (<3.5 ex-S), and who quit ≥3.5 years ago (≥3.5 ex-S) with COPD. Data are presented as box plots (median, IQR, range) of the number of cells /0.1 mm^2 tissue examined or ratios. * $p<0.05$: 2-tailed unpaired t-tests of log transformed data (square root in case of CD8/CD3).

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Table 1. Antibodies used for immunohistochemistry

Ab	Pretreatment	Species	Dilution	Clone	Origin*
CD3 (lymphocyte)	citrate	rabbit	1:500	polyclonal	DAKO
CD4 (T-helper)	citrate	mouse	1:300	CD4-368	Novocastra
CD8 (T-cytotoxic)	citrate	mouse	1:50	CD8-144B	DAKO
Neutrophil elastase (neutrophil)	protease	mouse	1:3200	NP57	DAKO
CD68 (macrophage)	citrate	mouse	1:3000	KP1	DAKO
Tryptase (mast cell)	citrate	mouse	1:16000	AA1	DAKO
EG2 (eosinophil)	trypsin	mouse	1:150	EG-2	Pharmacia
CD138 (plasma cell)	EDTA	mouse	1:6400	B-B4	IQ products
Granzyme B	citrate	mouse	1:200	CLB-GB7	CLB/Sanquin

Footnote table 1.
* Dako, Glostrup, Denmark; Novocastra, Newcastle upon Tyne, United Kingdom; Pharmacia Diagnostics, Uppsala, Sweden; IQ products, Groningen, The Netherlands; CLB/Sanquin, Amsterdam, The Netherlands

Table 2. Patient Characteristics of current smokers and ex-smokers groups with COPD

	COPD Current smokers	COPD Ex-smokers, combined group	COPD Ex-smokers, quit <3.5 yrs	COPD Ex-smokers, quit ≥3.5 yrs
General				
Sex (M/F, n)	59 / 13	40 / 2 *	20 / 1	20 / 1
Age (yrs)	60 ± 8	64 ± 7 *	61 ± 8	67 ± 4 *†
Pack-years (yrs)	43 (32-56)	37 (28-53)	45 (29-65)	35 (26-41) *
Duration of smoking cessation (yrs)	-	3.5 (1-10)	1.0 (1.0-2.0)	10 (6.5-14.5)
Smoking duration (yrs)	44 ± 8	41 ± 10	43 ± 11	39 ± 8
Chronic bronchitis (%)	55.6	31.0 *	23.8 *	38.1
Lung Function				
Postbronchodilator FEV_1 (%pred)	63.3 ± 8.3	62.5 ± 9.6	62.6 ± 10	62.5 ± 9.4
Postbronchodilator FEV_1/IVC (%)	49.5 ± 8.5	46.0 ± 8.3 *	45.3 ± 8.6 *	46.7 ± 8.1
ΔFEV_1 (%pred)	6.9 ± 5.2	6.8 ± 4.5	6.9 ± 3.9	6.8 ± 5.1
K_{CO} (%pred)	73.3 ± 25.1	80.4 ± 25.9	75.3 ± 24.9	85.7 ± 26.5 *

Footnote table 2.

Data are presented as mean ± standard deviation or median (IQR: 25^{th} - 75^{th} percentile), ex-smokers divided in two groups based on median duration of smoking cessation (3.5 years). FEV_1= Forced expiratory volume in one second, IVC= Inspiratory vital capacity, ΔFEV_1= Reversibility to salbutamol (change in FEV_1 as percentage of predicted), K_{CO}= Diffusing capacity for carbon monoxide per liter alveolar volume, pred= predicted.

