Differential expression of mast cells in the small airways and alveolar septa of current smokers and patients with small airway disease and COPD

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Mast cells in smokers and patients with small airway disease and COPD may be associated with airway wall changes and impaired lung function, indicating an active role in maintaining normal tissue homeostasis and, indirectly, in airway architecture https://bit.ly/3vKxxab

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Abstract

Background COPD patients suffer from dysregulated and suppressed immune functionality, determined by their loss of degranulating capacity. Here we provide crucial information on the presence of degranulated mast cells (MCs) in COPD airways and demonstrate their relationship to lung physiology and airway remodelling.

Methods Small airway lung resections from non-smoking controls (NC), normal lung function smokers (NLFS), small airway disease (SAD), and mild-to-moderate COPD current smokers (COPD-CS) and ex-smokers (COPD-ES) were dual immuno-stained with MC tryptase and degranulation marker lysosome-associated membrane protein (LAMP)-1. Total MCs, degranulating MCs and non-MCs were enumerated in small airway epithelium and subepithelium, and in alveolar septa.

Results In the small airway wall subepithelial areas, COPD-CS and COPD-ES patients had significantly lower MCs than the NC group ($p<0.05$), although the numbers were considerably higher in the small airway epithelium ($p<0.01$). Degranulating non-MCs were higher in SAD ($p<0.05$) than in COPD in the small airway subepithelium. In contrast, there were significant increases in total MCs (degranulated and non-degranulated) and degranulated non-MCs in the alveolar septum of COPD patients compared with the NC group ($p<001$). The lower numbers of MCs in the subepithelium correlated with lower forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) and forced expiratory flow at 25–75% of FVC ($FEF_{25–75\%}$), higher smoking rates in COPD patients, and increased small airway wall thickness and extracellular matrix. The increase in MCs in the alveolar septum negatively correlated with $FEF_{25–75\%}$.

Conclusions This study is the first to assess the differential pattern of MC, degranulating MC and non-MC populations in the small airways and alveoli of COPD patients. The spatial positioning of the MCs within the airways showed variable correlations with lung function.

Introduction

COPD is a devastating disease, with a global prevalence of 300 million and increasing, causing at least 3 million deaths annually, and is currently ranked the third leading cause of mortality [1, 2]. It is primarily a

slow progressive pan-airway disease with partial reversibility at best. Small airways are thickened through fibrotic remodelling changes, which causes their narrowing and subsequent obliteration [3]. Inflammatory changes are variable and increasingly abnormal over time in affected patients [4, 5]. People with COPD also suffer severe secondary bronchitis and 50% also have a varying degree of parenchymal lung destruction (emphysema), adding to airflow obstruction and symptoms.

Mast cells (MCs) play a crucial role in innate immune responses, recognising pathogens and allergens. Granulated innate cells differentiate from haematopoietic stem cells in the bone marrow and mature into MCs in the target tissue [6]. Their granule contents are released on activation and include pro-inflammatory, pro-fibrotic and pro-angiogenic mediators, which are crucial in regulating vasculature and lung remodelling [3, 7]. MCs have been classically associated with allergic reactions [8, 9], whereby they are activated by cross-linking of IgE molecules attached to specific surface receptors by their Fc fragment, but it is now known that there are other ways that degranulation can also be stimulated [10, 11].

We describe the variability in critical immune cells in the airway wall, including both innate and adaptive populations [5]. Previously, our group reported an increase in the MC population in the large airways of COPD patients in both the reticular basement membrane (RBM) and lamina propria in endobronchial biopsy samples [7]. Our recent assessment has also demonstrated the critical roles of MC tryptase-β, tryptase-γ and chymase-1 in the experimental mouse model of COPD pathogenesis and in human samples *ex vivo* [12–14]. Furthermore, recent findings by Faiz *et al*. [15] through gene expression profiling studies in large airway bronchial biopsies and sputum of moderate-to-severe COPD patients showed an association of MC tryptase and chymase as critical drivers of COPD pathology, with increased suppression seen in the inhaled corticosteroid-treated group of patients. A similar association was also drawn between MC gene expression in the eosinophil^{high} subpopulation of COPD patients [16]. These responses are reminiscent of the type 2 immune environment typically observed in COPD and asthma patients [4]. Gosman *et al*. [17] also identified increases in MC numbers expressing tryptase and chymase in COPD patients' central and peripheral airways compared with smoker controls. Their study also provided an enumeration of the degranulating MCs; however, these observations relied on the morphometric appearance of granules within the cells rather than specific biomarkers. We also reported that smoking decreased large and small airway subepithelial lamina propria total cellularity in mild-to-moderate COPD patients [2, 4, 7, 18]. However, we did not previously enumerate the contribution of MCs in the small airway and in the alveolar areas of smokers and COPD patients.

