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Increased number of B-cells in bronchial biopsies in COPD

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ABSTRACT: Recently, it has been shown that the accumulated volume of B-cells in small airways is increased in chronic obstructive pulmonary disease (COPD) Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages 3 and 4. Little is known about the number of B-cells in central airways in COPD.

The present authors hypothesised that the number of B-cells in bronchial biopsies of large airways is higher in patients with COPD than in controls without airflow limitation and higher in more severe COPD. Therefore, bronchial biopsies were collected from 114 COPD patients (postbronchodilator forced expiratory volume in one second (FEV_1) 63±9 % predicted value, FEV_1/inspiratory vital capacity (IVC) 48±9%) and 28 controls (postbronchodilator FEV_1 108±12 % predicted value, FEV_1/IVC 78±4%).

Paraffin sections were stained for B-cells (CD20+) and their number was determined in the subepithelial area (excluding muscle, glands and vessels). B-cell numbers were higher in patients with COPD *versus* controls (8.5 *versus* 3.9 cells·mm^{-2}, respectively) and higher in patients with GOLD severity stage 3 (n=11) than stage 2 (n=103; 22.3 *versus* 7.8 cells·mm^{-2}).

No relationship was found between the number of B-cells and clinical characteristics within the chronic obstructive pulmonary disease group. The authors suggest that these increased B-cell numbers may have an important contribution to the pathogenesis of chronic obstructive pulmonary disease.

KEYWORDS: Autoimmunity, biopsies, inflammation, pathophysiology, smoking

Chronic obstructive pulmonary disease (COPD) is characterised by an abnormal inflammatory response of the lungs to noxious particles or gases. In addition, there seems to be an abnormal and insufficient tissue repair to counteract the destructive effects of cigarette smoke and accompanying inflammation. The inflammation persists even after smoking cessation [1]. Altogether, this results in epithelial changes, airway wall fibrosis and emphysematous lesions in lung parenchyma. Although exposure to cigarette smoke is the main risk factor for the development of COPD in the industrialised world, only a minority of smokers develops the disease indicating that other factors must play a role.

Recently, the hypothesis of an autoimmune component in the pathogenesis of COPD was put forward [2]. Such an autoimmune response could include activation of (autoreactive) B-cells [3]. Additionally, it has recently been shown that the accumulated volume of B-cells in small airways is increased in Global Initiative for Chronic Obstructive Lung Disease (GOLD) [4] stage 3 and 4 COPD [5]. These small airways can only be assessed in surgical resection material. Little is known about the number of B-cells in the central airways of patients with COPD. Therefore, the presence of B-cells in bronchial biopsies was evaluated. It was hypothesised that the number of B-cells in central airway biopsies is higher in patients with COPD than in smoking and ex-smoking controls without airflow limitation, and higher in more severe COPD.

METHODS

Patients

A total of 142 patients was included. Of these, 114 were clinically stable patients with COPD participating in an ongoing study (the Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease (GLUCOLD) Study) [6] and 28 were controls without airflow limitation. All participants met the following criteria: aged

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45–75 yrs; ex- or current smoker with ⩾10 pack-yrs; and no history of asthma. Furthermore, patients with COPD had irreversible airflow limitation, *i.e.* postbronchodilator forced expiratory volume in one second (FEV_1) and FEV_1/inspiratory vital capacity <90 % confidence interval of the predicted value, FEV_1 ⩾1.3 L and >20 % of the predicted value, as well as one or more of the following symptoms: chronic cough; chronic sputum production; or dyspnoea on exertion. Patients had not used a course of steroids during the last 3 months prior to randomisation, and had not had maintenance treatment with inhaled or oral steroids during the last 6 months. The study was approved by the medical ethics committees of both university hospitals (The Netherlands). All participants gave their written informed consent.

Pulmonary function tests

Spirometry was performed as described previously [6]. Airway responsiveness was measured as the provocative concentration of methacholine causing a 20% fall in the FEV_1 (PC_{20}) using the 2-min tidal breathing method. In four patients with COPD, a PC_{20} was not performed because of an FEV_1 <1.2 L. Two controls were unable to complete the test because of discomfort.

