Increased intraepithelial T-cells in stable COPD[☆]

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Summary

Background: The airway epithelium is the first line of defence in the response to inhaled particles and irritants. Chronic obstructive pulmonary disease (COPD) is an inflammatory disease characterised by an irreversible loss of lung function, with cigarette smoking as a major risk factor. Here, we address intraepithelial T-cells in COPD, as these cells are a distinct T-cell subtype thought to have important regulatory functions. We hypothesised that intraepithelial T-cells play a role in the response to lung irritants and that the T-cell populations would be altered and associated with signs of inflammation in COPD.

Methods: Bronchoscopy with endobronchial mucosal biopsy sampling was performed in 22 patients (mean age; 57) with stable COPD (median FEV_1% predicted: 51). Age- and smoking- matched smokers (S) with normal lung function ($n = 14$) and age-matched non-smokers (NS) ($n = 15$) served as controls. Airway inflammation was recorded visually using bronchitis index (BI). Biopsy specimens were processed into glycol methacrylate resin and inflammatory cells were stained immunohistochemically.

Results: The number of intraepithelial CD4+ T-cells were significantly higher in COPD patients compared to smokers as well as trend towards significance in non-smokers ($p = 0.005$ and $p = 0.036$, respectively), whereas intraepithelial CD8+ T-cells number were increased in patients with COPD compared to non-smokers ($p = 0.017$). Both

patients with COPD and smokers had a higher BI than non-smokers ($p < 0.001$ for both).
Conclusions: The present data suggest a role for intraepithelial CD4+ and CD8+ T-cells in stable COPD and indicate that T-cells are of importance in the long-term inflammatory response in COPD or, alternatively, play a regulatory role.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by a gradual and mainly irreversible loss of lung function. The most important risk factor for developing COPD is tobacco smoking, which leads to an inflammatory response in the lung.[1] In some smokers, this inflammation induces structural changes in the lung and, as a consequence, the development of COPD. Previous studies have shown an increased number of inflammatory cells, such as neutrophils and macrophages, in the bronchial mucosa of patients with mild to severe COPD compared to control subjects.[2] This has generated the hypothesis that COPD is associated with an amplified inflammatory response compared to smokers with normal lung function.[1]

Airway epithelial cells can produce mediators that initiate and modulate the host response to inhaled irritants.[3–5] This inflammatory response can be further amplified by cells infiltrating the epithelium. Intraepithelial T-lymphocytes seem to be of specific interest in this context, as they constitute a distinct T-cell subset with specific activation, regulatory capacity and subsequent capability of adhering to the bronchial epithelium, suggesting a role in an initial defence mechanism and T-cell cytotoxicity.[6] Ligands and receptors, such as CXCR3, might also play an important role in the inflammatory response.[7] Previous studies aiming to characterize the bronchial mucosa in COPD have mainly focused on subepithelial T-cells, reporting increased numbers of T-lymphocytes in this compartment in COPD.[8–10] In contrast, data on the intraepithelial T-cell population in COPD are sparse.

In the current study, we, therefore, addressed intraepithelial T-lymphocytes in the chronicity of COPD, since these cells are thought to play an important role in the first line of defence in the airways and may provoke long-term immune responses. We hypothesised that intraepithelial T-lymphocytes modulate the response to inhaled cigarette smoke and contribute to the pathogenesis of COPD. In addition, we sought to determine whether any association between intraepithelial inflammation, lung function and signs of bronchitis were present in COPD.

Methods

Patients and control subjects

Twenty-two COPD patients aged 39–69 years (mean age 57) were recruited (Table 1). All patients had a post-bronchodilator $FEV_1/FVC < 70\%$ and $FEV_1 < 80\%$ of predicted, according to GOLD criteria (www.goldcopd.org), as well as a smoking history of more than 10 pack-years. None had clinical or radiological signs of any other lung disease than COPD. Age-matched smokers with normal lung function ($n = 14$), and non-smokers ($n = 15$) served as controls. In addition, the control group of smokers with normal lung function was matched regarding smoking history assessed as pack-years. None of the control subjects had clinical signs of any lung disease and they all had a normal chest X-ray. No patient or control subject had a history of allergy or asthma and *in vitro* screening for the presence of specific IgE antibodies against common inhaled allergens (Phadiatop®, Pharmacia–Upjohn, Uppsala, Sweden) was negative in all participants. All subjects were in a stable condition (i.e., none had a respiratory tract infection within three months prior to the study), and no participant had received oral or inhaled corticosteroids during the three months preceding inclusion.