We also previously demonstrated the increase in airway wall thickness, especially in the lamina propria and smooth muscles, along with the increase in collagen and fibronectin deposition in smokers and COPD patients [3]. MC secretions have been observed to regulate the extracellular matrix (ECM) in airway epithelial cells [19, 20]. However, few studies have critically assessed MCs and their association with airway remodelling and ECM changes in the small airways of smokers and COPD patients. In this study, we provide this vital insight into their relationship, having examined lung tissue from the same cohort of patients and normal controls.

Lysosome-associated membrane protein (LAMP)-1 is an established marker for active cell degranulation [21–23]. LAMP-1 is a transmembrane lysosomal glycoprotein expressed on activated lysosomes and endosomes with essential links to autophagy and lysosomal storage diseases [11, 13]. LAMP-1 expression is known to be increased in MCs when activated with IgE [24]. It is also activated in $CD8^+$ lymphocytes and the suppression of $CD8^+$ cell degranulation is associated with LAMP-1 reduction [24, 25]. In the current study, we investigated the coexpression of MC tryptase and LAMP-1 to determine their degranulation capacity. In addition, we determined the total degranulated cells observed in COPD patients.

Here, we provide a detailed analysis of the contribution of MCs, their degranulation capacity and other degranulating cells in the small airway wall and alveolar septum of smokers and patients with small airway disease (SAD) and COPD. We examined the cellular expression of LAMP-1 as a biomarker for degranulation [21–23]. The study also provides the critical association of MCs with lung physiological parameters and airway remodelling changes. We identified differential MC numbers and activity in the small airways than in the alveolar areas in the patient groups.

Methods

Subject classification and small airway tissue

Pathological small airway resected tissue was available from our biobank (Tasmanian Health & Medical Human Research Ethics Committee; H0012374). In addition, healthy non-smoking control tissues (NC group) were obtained from the James Hogg Lung Registry, University of British Columbia (Vancouver,

BC, Canada), with approval from the Providence Health Care Research Ethics Board (H00-50110), from subjects who had died of causes other than pulmonary diseases. We assessed tissues from 42 patients (table 1). All subjects had primary non-small cell lung cancer, with an approximately equal distribution of squamous and adenocarcinoma, and were approved for their surgical tissue to be used for research at Royal Hobart Hospital (Hobart, Australia). 16 patients had demonstrated mild-to-moderate Global Initiative for Obstructive Lung Disease (GOLD) stage I and II COPD, of which eight were current smokers (COPD-CS) and eight were ex-smokers (COPD-ES) (>6 months smoking cessation). Seven donors were normal lung function smokers (NLFS) and nine (two current smokers and seven ex-smokers) were categorised as patients with SAD based on their normal lung function forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) (>70%) but had lower forced expiratory flow at 25–75% of FVC ($FEF_{25–75\%}$). 10 non-smoking tissues were included as the NC group for comparison. Subjects with other respiratory diseases, a history of recent acute exacerbation of COPD and those on systemic or inhaled corticosteroids were excluded from the study. The surgically resected material was taken far away from the primary tumour and contained non-cancer-affected small airways.

Immunostaining for MCs, degranulating MCs and degranulating cells

To enumerate degranulated MCs in the small airway, dual staining was performed using MC tryptase AA1 and LAMP-1. For the antigen retrieval step, tissues were first boiled in a high pH 9 buffer for 3 min, followed by treatment with 3% H_2O_2 in water for 3 min. Tissue sections were washed three times with Tris–HCl, pH 7.5 (wash buffer) at 2 min intervals. Primary antibodies were a mouse anti-human MC tryptase (AA1) monoclonal antibody (M7052; Dako, Glostrup, Denmark; 1:1500 dilution) and a rabbit anti-LAMP-1 antibody (ab24170; Abcam, Cambridge, UK; 1:200 dilution), and were sequentially added with incubation for 1 h. Matched negative controls were used: isotype-matched immunoglobulin IgG1κ (X0931; Dako) and rabbit serum (X0903; Dako) at appropriately adjusted concentrations. Bound tryptase AA1 antibodies were elaborated using the REAL detection system (K5005; Dako) and were visualised with 5-bromo-4-chloro-3′-indolyphosphate and nitroblue tetrazolium in a ready-made substrate system (K0598; Dako). Endogenous alkaline phosphatase activity was inhibited by adding levamisole to the visualisation substrate (X3021; Dako). Sections were further rinsed using wash buffer (three times, 5 min intervals) and bound LAMP-1 before the addition of anti-mouse secondary antibodies (Envision+ System horseradish peroxidase (HRP)-labelled polymer reagent; K4001; Dako) for 30 min. After three washes, DAB+ (K3468; Dako) was applied to sections for 10 min and rinsed in wash buffer (twice), followed by once with distilled water. Tissue sections were counterstained with Nuclear Fast Red to elaborate nuclei for ∼2 min, then rinsed thoroughly in running water to remove the excess stains. Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 min each). The clearing was achieved with two changes of xylene (2 min each). Sections were then mounted in Depex using Dako Cover-slipper (Dako) and dried on a hotplate overnight.

