Bronchial mucosal dendritic cells in smokers and ex-smokers with COPD: an electron microscopic study

Short title: Smoking & bronchial dendritic cells in COPD

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Keywords: dendritic cell, COPD, smoking, ultrastructure

Word count (Text): 3491

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Abstract (words: 286)

**Background:** Bronchial mucosal dendritic cells (DCs) initiate and regulate immune responses to inhaled antigens, viruses and bacteria. Currently, little is known of their numbers in patients with chronic obstructive pulmonary disease (COPD). While reductions in their numbers have been reported recently in smokers with asthma, nothing is known of the effects of cigarette smoking on bronchial DCs in COPD. The present study compares DC numbers in smokers and ex-smokers with COPD.

**Methods:** Endobronchial biopsies were obtained from 15 patients with moderate to severe COPD [10 current smokers (median FEV$_1$ 45.5 [range 23-68] %) & 5 ex-smokers (median FEV$_1$ 30 [range 21-52] % of predicted), 11 non-smoker asthmatics (median FEV$_1$ 102 [range 89-116] %) and 11 non-smoker healthy controls (median FEV$_1$ 110 [range 92-135] %). We applied transmission electron microscopy (TEM) in order to identify the total population of DCs by their ultrastructure and counted them in both epithelium and subepithelium.

**Results:** DC numbers (median & range) were significantly lower in current smokers with COPD at 0.0 (0.0-156.8) cells/mm$^2$ in the epithelium and 4.5 (0.0-63.6) cells/mm$^2$ in the subepithelium compared with ex-smokers with COPD that had 97.9 (93.5-170.3) cells/mm$^2$ in their epithelium (p < 0.05) and 91.8 (38.2-283.3) cells/mm$^2$ in their subepithelium (p < 0.01). DCs numbers in ex-smokers with COPD were similar to those in the atopic asthmatics and in the healthy controls: i.e. for the latter 131.6 (33.3-235.5) cells/mm$^2$ in the epithelium and 64.4 (0.0-182.4) cells/mm$^2$ in the subepithelium.

**Conclusions:** In COPD, bronchial mucosal DC numbers are fewer in current smokers whereas in those who quit, numbers are similar to non-smoking asthmatics and non-smoking healthy controls. The functional consequences of the reduction in mucosal DC numbers in smokers with COPD have yet to be determined.
Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory condition of the conducting airways and lung parenchyma and a major cause of death and increasing long-term care costs. Exposure to cigarette smoke in predisposed individuals is a major factor in the development of COPD. In COPD, increased numbers of CD8+ (cytotoxic) T-lymphocytes are associated with reduced lung function. While inflammation is also increased in atopic asthma, the predominant T-lymphocyte phenotype differs and is of the CD4+ (helper) phenotype. Bronchial dendritic cells (DCs) provide surveillance against inhaled allergen, viruses and/or bacteria that may breach the mucosal/environmental interface: they are the key antigen-presenting cells (APCs) and influence the lung CD4+/CD8+ T cell ratio.

In smokers, the bronchial mucosa and its transient DC population are exposed to the complex constituents of cigarette smoke. Nicotine has an immunosuppressive effect in vitro, cigarette smoke reduces the number of lung DCs experimentally and impairs the DC response to virus. In humans, cigarette smoking reduces DC precursors in cord blood and DCs have been shown to have a role in asthma. We have recently demonstrated, by immunohistology, a reduction of mature (CD83+) DCs in asthmatics who smoke. But it is unclear whether these effects are due to smoking per se or require an interaction between cigarette smoke and the asthma phenotype. Recently, two immunohistological studies have identified airway DCs in the airways of lungs surgically resected from patients with COPD and an immunohistologically defined subset of DCs has been shown to be increased in the small airways (i.e. airways <2mm) of COPD patients. In contrast, nothing is known of their mucosal distribution in large airways and whether current smoking alters mucosal DC numbers in COPD.