Ten of the 22 COPD patients and one of the smokers with normal lung function, met the criteria of chronic bronchitis, defined as cough and production of mucus most days of the months for at least three months in the previous two years.[11] Characteristics of patients with chronic bronchitis did not differ compared to those without.

Each participant gave written informed consent and the study was approved by the local ethics committee, Karolinska University Hospital, Stockholm, Sweden.

Table 1 Characteristics and lung function data on COPD patients, smokers with normal lung function (S) and non-smokers (NS)

	COPD (n = 22)	S (n = 14)	NS (n = 15)
Age (years)	57 ± 6	54 ± 7	56 ± 5
Sex (male/female)	12/10	6/8	6/7
Tobacco (pack-years)	34[###] (23–43)	34[§§§] (26–38)	0
Current smokers (n)	18	14	0
Chronic bronchitis (n)	10	0	0
FEV_1 (% of predicted)	51[***,###] (47–60)	100 (94–114)	109 (105–123)
FEV_1/FVC (%)	56[***,###] (52–62)	80 (76–84)	84 (82–84)
Reversibility (% of predicted FEV_1)	7[#] (1–8)	3 (0–5)	2 (0–3)

Data are shown as mean ± standard deviation for age, median and inter-quartile range for all others.

Significant difference between groups is marked with * (COPD vs S), # (COPD vs NS) and § (S vs NS). The considered levels of significance were $p < 0.05$ (*, # or §), $p < 0.01$ (**, ##, §§) and $p < 0.001$ (***, ###, §§§).

FEV_1: forced expiratory volume in one second, measured post-bronchodilation; and FVC: forced vital capacity.

Pulmonary function test

All subjects performed a dynamic spirometry in a standardised manner (Vitalograph®, Buckingham, UK), according to the recommendations of the American Thoracic Society.[12] Both slow vital capacity and forced vital capacity were performed before and 10 min after inhalation of two doses of 0.5 mg terbutaline (Bricanyl® Turbuhaler®; AstraZeneca, Södertälje, Sweden), and reversibility was calculated.

Bronchoscopy, bronchitis index and bronchial biopsies

Pre-medication with morphine—hyoscine (Morfin-skopolamin, Meda, Solna, Sweden) was given intramuscularly and lidocaine (Xylocain® AstraZeneca, Södertälje, Sweden) was applied topically whereupon bronchoscopy was performed with a flexible fiber-optic bronchoscope (Olympus F Type P30, Tokyo, Japan). During bronchoscopy the degree of inflammation was visually assessed as bronchitis index (BI).[13] This was defined as the sum of scores of the visual appearance of airways according to the presence or absence of abnormal edema, erythema, secretions and friability (0 = normal, 3 = remarkably abnormal). Consequently, the visual appearance of airway inflammation was assessed in a semi-quantitative manner on a scale ranging from 0 to 12 with the investigator blinded to the subject's status (COPD, healthy smokers or normal control).

Biopsy specimens were taken by use of pulmonary biopsy forceps with smooth edge jaws (Radial Edge® Biopsy Forceps, Boston Scientific, Boston, MA). From each subject, four to six endobronchial mucosal biopsies were taken from the sub-segmental carinae of the upper left lobe or the apical segment of the lower left lobe.