Immunohistochemical staining was performed for MC chymase-1 (CMA1). In brief, tissue was subjected to heat retrieval (pH 6) (S169984; Dako) in the decloaking chamber at 110°C for 15 min. The tissue was stained with primary antibody CMA1 (ab2377; Abcam; 1:00 dilution) which was applied and kept at ambient temperature for 60 min, followed by the HRP-conjugated polymer-carried secondary antibody for

TABLE 1 Donor demographics

	NC	NLFS	SAD	COPD-CS	COPD-ES[#]
Subjects	10	7	9	8	8
Sex					
Female	6	4	7	5	3
Male	4	3	2	3	5
Age, years	39 (19–63)	72 (52–79)	59 (42–84)	63 (59–78)	70.5 (56–85)
Smoking, pack-years	0	22.5 (0.3–40)	40 (0–72)	32.5 (20–67)	33 (18–60)
FEV_1/FVC, %		81 (70–90)	73 (69–78)	66 (59.9–70)	65 (55–69)
$FEF_{25–75\%}$, $L{\cdot}s^{-1}$		84 (71–116)	46 (31–69)	35.5 (28–47)	40.5 (20–55)
D_{LCO}, % pred		78 (50–95)	78 (54–91)	68.9 (34–84)	74 (51–114)

Data are expressed as n or median (range). NC: normal control; NLFS: normal lung function smoker; SAD: small airway disease; COPD-CS: COPD current smoker; COPD-ES: COPD ex-smoker; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; $FEF_{25–75\%}$: forced expiratory flow at 25–75% of FVC (post-bronchodilator); D_{LCO}: diffusing capacity of the lung for carbon monoxide. [#]: COPD-ES patients had quit smoking for >6 months.

30 min, and then visualised by DAB+ staining (Envision Detection System, peroxidase/DAB, K5007; Dako) for 10 min followed by counterstaining with haematoxylin for nuclear staining.

Image analysis

Computer-assisted image analysis was performed using a Leica DM 2500 microscope and a Leica DFC495 camera (Leice, Wetzlar, Germany) with Image-Pro Plus version 7.0 (Media Cybernetics, Rockville, MD, USA) software. Eight random fields of small airways <2 mm thick (minimum of two airways per subject) were chosen for comprehensive analysis without *ad hoc* area selection. However, muscle bundles and glands were excluded from the area surveyed. Small airway subepithelium <100 µm deep was quantitated. Stained MCs (MC tryptase$^+$) and degranulated cells (LAMP-1$^+$) in the subepithelium and epithelium were separately counted, and data are presented as per mm^2 of the area surveyed and per mm of RBM length, respectively. Similarly, positive MCs and active degranulated cells in the parenchymal region were counted, and data are presented as a percentage of total alveolar tissue area cells.

Statistical analysis

Following a check for normal distribution, the analysis is presented as median (range). Non-parametric (Kruskal–Wallis) ANOVA with multiple comparisons was performed using Dunn's test. Linear regression and Spearman's rank test was used for correlation analysis. All analyses were performed using Prism version 9 (GraphPad, Boston, MA, USA) and $p<0.05$ was considered significant.

Results

Total MCs in small airway walls and alveolar septa

In the small airway wall subepithelial areas, we found fewer MC tryptase$^+$ and LAMP-1$^+$ single-stained cells in patients with COPD compared with the NC group (figure 1a and b). In contrast, these patients had a significantly higher number of single-stained MC tryptase$^+$ and LAMP-1$^+$ cells in the small airway epithelium and alveolar septa than the NC group (figure 1a–d). We found that the dual stain was significantly higher in the alveolar spaces in COPD patients compared with their small airways. Similarly, we observed a higher level of MC chymase expression in the alveolar spaces compared with the small