Examination of airway tissues by transmission electron microscopy (TEM) potentially identifies all DCs, including the various subsets previously defined immunohistologically. TEM has already revealed the ultrastructural morphology of DCs in the lung and other tissues. Additionally, we have validated the ultrastructural criteria required for the secure identification of DC by TEM examination of a model system in which we have co-cultured DCs and bronchial epithelial cells. Thus, we applied TEM herein to identify the entire population of DC in bronchial biopsies, free of the complications of other pathologies and changes associated with end-stage disease. We
have quantified DCs in endobronchial biopsies from current and ex-smoker COPD subjects and, as there has been no comparative study, we have included non-smoker asthmatics and normal healthy non-smoker subjects. We test the hypothesis that smoking reduces bronchial mucosal DC number, independent of the asthma phenotype.

**Subjects and methods**

**Study population**

The study complied with the declaration of Helsinki. (See online supplement (OLS) for further details). All subjects involved in the study gave written informed consent for endobronchial biopsies to be taken for analyses of inflammatory cells.

Four groups of subjects were studied (see Table1): (1) 11 non smoker non-atopic healthy control volunteers with normal lung function, (2) 5 ex-smokers with COPD who had moderate to severe airway obstruction by GOLD criteria $^3$, (3) 10 current smokers with COPD who had moderate to severe airway obstruction and (4) 11 non-smoker mild atopic asthmatics who required occasional bronchodilator treatment only. COPD patients and normal subjects were non-atopic. Atopic asthmatics had a positive response to one or more skin prick tests with extracts of nine common allergens which included dog, cat, timothy, mugwort, birch, *Dermatophagoides farinae*, *D. pteronyssinus*, *Cladosporium* and *Alternaria* (Phazet, Pharmacia, Uppsala, Sweden). Asthmatics were biopsied late autumn and winter to avoid the hay fever season and were excluded if exposed to a sensitising allergen within the previous 2 months. The mild asthmatics were steroid naïve and none of the normal healthy controls had received such medication. COPD subjects taking inhaled corticosteroids at the time of recruitment had the drug withdrawn and were stable for at least 8 weeks prior to biopsy. Patients with a chest infection within 8 weeks were excluded from the study. All the remaining patients did not receive antibiotics.
### TABLE 1. - Clinical and functional characteristics of subjects

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>COPD ex-smokers</th>
<th>COPD Smokers</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>11</td>
<td>5</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Male/female</td>
<td>5 / 6</td>
<td>5 / 0</td>
<td>10 / 0</td>
<td>9 / 2</td>
</tr>
<tr>
<td>Age (years)</td>
<td>37 (21 - 42)</td>
<td>69 (61-73)</td>
<td>63 (53 – 71)</td>
<td>27 (18 – 41)</td>
</tr>
<tr>
<td>Atopy</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PC$_{20}$ (FEV$_1$) (mg/ml)</td>
<td>&gt; 16</td>
<td>N/A</td>
<td>N/A</td>
<td>2.72 (0.52 – 6.96)</td>
</tr>
<tr>
<td>FEV$_1$ (% predicted)</td>
<td>110 (92 – 135)</td>
<td>30 (21 – 52)</td>
<td>46 (23 – 68)</td>
<td>102 (89 – 116)</td>
</tr>
<tr>
<td>FEV$_1$/FVC (%)</td>
<td>86 (72 – 92)</td>
<td>41 (24 - 46)</td>
<td>56 (31 – 63)</td>
<td>82 (71 – 100)</td>
</tr>
<tr>
<td>Smoking history (pack years)</td>
<td>Non-smokers</td>
<td>98 (45 - 129)</td>
<td>43 (24 – 102)</td>
<td>Non-smokers</td>
</tr>
<tr>
<td>Stop years (years)</td>
<td>N/A</td>
<td>3 (1 - 12)</td>
<td>0</td>
<td>N/A</td>
</tr>
</tbody>
</table>

FEV$_1$ forced expiratory volume in 1 second; FVC, forced vital capacity; PC$_{20}$, concentration of histamine that causes a 20 % fall in FEV$_1$.