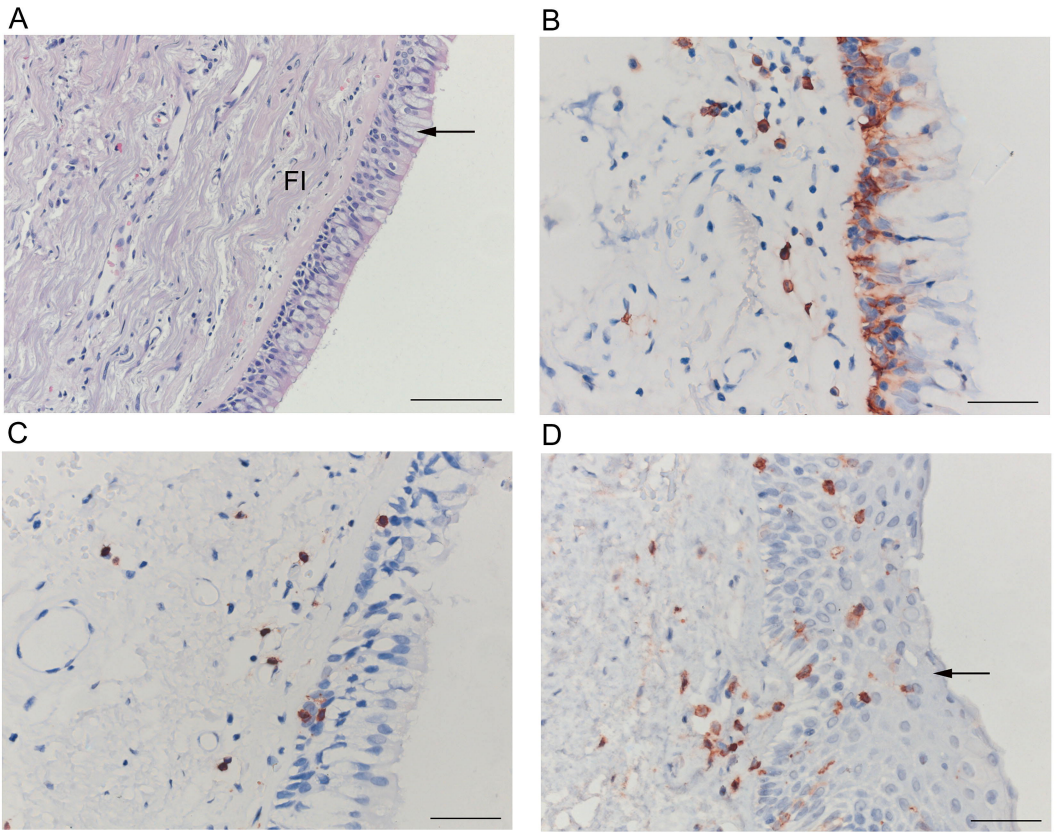
* $p<0.05$: significant difference from COPD current smokers [Chi-square tests for sex differences, 2-tailed unpaired t-tests for other (log-transformed) data];

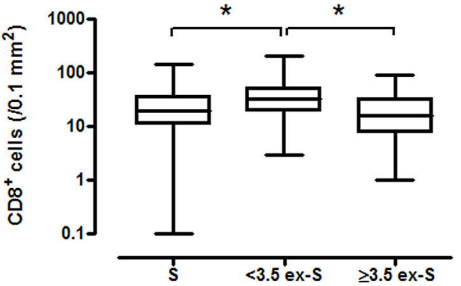
† $p<0.05$: significant difference from COPD ex-smokers who quit <3.5 yrs (2-tailed unpaired t-tests).

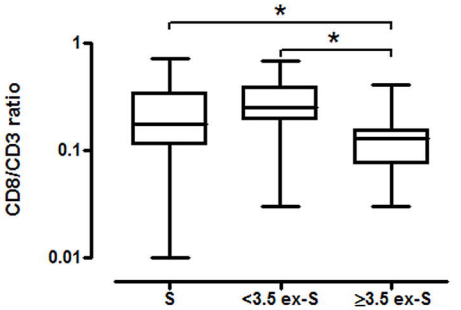
Table 3. Bronchial inflammatory cell counts of current smokers and ex-smokers groups with COPD