Processing and staining of bronchial biopsies

The mucosal biopsies were immediately fixed in ice-cold acetone with the inclusion of the protease inhibitors phenylmethylsulphonyl fluoride (2 mM) and iodoacetamine (20 mM). The biopsies were subsequently processed into glycol methacrylate (GMA) resin according to a previously described protocol.[14] The GMA blocks were stored in airtight containers at $-20\,^{\circ}$C until the staining procedures were initiated. In order to detect mucus-secreting cells (goblet cells) in the epithelium, two sections from each subject were stained with Alcian blue. Immunohistochemical staining with monoclonal antibodies (MoAbs) was performed as described in detail elsewhere.[15] Briefly, 2 μm sections were treated to inhibit endogenous peroxidases by applying a solution of 0.3% hydrogen peroxide in 0.1% sodium azide. Additionally, non-specific antibody binding was blocked by use of Dulbecco's minimal essential medium containing 10% foetal calf serum and 1% bovine serum albumin. Undiluted blocking medium was applied for 30 min and then poured off, whereupon mouse MoAbs (Table 2) were applied and incubated for 16—20 h at room temperature. Biotinylated rabbit anti-mouse immunoglobulin F(ab')$_2$ (Dako, Glostrup, Denmark) was added to each section and incubated for 2 h, and subsequently a complex of streptavidin—biotin—horseradish and peroxidase (Dako) was added

for 2 h. Aminoethyl carbazole in acetate buffer (pH 5.2) and hydrogen peroxide were used to induce a red colour, in this manner marking positive immunoreactions. All sections were counterstained with Mayer's haematoxylin. Primary antibody was omitted on sections serving as negative controls. Two stained sections from each participant were estimated with respect to epithelium quality, and the best of these was used for quantifying positively stained cells.

Quantification of staining in mucosal biopsy specimens

The intact epithelium was defined as the area above the basement membrane. The total length was calculated by use of computer assisted image analysis (Qwin, Leica Q500IW; Leica, Cambridge, UK). Quantification of positive stained cells was performed using a light microscope. The number of positive stained cells was expressed as cells per millimeter of epithelium as previously described.[15]

Statistical analysis

Statistical comparisons between the three groups were made using Kruskal—Wallis test and a p-value of less than 0.05 was considered significant. If the Kruskal—Wallis test indicated significance, the Mann—Whitney U-test was used for post hoc analysis for comparison between two groups, with corrections of p-values according to Bonferroni (a p-value less than 0.017 was considered significant). Correlations were calculated using Spearman's rank correlation test. The level of significance considered was 0.01. In case of a statistically significant result, the probability value (p-value) has been given.

Results

Bronchitis index

The COPD group as well as smokers with normal lung function displayed higher ($p < 0.001$ for both) bronchitis index than the non-smoker group (COPD 4, 3—6 (median and interquartile range); smokers with normal lung function 3, 3—4; non-smokers 1, 0—2). Bronchitis index did not differ significantly between COPD patients and smokers with normal lung function ($p = 0.06$). There was no difference in bronchitis index when comparing the COPD patients who fulfilled the criteria for chronic bronchitis to those without.

Biopsy findings

There was no significant difference in analysed epithelial length, expressed in mm, between the groups.[16] Positive staining for monoclonal antibodies revealed a ring-staining pattern for CD3+, CD4+ and CD8+ lymphocytes (Fig. 1). The number of intraepithelial CD3+ lymphocytes mm^{-1} epithelium in the COPD group (9.1, 0.7—27) (median, inter-quartile range) was higher compared to the number of intraepithelial CD3+ lymphocytes in smokers with normal lung function (1.5, 0—6.2) and to non-smokers (3.2, 0—8.3), but the difference did not reach statistical

Table 2 Monoclonal antibodies to epithelial inflammatory cells used in the present study

Antibody	Marker	Dilution	Clone	Source
Anti-CD3	T-lymphocytes CD3	1:1000	UCHT 1	DAKO, Glostrup, Denmark
Anti-CD4	T-helper cells CD4	1:15	MT310	DAKO, Glostrup, Denmark
Anti-CD8	Cytotoxic T-cells CD8	1:100	DK 25	DAKO, Glostrup, Denmark
Anti-CXCR3	Expression of interferon inducible CXC chemokines receptor	1:150	49801	R&D, Abingdon, UK
Anti-CD68	Macrophages CD68	1:100	PG-M1	DAKO, Glostrup, Denmark
Anti-NE	Neutrophil elastase	1:800	NP57	DAKO, Glostrup, Denmark
Anti-EG-2	Eosinophils ECP	1:150	EG2	Pharmacia—Upjohn Diagnostic AB, Sweden