N/A = not applicable. Data presented as median (range) and absolute values where appropriate.
Bronchoscopy
After an overnight fast and baseline spirometry tests, bronchoscopy was performed in the morning. In COPD patients, biopsies were taken from the carinae of the second-order bronchi of the right middle and lower lobes. Asthmatic and normal control subjects were biopsied at three sites in the left lung (1) lobar carinae, (2) carinae of the basal segment and (3) carinae of the lingular and upper divisions. A previous study of COPD has shown no differences in the counts of inflammatory cells between segmental and sub-segmental airway generations 6.

Bronchial epithelial cell & DC co-culture
As myeloid CD1c+ DCs have been reported to predominate in the lung 25;26 we aimed to isolate and characterise ultrastructurally this population. Peripheral blood was obtained from a single non-atopic, non-smoker male donor (age 38 yrs) and CD1c (anti-BDCA-1) myeloid DC (CD1c+ LinnegCD11c+HLA-DR+) precursors 27 were isolated by magnetic-activated cell sorting using a commercially available kit (VarioMACS; Miltenyi Biotec, Bisley, Surrey UK) (see OLS for details of CD1c cell isolation). The isolated DCs were then co-cultured with epithelial layers formed form an immortalised bronchial epithelial cell line, BEAS-2B (a gift of Dr Curtis Harris, National Cancer Institute, Bethesda, MD USA) grown on 5.0 μm pore size 6.5 mm polycarbonate Transwell membrane inserts (Corning Costar, Cambridge MA, USA) (refer to OLS for details).

Transmission electron microscopy
Bronchial biopsies obtained from two centres were prepared by a standard method for TEM. All the samples were fixed in 2.5 % glutaraldehyde, post-fixed in 1.0 % osmium tetroxide, dehydrated and embedded in epoxy resin and all were subsequently examined at the Royal Brompton Hospital, London. Co-cultures were prepared in a similar manner. Areas of bronchial biopsy with intact epithelium and co-cultures were selected from 1.0 μm thick toluidine-blue stained survey sections. Ultrathin sections (70 - 80 nm) were contrast stained and examined by TEM (Hitachi 7000; Nisei Sangyo, Japan).

Cell counts and image analysis
Nucleated cell profiles of DCs were counted (by AVR) in the epithelium and the entire subepithelium below the basement membrane excluding areas with bronchial smooth muscle and submucosal gland. Whilst not formally blinded, coded sections were used
throughout and assessed randomly such that the investigator was unaware of smoking status or study group. TEM micrographs of bronchial mucosa at x300 final magnification were scanned (ScanMaker X6; Microtek, CA, USA) for determination of length and area by image analysis (NIH Image-based software, National Institute of Health, MD, USA).

**Statistical analyses**

The coefficient of variation (CV) for three repeat counts by one observer (AVR) of DC number by TEM was 3.5 %. As the data were not normally distributed non-parametric tests were applied (StatView 5 software; SAS Institute Inc., NC, USA). The epithelial and subepithelial data from all four subject groups were compared first using the Kruskal-Wallis test. Where values of probability were $\leq 0.05$, selected pairs of groups were then investigated by Mann-Whitney $U$ test. Data are presented as median and range.
Results

Ultrastructural identification and description of biopsy DCs

CD1c+ myeloid DCs in bronchial epithelial cell co-culture had indented nuclei with a peripheral distribution of heterochromatin, an abundance of cytoplasmic vesicles and, in those that migrated into the epithelial cell layer, cell body extensions/pseudopodia gave it a characteristic dendritic shape (Figs. 1a & b). The following features were used to identify myeloid DCs in the endobronchial biopsies: (1) the presence of pseudopodia; (2) nuclear indentation with peripheral distribution of heterochromatin, and (3) abundance of cytoplasm which was electron-lucent and containing numerous micropinocytic vesicles, profiles of endoplasmic reticulum, and free ribosomes. Mucosal DCs also contained a Golgi apparatus, lysosomes and dense bodies (Figs. 2 – 5). Birbeck granules, the unique identifier of Langerhans cells (LC), were found in some, but not all, of the DCs present in two of the five ex-smokers with COPD. In all other respects, LCs shared the ultrastructural characteristics of DCs and were included in their counts (Figs. 3a - c). In the present study no cell with the features of plasmacytoid DCs was found.