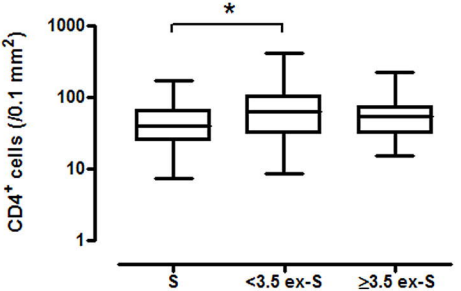
Cell marker	COPD Current smokers	COPD Ex-smokers, combined group	COPD Ex-smokers, quit <3.5 yrs	COPD Ex-smokers, quit ≥3.5 yrs	p value ‡
CD3	108 (61-164)	149 (88-225) *	137 (93-229)	170 (62-221)	0.097
CD4	40 (25-66)	58 (32-90) *	64 (30-111) *	54 (32-75)	0.045
CD8	20 (11-37)	24 (8.8-41)	34 (18-54) *	16 (7.8-32) †	0.023
CD4/CD8	2.0 (1.1-3.7)	2.7 (1.3-5.0)	2.1 (1.2-3.3)	3.1 (1.8-6.4)	0.065
CD4/CD3	0.4 (0.3-0.6)	0.4 (0.3-0.8)	0.5 (0.3-0.8)	0.4 (0.3-0.6)	0.680
CD8/CD3	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.3 (0.2-0.4)	0.1 (0.1-0.2) *†	0.008
NE (neutrophils)	4.0 (2.0-7.8)	4.5 (2.0-9.0)	5.0 (2.6-9.0)	4.0 (1.8-9.8)	0.289
CD68 (macrophages)	8.5 (4.1-12)	11 (5.8-18)	11 (5.8-17)	8.5 (5.8-18)	0.124
EG2 (eosinophils)	1.3 (0.5-3.0)	2.0 (0.5-5.5)	1.5 (0.5-2.8)	3.5 (0.5-11)	0.183
AA1 (mast cells)	28 (20-34)	26 (18-35)	28 (22-36)	24 (14-33)	0.303
CD138 (plasma cells)	7.5 (3.1-14)	9.0 (5.5-20)*	7.8 (4.0-11)	12 (7.8-24) *	0.013

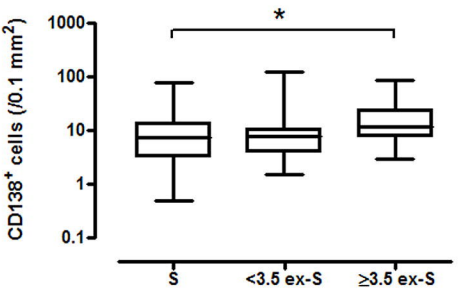
Footnote table 3.
Data are presented as median cell number /0.1 mm^2 (IQR), ex-smokers divided in two groups based on median duration of smoking cessation (3.5 years).
* $p<0.05$: significant difference from COPD current smokers (2-tailed unpaired t-tests of log-transformed data, square root in case of CD8/CD3);
† $p<0.05$: significant difference from COPD ex-smokers who quit < 3.5 yrs (2-tailed unpaired t-tests of log-transformed data, square root in case of CD8/CD3);
‡ p value from one-way ANOVA (Kruskal-Wallis test in case of EG2) of log-transformed data (square root in case of CD8/CD3) between current smokers, < and ≥3.5 yrs ex-smokers with COPD.


A
FI
B
C
D


CD8+ cells (/0.1 mm2)
1000
100
10
1
0.1
*
*
S
<3.5 ex-S
≥3.5 ex-S


CD8/CD3 ratio
1
0.1
0.01
*
*
S
<3.5 ex-S
≥3.5 ex-S


CD4+ cells (/0.1 mm2)
1000
100
10
1
*
S
<3.5 ex-S
≥3.5 ex-S


CD138+ cells (/0.1 mm2)
1000
100
10
1
0.1
*
S
<3.5 ex-S
≥3.5 ex-S