Biopsies and immunohistology

Four bronchial biopsies were collected from each patient by flexible bronchoscopy from the lower lobe sub-segmental carinae, following local anaesthesia with lidocaine. For the current analysis, the best paraffin biopsy out of these four was selected (*i.e.* of sufficient volume and without crushing artefacts or large blood clots) and then one paraffin section of 4 μm per patient was stained with monoclonal anti-CD20 (L26; Dako, Glostrup, Denmark), using an immunoperoxidase streptavidin-biotin method (LSAB+ Kit; Dako). Together with hydrogen peroxide, 3-amino-9-ethyl carbazole (AEC+ Substrate-Chromogen; Dako) was used as a substrate. Computer-aided image analysis was performed to determine and mark the total subepithelial surface area of each bronchial section, thereby excluding the area occupied by muscle, glands and vessels. The number of CD20+ cells in the marked area was counted by the same, blinded observer using light microscopy and was expressed as the number of cells per square millimetre of subepithelial area (cells·mm^{-2}). Clusters of CD20+ cells, defined as a collection of cells in which individual cells could not be discriminated and therefore counted, were not included in the total CD20+ cell counts, but were documented separately. Finally, a random selection of 30 sections was recounted to determine the intra-observer variation.

Statistical analysis

Comparisons between groups were performed using the non-parametric Mann-Whitney U-test. Correlations were calculated by Spearman's rank correlation test. Intra-observer variation was assessed by the intraclass correlation coefficient [7], which reflects the level of agreement for repeat counts by the same observer.

RESULTS

Clinical findings

In total, 114 patients with clinically stable COPD (GOLD stage 2, n=103; GOLD stage 3, n=11) [4], and 28 controls without airflow limitation were included in the study. Patient characteristics are presented in table 1. Patients with COPD were significantly older, had more pack-yrs, a lower PC_{20} methacholine, were more often male and more often ex-smoker compared with control subjects (all $p<0.05$).

Biopsy findings

The biopsy material of one COPD patient was excluded because of poor quality. From the other 113 COPD subjects and 28 control subjects, a section of a bronchial biopsy with a median (interquartile range (IQR)) subepithelial surface area of 1.43 mm^2 (1.03–1.81) and 0.94 mm^2 (0.62–1.08), respectively ($p<0.05$), was stained for B-cells. Figure 1 illustrates the CD20+ staining of a bronchial section from a COPD patient. The intra-observer variation assessed by the intraclass variation coefficient was 0.98.

One to three clusters of CD20+ cells were observed in bronchial sections of eight (7%) COPD patients and two (7%) controls.

TABLE 1 Patient characteristics

	COPD	Controls
Subjects n	114	28
Male/female n	99/15	11/17
Age yrs	62±8	51±4
Current/ex-smokers n	72/42	27/1
Smoking history pack-yrs#	42 (31–55)	31 (25–37)
Postbronchodilator FEV_1 % pred	63±9	108±12
Postbronchodilator FEV_1/IVC %	48±9	78±4
PC_{20} methacholine mg·mL^{-1}¶	0.6±2.8	26.7±1.7

Data are presented as mean±SD, unless stated otherwise. #: median (25th–75th percentile); ¶: geometric mean±doubling dose. COPD: chronic obstructive pulmonary disease; FEV_1=forced expiratory volume in one second; % pred=percentage of predicted value; IVC=inspiratory vital capacity; PC_{20} methacholine=the provocative concentration of methacholine causing a 20% fall in FEV_1.

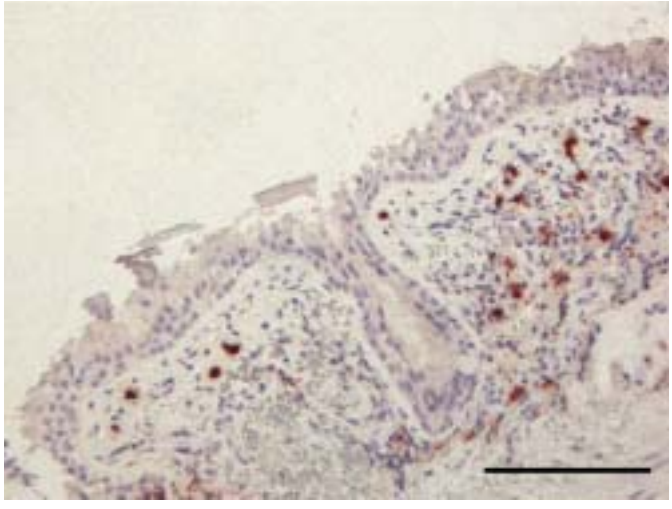

FIGURE 1. CD20+ B-cells (red) in a bronchial biopsy from a patient with chronic obstructive pulmonary disease (immunoperoxidase). Scale bar=100 μm.