Bronchial DCs were distinguished from other transient cells, for example: (a) lymphocytes with low cytoplasm to nuclear ratio and a non-peripheral distribution of nuclear heterochromatin, (b) plasma cells with abundant profiles of RER and a 'clock-face' arrangement of nuclear heterochromatin, (c) mast cells with intracytoplasmic granules containing characteristic scrolls, (d) polymorphonuclear neutrophils with two types of small intracytoplasmic granules, (e) polymorphonuclear eosinophils with larger granules with crystalline cores, (f) basophils with large granules of homogenous content, (g) macrophages with numerous lysosomes and apoptotic bodies, (h) monocytes (rare) with their horseshoe-shaped nucleus and (i) fibroblasts, elongate, bi-polar, without the stellate cell body shape of DCs and present only in the subepithelium.

Distribution and counts of biopsy DCs

Epithelial DCs were distributed at the level of the basal cells, close to the basal lamina (Figs. 2a and 4) and subepithelial DCs (Fig. 2b and 3a & b) were found adjacent to the reticular basement membrane. In healthy non-atopic individuals, DCs were identified traversing the epithelial basal lamina (Fig. 5) and were also observed in the lymphatics.
Tables 2 & 3 summarize the counts of DC and Figures 7a - c show dot plots of the individual subject data.

**TABLE 2.** – Number (median & range) of dendritic cells per mm² bronchial mucosa

<table>
<thead>
<tr>
<th></th>
<th>Normal Ex-smokers</th>
<th>COPD Smokers</th>
<th>COPD Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 11</td>
<td>131.6 (33.3 - 235.5)</td>
<td>97.9 (93.5 - 170.3)</td>
<td>0.0** † † † † † (0.0 - 156.8)</td>
</tr>
<tr>
<td>Subepithelium</td>
<td>64.4 (0.0 - 182.4)</td>
<td>91.8 (38.2 - 283.3)</td>
<td>4.5** † † † † † (0.0 - 63.6)</td>
</tr>
</tbody>
</table>

* indicates a significant difference from the normal control group
† indicates a significant difference between the COPD ex-smoker and COPD smoker groups.
▲ indicates a significant difference between the COPD and asthma groups

Mann-Whitney U-test *p < 0.05 **p < 0.01 ***p < 0.001

Data presented as median and range
**TABLE 3.** – Number (median & range) of dendritic cells per mm length bronchial epithelium

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>COPD ex-smokers</th>
<th>COPD smokers</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 11</td>
<td>n = 5</td>
<td>n = 10</td>
<td>n = 11</td>
</tr>
<tr>
<td><strong>Epithelium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0 (1.6 – 13.4)</td>
<td>6.3 (3.9 – 9.5)</td>
<td>0.0** †▲▲ (0.0 – 9.3)</td>
<td>6.5 (2.9 – 15.6)</td>
</tr>
</tbody>
</table>

* indicates a significant difference from the normal control group
† indicates a significant difference between the COPD ex-smoker and COPD smoker groups.
▲ indicates a significant difference between the COPD and asthma groups.

Mann-Whitney U-test  
* p < 0.05  ** p < 0.01  *** p < 0.001

Data presented as median and range

In smokers with COPD, there were at least 100-fold fewer intra-epithelial DCs cells/mm² compared with the ex-smokers with COPD (p < 0.05). This was mirrored in the subepithelium where current smokers with COPD had 20-fold fewer cells/mm² than the ex-smokers with COPD (p < 0.01). Intra-epithelial DC numbers in smokers with COPD were 100-fold fewer than in the healthy non-atopic controls (p < 0.01) or atopic asthmatics (p < 0.001). DCs (cells/mm²) in the subepithelium of current smokers with COPD were significantly lower than that of both normal non-atopic controls (p < 0.01) and the asthmatics (p < 0.01) with the differences, 15 and 20-fold, respectively.