significance ($p = 0.058$; Kruskall—Wallis). The number of intraepithelial CD4+ lymphocytes mm^{-1} epithelium was significantly higher in the COPD group (3.0, 0—7.1) compared to smokers with normal lung function (0, 0—0.2) as well as trend towards significance in non-smokers (0, 0—1.7) ($p = 0.005$ and $p = 0.036$, respectively). However, there was no difference in the number of intraepithelial CD4+ T-cells of COPD patients with a history of chronic bronchitis compared to those without ($p = 0.56$). The number of intraepithelial CD8+ lymphocytes mm^{-1} epithelium

was significantly higher ($p = 0.017$) in the COPD group (7.3, 0.8—17) compared to the non-smoking controls (0, 0—6.3), without a significant difference compared to smokers with normal lung function (1.8, 0—7.1) (Fig. 2).

Within the group of COPD patients, the majority of patients did not exhibit cells positive for CXCR3 within the epithelium. Notably, only the three COPD patients with the highest numbers of intraepithelial CD4+ T-lymphocytes (ranging from 11 to 18 CD4+ T-cells mm^{-1} epithelium) showed positive staining for CXCR3. No similar pattern was present for the COPD patients with high number of CD8+ T-cells.

Additionally, within the COPD group, the number of intraepithelial CD4+ T-cells was positively associated with the number of intraepithelial CD8+ T-cells; $R_s = 0.85$ ($p < 0.001$).

There was no difference in the CD4+/CD8+ ratio between the three groups. The number of epithelial airway inflammatory cells such as neutrophils, macrophages, eosinophils did not differ between the groups, neither did mucus staining with Alcian blue (data not shown). In COPD, no association was found between T-lymphocytes, lung function parameters or BI.

Discussion

In this study, we demonstrate differences in intraepithelial T-cell subsets in stable COPD patients compared to smokers with normal lung function and non-smokers. The visual appearance of the bronchial mucosa indicated signs of bronchitis, assessed as BI, in the two smoke-exposed groups compared to the non-smoking controls. However, no association was found between BI, lung function and the presence of inflammatory cells in the bronchial epithelium.

We collected endobronchial mucosal specimens using bronchoscopy in stable steroid-naïve COPD patients, i.e. the patients had no clinical history of present or recent exacerbation. In addition, the patients were recruited to this project solely for research purposes. They had no other diseases than COPD and they were not investigated for any other reasons than research. This gives an opportunity to include an age- and smoking- matched patient and control material, and it minimizes the risk of influence by a number of variables such as co-morbidity, medications and exacerbations. By the use of bronchoscopy it is also possible to sample endobronchial mucosal biopsies at predetermined sites in the airways reducing the risk of sampling error. Moreover bronchial biopsies sampled proximally through
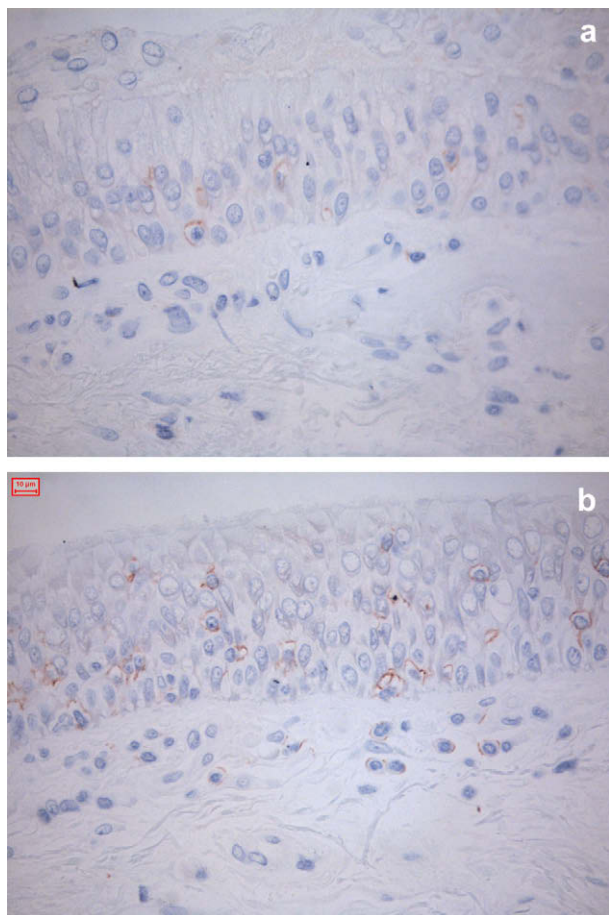