The observed clusters did not resemble bronchus-associated lymphoid tissue, as judged by the absence of centroblasts and centrocytes.

The number of CD20+ cells·mm^{-2} subepithelial area was significantly higher in patients with COPD than in controls (median (IQR) 8.5 cells·mm^{-2} (3.8–18.3) *versus* 3.9 cells·mm^{-2} (1.6–10.3) respectively, p=0.007; fig. 2a). Results were similar when only current smokers (with and without COPD) were considered (8.1 cells·mm^{-2} (3.7–14.5) *versus* 3.6 cells·mm^{-2} (1.5–10.5) respectively, p=0.04; fig. 2b). Within the COPD group, the number of CD20+ cells tended to be higher in ex-smokers than in current smokers (9.6 cells·mm^{-2} (5.1–42.5) *versus* 8.1 cells·mm^{-2} (3.7–14.5) respectively, p=0.06; fig. 2c). Excluding the 10 subjects with CD20+ clusters did not affect the results (data not shown).

Relationship with COPD severity and other clinical characteristics

B-cell numbers were significantly higher in individuals with COPD stage 3 than stage 2 (22.3 cells·mm^{-2} (11.8–60.7) *versus* 7.8 cells·mm^{-2} (3.6–16.9), respectively, p=0.004; fig. 2a) and higher with COPD stage 2 than in controls (7.8 cells·mm^{-2} (3.6–16.9) *versus* 3.9 cells·mm^{-2} (1.6–10.3), respectively, p=0.02; fig. 2a). Within the total group of both patients with COPD and controls, higher numbers of CD20+ cells correlated with lower values of postbronchodilator FEV1/vital capacity (%; r_s=-0.18; p=0.03) and more severe airway hyperresponsiveness (r_s=-0.19; p=0.03), and tended to correlate with lower values of postbronchodilator FEV1 % predicted value (r_s=-0.15; p=0.08). The number of CD20+ cells was not significantly related to age or pack-yrs and was not different between sexes. Within the group of COPD patients the number of CD20+ cells·mm^{-2} subepithelial area showed no significant correlation with any of these variables.

DISCUSSION

In the present study, it was demonstrated that the number of B-cells in bronchial biopsies of central airways is higher in patients with moderate-to-severe COPD than in controls without airflow limitation. These results were similar when only currently smoking subjects were considered.

The number of B-cells in central airways in a large number of COPD patients and controls was investigated. The present findings contrast with the results of O'SHAUGNESSY *et al.* [8], who did not find a difference in the number of B-cells in bronchial biopsies of large airways between patients with COPD and healthy non-smoking controls. They included 29 individuals, whereas the current authors investigated 114 COPD patients and 28 controls, thus having more power to observe differences in B-cell numbers. In resected lung tissue, BOSKEN *et al.* [9] demonstrated more B-cells in the adventitial layer of small airways in smokers with airflow limitation *versus* smokers without airflow limitation [9]. In lung tissue, HOGG *et al.* [5] recently showed an increase in the accumulated volume of B-cells in small airways with increasing severity of COPD. These B-cells were partially arranged in follicles [5]. B-cell clusters were present in bronchial biopsies of only 7% of the total study population, whereas HOGG *et al.* [5] demonstrated B-cell follicles to be present in ~5–35% of the small airway examined. There are two important differences between the current study and the study by HOGG *et al.* [5]. Firstly, demographic characteristics of the study subjects were different. The present COPD patients had less severe COPD