Expressed as cells/mm length in order to allow comparison with previously published data (see Table 3, Fig. 7c), smokers with COPD had at least 6-fold fewer (cells/mm) intra-epithelial DCs compared with ex-smokers with COPD (p < 0.05). They also had 5-fold fewer intra-epithelial DCs than both the normal non-atopic control (p < 0.01) and atopic asthmatic groups (p < 0.01). DC numbers in atopic asthmatics were similar to non-atopic controls in both the epithelium and subepithelium. Thus, the findings are consistent no matter which way the data are expressed.
Discussion

We have applied an ultrastructural approach in order to identify all bronchial mucosal dendritic cells (DCs), irrespective of their immuno-phenotype. We demonstrate for the first time in COPD the ultrastructure of bronchial DCs and show that current smoking is associated with abnormally low numbers of these sentinel cells. Current smokers with COPD had 100-fold fewer intra-epithelial DCs and 20-fold fewer DCs in their subepithelium as compared with the bronchi of ex-smokers with COPD. Moreover, the effect appears to be driven primarily by current smoking per se and is not dependent on its interaction with an asthma phenotype, a possibility considered recently by Tsoumakidou and colleagues.

Our counts of DCs in normal healthy individuals were of similar order to those of CD1a+ cells/mm reported previously for normal lung tissue of non-smokers. Also in agreement with Moller and co-workers, we found no difference in counts of DCs, expressed per mm epithelium, between healthy controls and asthmatic epithelium. However, in absolute terms, our asthma count of 6.5 (3.9 – 9.5) DCs/mm by electron microscopy was higher than theirs of 0.4 (0.0 – 5.3) CD1a+ cells/mm. In asthmatics, Jahnsen and coworkers reported bronchial epithelial DC counts of 1.7 cells/mm increasing to 3.0 cells/mm after challenge, considerably less than our counts. This was mirrored in the subepithelium (baseline DC number of 7.8 CD1c+ HLA-DR (Ia)+ cells/mm2 increasing to 22.4 cells/mm2 following allergen challenge). In contrast, the subepithelium of our stable asthmatics, though biopsied outside the hayfever season, had more than 10 fold this number. These divergent results support the idea that the number of DCs identified by existing immuno-markers underestimates the total DC population and emphasises the need to find and validate a pan-specific immuno-marker that will identify all DCs. While DC-SIGN has been considered a putative pan-specific marker, even this has been recently thrown into question as its epitope may also be expressed by B-lymphocytes.

The recent immunohistological study of small airways by Demedts and colleagues and earlier studies in vivo reported an increased accumulation of bronchial DCs in smokers whose lungs had either been resected primarily for cancer or sampled by bronchoalveolar lavage. The antibody used by Demedts and co-workers was directed against Langerin expression, which is lost as DCs mature, suggesting that their data does not reveal the total population of DCs. The earlier study of Soler and colleagues applied an
antibody (i.e. OKT6), that identifies the CD1a DC subset, and found that their numbers in the large airways were unchanged by smoking whereas there were greater numbers in the lung parenchyma of smokers as compared with non-smokers\(^{20}\). In murine studies, D'hulst et al showed that cigarette smoke elevated putative DCs in both BAL and the lung parenchyma\(^{26}\). Also, Zeid and Muller studied smoke-induced Langerhans’ cell granulomatosis and observed accumulations of lung LCs but these returned to control levels after cessation of exposure\(^{32}\). We consider that the differences between these studies and ours lie in the distinct populations of DC quantified, the lack of exclusivity for DC by immunohistochemistry and/or the nature and site of the tissue examined (e.g. surgically resected tissue in which small airways and lung parenchyma are examined versus endobronchial biopsy of large airways)\(^{15;\,18}\). Moreover, in support of our present findings, Robbins and colleagues have demonstrated that exposure to cigarette smoke in mice reduces lung DC numbers and impairs the DC response to virus\(^{12}\). Nouri-Shirazi and Guinet demonstrate further, \textit{in vitro}, that nicotine contributes to the immunosuppressive effect of smoke on DCs by reducing their capacity to stimulate T-cells\(^{33}\) and to secrete IL-12, which promotes TH1 T-cell polarisation\(^{34-36}\). Accordingly, cigarette smoke has been shown \textit{in vitro} to suppress DC maturation\(^{37}\) and incubation of LPS-matured DCs with sputum from patients with COPD decreases expression of co-stimulatory molecules on mature DCs and inhibits DC maturation\(^{38}\). Additionally, another immunohistochemical study of endobronchial biopsies, whilst of asthmatics, has recently reported that current smokers have reduced numbers of mature (i.e. CD83+) DCs in their bronchial mucosa as compared with either never-smoker asthmatics or healthy never-smokers. The alteration of this mature DC subset was accompanied by a significant reduction of B lymphocytes and a trend towards decreased numbers of cells expressing the Th1 cytokine, IFN\(_\gamma\)\(^{15}\). Thus, we have added our ultrastructural approach to the apparent controversy and demonstrate that cigarette smoke is associated with lower overall numbers of DCs per se rather than modulation of their immuno-markers alone. We speculate that cigarette smoke exposure is associated with migration of DCs away from the mucosa to local lymph nodes or with suppression of their recruitment to and accumulation in the bronchial mucosa. Cigarette smoke may also induce apoptosis of DCs but ultrastructural evidence of apoptosis was not observed in our study.