Figure 1 Representative photomicrographs showing bronchial epithelium of patients in the COPD group. Immunohistochemical staining of bronchial biopsies for: (a) CD4+ cells and (b) CD8+ cells.
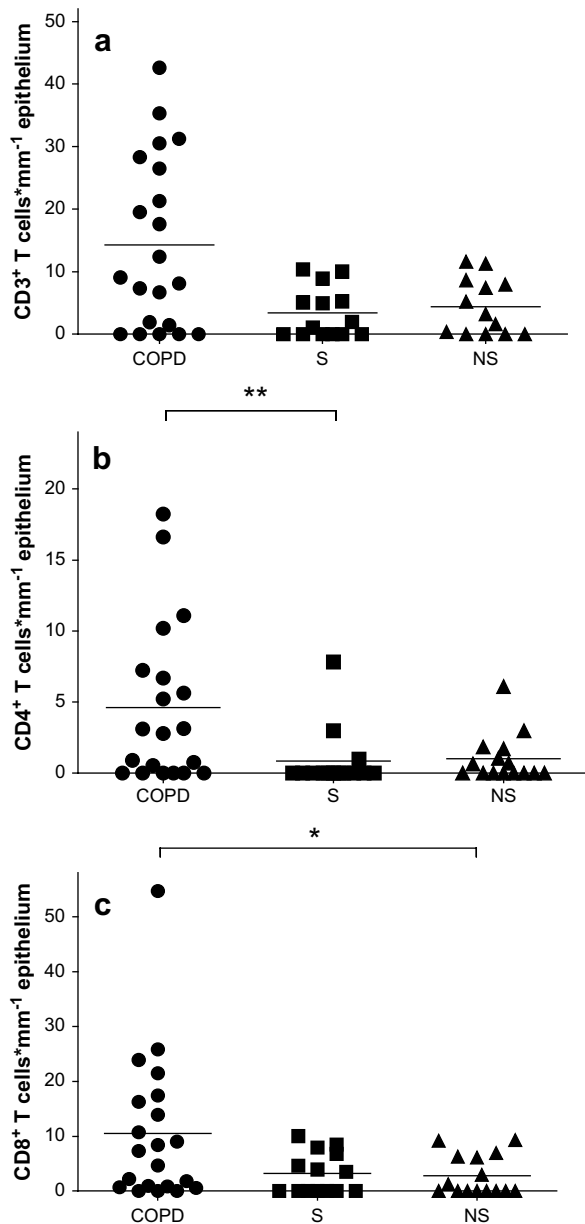
Figure 2 The number of T-lymphocytes mm^{-1} epithelium in bronchial biopsies, given for the three groups COPD, smokers with normal lung function (S) and non-smokers (NS). Each point depicts the result from one subject and the median of the group is marked with a horizontal bar. Significant difference between groups is marked with *($p < 0.017$) or **($p < 0.01$). Results given for: (a) CD3+ cells, (b) CD4+ cells, and (c) CD8+ cells.

the bronchoscope are suitable for the GMA embedding technique, which enables an excellent morphology, outstanding other immunohistochemical methods.[14]

The present data indicate increased CD4+ T-cells in bronchial epithelium in COPD compared to both control groups. In an early study by Fournier et al[17] it was reported increased numbers of intraepithelial lymphocytes in patients with chronic bronchitis compared to smoking and non-smoking controls. In that study, however, the degree

of airflow obstruction assessed as FEV$_1$ was not accounted for. Consequently, it was not clear whether the patients had COPD or not. In the present study, COPD patients with and without chronic bronchitis were compared and no differences in intraepithelial CD4 numbers were found between the two groups. Saetta et al[18] found no difference in intraepithelial T-lymphocytes when comparing patients with chronic bronchitis to asymptomatic smokers. The objective in the latter study was to address inflammation in the bronchial glands, and not primarily a difference in intraepithelial T-lymphocytes. In addition, Saetta et al included both patients with and without concomitant COPD. Regarding studies of populations consisting exclusively of COPD patients, the total number of leukocytes (CD45+) have been reported to be increased in the airway epithelium.[8]