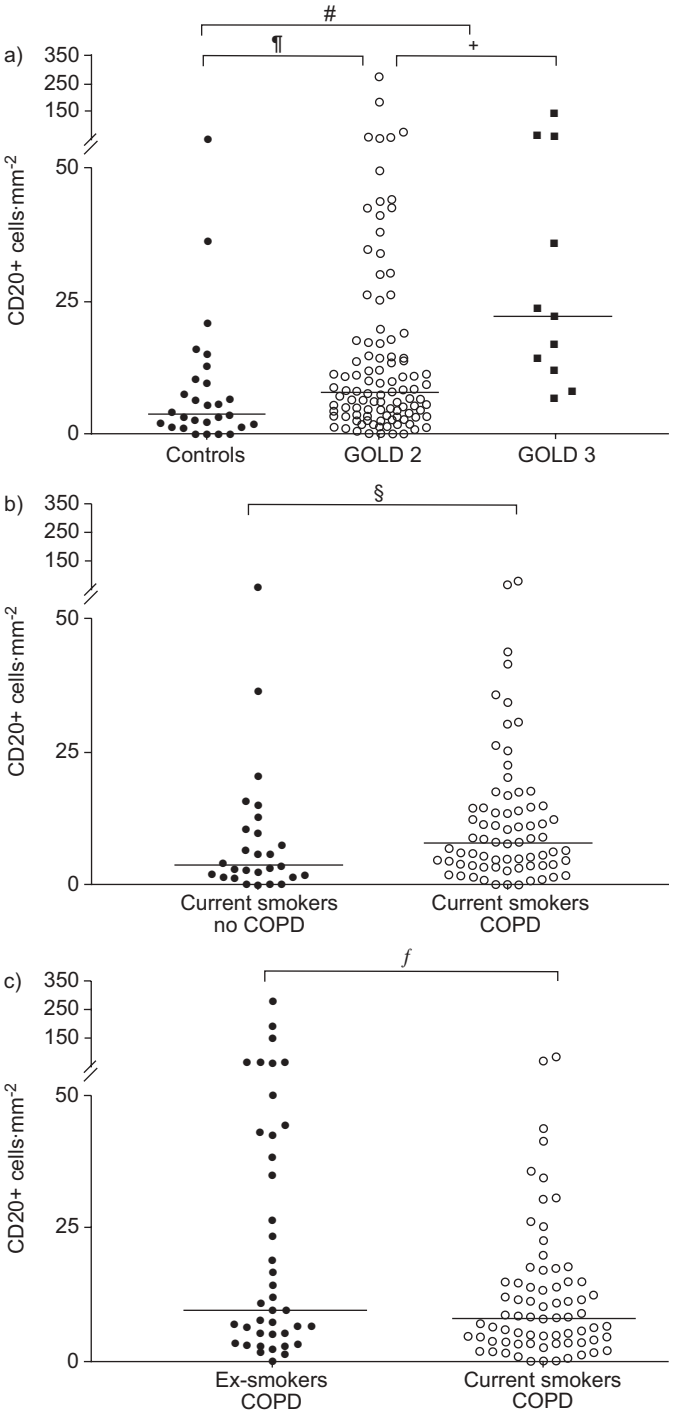

FIGURE 2. The number of B-cells (CD20+) per mm^2 subepithelial area in bronchial biopsies. a) Controls *versus* patients with stage 2 chronic obstructive pulmonary disease (COPD) *versus* patients with stage 3 COPD. b) Current smokers without COPD *versus* current smokers with COPD. c) Ex-smokers with COPD *versus* current smokers with COPD. GOLD: Global Initiative for Chronic Obstructive Lung Disease. The horizontal bar represents the median. $^{\#}$: p=0.007; ¶: p=0.02; $^{+}$: p=0.004; §: p=0.04; ^{f}: p=0.06.

than in the other study. HOGG *et al.* [5] demonstrated that the number of B-cell follicles increased in stage 3 and 4 COPD, whereas most of the present COPD patients had stage 2 COPD (n=103) and a minority had stage 3 COPD (n=11). Secondly, perhaps the most crucial difference is that the current authors studied large airways using bronchial biopsies, while HOGG *et al.* [5] examined small airways in resected lung tissue. It is important to emphasise that the present authors studied a different compartment and that bronchial biopsies in general are small and do not extend very deeply into the airway wall. Although it may very well be that B-cells in large airways do not show an arrangement of B-cells in follicles similar to small airways, the use of mucosal biopsies is not adequate to obtain this particular information. Nevertheless, the present study shows additional new data on significant differences in numbers of B-cells that are not aggregated in follicles, which extends and supports the previously published data.