\textbf{Immunohistochemical studies alone are likely to underestimate the total numbers of bronchial DCs. Immuno-markers such as CD1a identify DCs but this marker is expressed}
only by approximately 30% of all bronchial epithelial DCs: these are primarily within the surface epithelium and include Langerhans cells (LC) \(^{18,39}\). An immuno-marker identifying CD1c (blood dendritic cell antigen (BDCA-1)) has also identified a further DC subset in human bronchial mucosa but this subset is primarily located within the subepithelium \(^{18,20}\). Other light microscopic investigations of the bronchial mucosa in normal subjects have demonstrated that HLA-DR (Ia)+ cells of dendritic morphology form a contiguous network within the epithelium of conducting airways \(^{40,41}\) but this marker is also expressed by macrophages/monocytes \(^{42}\) and by epithelial cells \(^{43}\). For these reasons, we have applied the ultrastructural approach to identify and count the total DC population, independent of their immunostaining characteristics. Thus, our data indicate that current cigarette smokers have fewer of these cells in their bronchial mucosa than ex- or never smokers, a consistent finding unrelated to whether the patients have COPD or asthma.

We acknowledge that the major site of pathology and obstruction in COPD are the small airways, whereas we and others (cited above) have sampled the large airways (i.e. bronchi). However, the inflammatory process and its pattern is known to be present throughout the entire tracheobronchial tree, for example in respect of CD8+ cells, B-lymphocytes and in the identification of DCs \(^{6-8,18}\). Thus, we consider that the alterations of DCs identified in the bronchial mucosa by TEM may also occur in small airways but are missed due to application of immunohistology alone. Alternatively, we have sampled large airways by bronchial biopsy which restricts our examination to the mucosal zone, where the effects and concentrations of smoke may be quite different to those occurring deeper in the bronchiolar wall. We also appreciate that differences in subject group ages might affect our interpretation of the data. However, both ex- and current smokers of our COPD group were of similar median age. Moreover, in the paper by Tsoumakidou & colleagues, asthma patients were matched for age, yet there were still fewer DCs found in the smokers \(^{15}\). Thus, we consider that the differences we report can not be accounted for by age alone. We accept that inclusion of a control group of smokers without COPD would have provided additional information as to the effects of smoking per se. Finally, this was an exploratory study and no formal sample size was calculated. Therefore, we acknowledge that small additional differences between our groups could have been missed. We hope that the observations of the present study will assist in the planning of future studies.
In summary, the application of TEM in the present study allowed enumeration of all DCs. Our novel findings demonstrate that there are markedly fewer DCs in the bronchial mucosa of COPD patients who currently smoke, whereas ex-smokers with COPD have DC numbers that do not differ from healthy subjects, or those with mild asthma. Thus, quitting smoking would appear to have the effect of returning DC numbers in smokers with COPD to levels similar to those seen in non-smoking healthy individuals. We consider it reasonable to speculate, that smoke-induced reduction of the overall number of DCs in COPD and of their mature subset in asthma together with the findings of reduced DC function, reported by others, would in consequence alter normal immunity and the pattern of inflammation. This may facilitate infection by virus and lead to increased exacerbation frequency. The relationships between DC number, the underlying chronic inflammation of COPD and susceptibility to acute infection and exacerbation require further study.