Most studies in the past decade addressing airway inflammation in COPD have focused on the submucosal compartment, but the importance of studies examining the airway epithelium in COPD has recently been underlined.[19] This is crucial since the bronchial epithelium is the first line of defence against inhaled particles and toxic compounds as well as in the immune defence against microorganisms. Furthermore, the bronchial epithelium play an important role in the secretion of pro-inflammatory mediators and by intraepithelial lymphocyte cell−cell interactions. Apart from the role in the defence against inhaled particles, pollutants, viruses, bacteria and other microorganisms, epithelial cells may also interact with inflammatory and structural cells important in resolution after injury.

The increase in intraepithelial T-cells in COPD may have the potential of either promoting a T-helper immune response or exaggerating cytotoxic activity against injured cells in the epithelium.[20] The tendency towards amplified numbers of CD8+ and CD4+ intraepithelial lymphocytes, along with a positive association between the two subtypes, suggest a chronic immune response involving both these cell types in patients with stable COPD, despite no increased numbers of clinical exacerbation increased neutrophil numbers in the epithelium. Various triggers of epithelial responses may induce immune activation that would result in T-cell infiltration. It is well documented that both bacterial[21] and latent viral infection[22] are common in patients with COPD. Lymphocytes situated intraepithelially have features distinct from lymphocytes in the subepithelium,[6] such as long survival and elevated expression of $\alpha_E \beta_7$ (CD103). This latter fact has been suggested to imply a capacity of firm adhesion to epithelial cells, which is required to mediate T-cell cytotoxicity.[23] Thus, the epithelial T-cell infiltration seen here may be consistent with either a response to a latent intracellular infection or a chronic trigger of intraepithelial lymphocytes through injured epithelial cells. It should be considered that the increased CD8+ cells may indicate a cytotoxic response against stressed or injured epithelial cells due to long-term exposure to cigarette smoke. Such responsiveness of activated CD8+ cells has previously been reported to be dependent on up-regulation of the MHC class 1-like molecules MIC A and MIC B on stressed epithelial cells, and the expression of the counter receptor NKG2D on cytotoxic cells.[24] However, it is not yet demonstrated that cigarette smoke can induce such T-cell activation.

We found that the three COPD patients with the highest number of CD4+ cells also had the highest expression of CXCR3, which is a receptor for interferon inducible CXC chemokines such as IP-10, MIG and I-TAC. Lung lymphocytes in patients with COPD secrete more interferon inducible proteins, and CXCR3 might be involved in the recruitment of CD4+ cells to the epithelium.[7,25] CXCR3 is a marker for Th1 cells and it is suggested that Th1 lymphocytes in the lungs of people with smoking-related damage, drive the progression of emphysema through CXCR3 ligands. Based on our data, it is not possible to conclude any Th1 bias of the increased intraepithelial CD4+ population.

In the COPD patients, in contrast to the findings by Saetta et al, who have shown increases in PMNs in the subepithelium of COPD patients,[26] no increased numbers of intraepithelial neutrophils were found in the present study. Neither were any signs of enhanced mucus secretion, assessed as goblet cells number detected. This might be due to the fact that the COPD patients in this study were stable, and without any clinical signs of exacerbations. The epithelial lymphocyte response in COPD might thus be a sign of a chronic immune response, rather than persistent inflammatory infiltrates in the bronchial epithelium.

In conclusion, we report increased numbers of intraepithelial lymphocyte subsets in bronchial biopsies from COPD patients. Based on our findings in the present report, we suggest that intraepithelial T-lymphocytes could be of importance in the long-term chronicity of the disease and may play a regulatory role in the inflammatory response.

Acknowledgements

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