There are a few issues in the current study that need to be addressed. The controls did not perfectly match the group of COPD patients. The latter were older, more often male and had more pack-yrs. The fact that B-cell numbers did not correlate with age, sex or pack-yrs argues against the reasoning that these differences will have biased the results. Additionally, the COPD patients had a lower PC_{20} than the controls. In the lung health study [10], it has been documented that approximately two-thirds of patients with COPD have increased airway responsiveness. Within the total study population (COPD and controls) a weak, but significant, association was found between PC_{20} and the number of B-cells. However, within the COPD group no such association was present. This probably implies that the association between PC_{20} and B-cells in the current study population is due to the differences in PC_{20} and B-cells between COPD patients and controls *per se*, rather than by an actual association of PC_{20} and B-cells. A previous study of 34 smokers with COPD demonstrated no association between the severity of airway hyperresponsiveness and the number of B-cells in bronchial biopsies [11]. Taken together, the current authors do not expect that the difference in PC_{20} between the COPD patients and controls has biased the results. The fact that a higher percentage of patients with COPD were ex-smokers than of controls did not influence the outcome of the present analysis, since the results were similar when only current smoking subjects were included. Ideally, multiple sections from multiple biopsies of each individual would be collected to better reflect the heterogeneity of the inflammatory response in COPD [12], but this is not always possible given the scarcity of pathological material. One histological section per subject was examined for the current analysis, but a large number of subjects were included in the current study and this should compensate for the overall variation. Moreover, the current authors believe that the fact of having examined one histological section instead of more would especially be of concern in case no difference in B-cell numbers between the two groups was found. If the histological sections were not representative of the levels of airway inflammation in the subjects, for reasons of variability the power to detect differences would diminish, increasing the risk of finding false-negative results. However, a difference in B-cell numbers between the two groups in the current study was demonstrated.

Higher numbers of B-cells can result from, *e.g.* a local inflammatory process, an altered T-helper (Th)1–Th2 balance or can reflect an antigen-specific reaction. At present, the first possibility cannot be excluded, *i.e.* that these B-cells are part of an innate response or represent a bystander effect in an inflammatory process. There is no conclusive literature available that assesses a Th1–Th2 imbalance in airway tissue, and inconsistent results have been reported in blood [13]. Regarding an antigen-specific role of these B-cells in COPD, it is tempting to speculate that they are directed against respiratory pathogens, such as viruses or bacteria [14], possibly resulting from stimulation by microbial superantigens [15]. Such stimulation leads to the activation and proliferation of different clones of B-cells. Information was not collected regarding respiratory infections, for example by sputum culture from the study patients. However, this is not regarded as a full explanation for the presence of B-cells since markedly more B-cells in mice with smoke-induced emphysema were observed when compared with control littermates, while all these mice were kept under the same controlled conditions [16]. An alternative explanation for the accumulation of B-cells in airway walls may be a local antigen-specific reaction to components of cigarette smoke. This could then be a consequence of an underlying mechanism that predisposes the susceptible smoker to a more pronounced B-cell reaction directed at smoke constituents than a nonsusceptible smoker. Related to the latter is a final possible explanation that the B-cell reaction is against one or more constituents normally present in the airway wall, such as extracellular matrix molecules, that may have been changed or broken down as a result of smoke exposure. These altered structures could then behave like auto-antigens, fitting in with the auto-immune hypothesis that has been put forward as a component of COPD pathogenesis [3]. The proposed concept of antigen specificity would be supported by further analyses to determine whether a clonal expansion of localised B-cells can be found. This could be done by performing variable heavy chain gene analysis after laser microdissection of the B-cells. Additionally, blotting of lung homogenate or specific extracellular matrix components with serum from patients with COPD might provide additional information on possible antigen specificity.

Similar to the results found in small airways by HOGG *et al.* [5], the present authors found a significant difference in the number of B-cells at the different COPD severity stages (GOLD), although the organisation of the B-cells was partially different in the present study and in that of HOGG *et al.* [5]. The association of B-cell numbers and COPD severity stages is certainly compatible with a pathogenetic role of these B-cells and is, therefore, very interesting, but the current authors do not think that this association proves such a role.

In conclusion, the present study demonstrated higher numbers of B-cells in bronchial biopsies from central airways of chronic obstructive pulmonary disease patients than of (ex-)smokers without airflow limitation. The current authors hypothesise that B-cells have a role in the pathogenesis of the disease. Further identification of antigen specificity of these B-cells should aid in defining their role in chronic obstructive pulmonary disease.

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