Acknowledgement: We thank the subjects and are grateful to Professors Neil Barnes and Paul O’Byrne for their help and support

Declaration: All authors declare that they have no competing interest.

Funding: The study was supported by Lung Pathology departmental funds.
Reference List


Figure Legends

**Figures 1a & b:** Transmission electron microscopy (TEM) micrographs of isolated myeloid-derived dendritic cells (DC) from a non-atopic subject in co-culture with bronchial epithelial cells. (a) demonstrates a DC, with extended pseudopodia (arrows), that has migrated at 24h through the porous support membrane (* indicates membrane position) into the epithelial cell layers. (b) shows a cluster of DCs in suspension which exhibits areas of vesicles, dense bodies and endoplasmic reticulum. In both micrographs, the DCs possess a peripheral arrangement of the heterochromatin. Scale bars = 5 µm.

**Figures 2a & b:** TEM micrographs showing DCs in the bronchial mucosa of a stable asthmatic. (a) in the epithelium, in close proximity to the reticular basement membrane (RBM) and cytoplasmic projections/pseudopodia (arrows) extend amongst the basal cells (BC). An associated lymphocyte (L) is indicated. (b) in the subepithelium, the distinctive blunt pseudopodia (arrows) of the DC contrasts with the adjacent fibroblast (F), which has thin bipolar cell extensions (arrowheads). The DCs exhibit a characteristic electron-lucent cytoplasm and nuclei with a distinctive narrow rim of heterochromatin. Scale bars = 5 µm.

**Figures 3a - c:** TEM micrographs of two adjacent sections through a DC in the subepithelium of an ex-smoker with COPD showing the change in the branching profile and its compartmentalisation in the cytoplasm. In (a) an area (see box) of the cytoplasm contains small electron-dense lysosomes and in (b) an area of the electron-lucent cytoplasm contains Birbeck granules (enlarged in (c)). An adjacent fibroblast (F) is indicated. Scale bars = 10 µm. (c) demonstrates the characteristic Langerhans cell identifier, the pentalaminar Birbeck granule, one of which has a ‘tennis racquet shaped’ head (white arrows). Mitochondria with dense cristae (black arrows), dense bodies (black arrowheads) and micropinocytic vesicles (white arrowheads) are also evident. Scale bar = 0.5 µm.

**Figure 4:** TEM micrograph montage of a DC in two consecutive tissue sections from the epithelium of an asthmatic subject. The dotted line (----) indicates the boundary between sections. The cell body extensions or pseudopodia (see outline & arrows), observed in this plane of sectioning of the stellate DC, extend between the basal cells.
A lymphocyte (L) is present at the reticular basement membrane (RBM). Scale bar = 5 µm.

**Figure 5:** TEM micrograph of a DC in the mucosa of a normal non-atopic adult with a cytoplasmic extension (pseudopod) which straddles the basement membrane (arrows) with its nucleus within the epithelium. A mast cell (M) and fibroblasts (F) in the subepithelium. Scale bar = 10 µm.

**Figures 6a - c:** TEM micrographs of a bronchial lymphatic (walls indicated by arrows) in the subepithelium of a normal non-atopic adult. In (a), the lumen contains DCs and lymphocytes (L) and at higher power micrographs (b & c) the DCs demonstrate the cell projections which give the characteristic ‘veil cell’ appearance described by light microscopy. A peripheral arrangement of heterochromatin is apparent in the DCs. Scale bars = 10 µm.

**Figures 7a - c:** Plots showing (a) the number of DCs per mm$^2$ of bronchial epithelium, (b) number of DCs per mm$^2$ of bronchial subepithelium and (c) the number of DCs per mm of bronchial epithelium of the four groups studied (line indicates median value). COPD patients - ex-smoker (COPD-Ex) and current smokers (COPD-Cu). Significant differences determined by Mann-Whitney U-test.
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Thorax  published online September 17, 2007

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