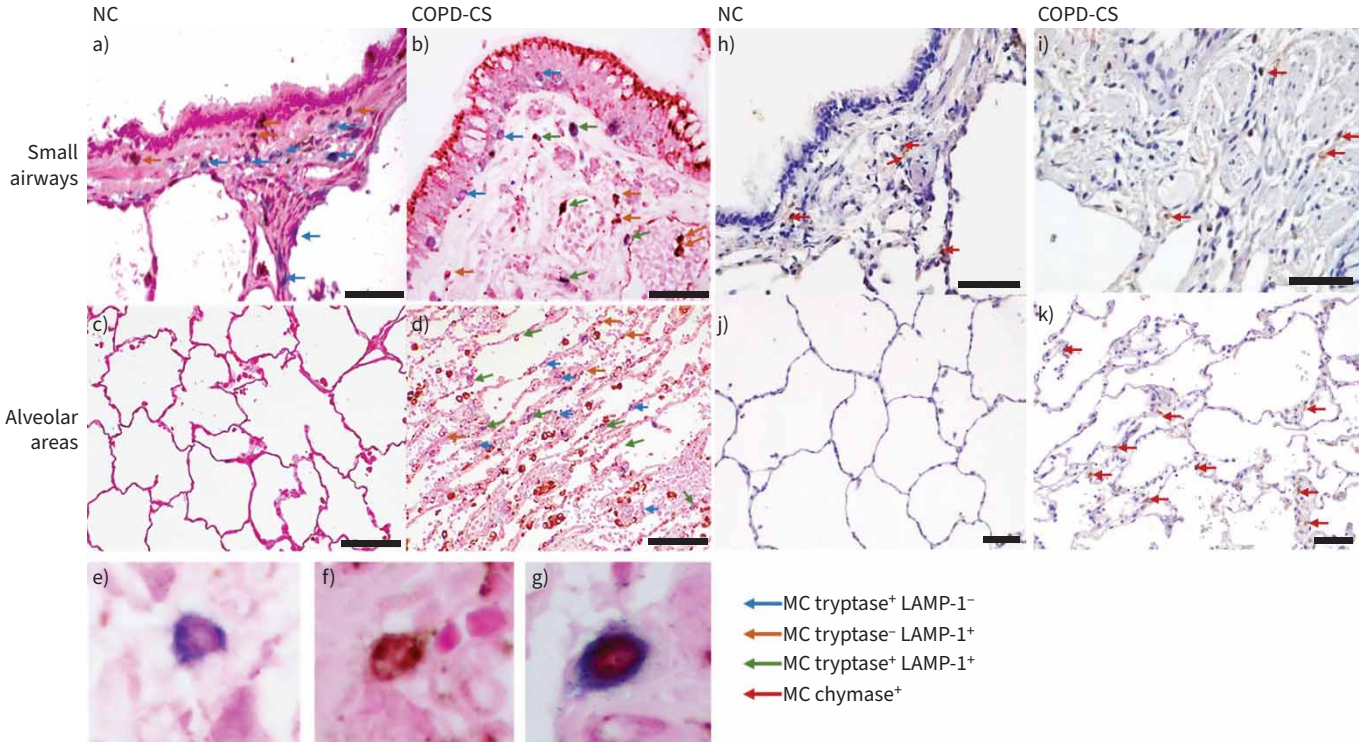

FIGURE 1 a–d) Representative images for dual-stained mast cells (MCs) (MC tryptase$^+$ and LAMP-1$^+$ cells) in the small airways and alveolar spaces of a, c) normal controls (NC) and b, d) COPD current smokers (COPD-CS). e, f, g) Enlarged images of cells captured from the analysed tissue: e) MC tryptase$^+$ cells, f) only lysosome-associated membrane protein (LAMP)-1$^+$ (degranulated) cells and g) dual-stained MC tryptase$^+$ LAMP-1$^+$ cells. h–k) MC chymase expression in the small airways and alveolar spaces of h, j) NC and i, k) COPD-CS. Scale bars: a, d, h, i) 100 µm; c, d, j, k) 50 µm.

airways of COPD patients (figure 1i and k). The difference between MC chymase in the normal airway and COPD patients was not obvious in our observation, although these differences were evident in the alveolar spaces (figure 1h–k).

Quantitative analysis demonstrated a significant increase in MC tryptase$^+$ cells in COPD-CS ($p<0.05$) and a moderate rise in SAD subjects ($p=0.08$) in the epithelium compared with the NC group (figure 2a). There was no significant change between COPD-CS and COPD-ES ($p=0.07$) (figure 2a). Interestingly, there were significant decreases in total MCs in the subepithelium in COPD-CS ($p<0.05$) and COPD-ES ($p<0.05$) compared with the NC group (figure 2b). However, no significant changes were observed compared with NLFS and SAD. Analysis of alveolar areas showed an increase in MCs across the pathological groups, with substantial increases observed in COPD-CS ($p<0.001$) and NLFS ($p<0.01$) compared with the NC group. Interestingly, there was a significant decline ($p<0.05$) in alveolar MC numbers in COPD-ES compared with COPD-CS.

Degranulated MCs in the small airway wall and alveolar septa

In the epithelium, an increase in the absolute numbers of LAMP-1$^+$ MCs was observed in COPD-CS patients compared with COPD-ES ($p<0.05$) and the NC group ($p<0.05$). However, compared with the NC group no change was observed in COPD-CS and COPD-ES for the subepithelium (figure 3a and b). We did, however, observe a specific increase in degranulated MCs in SAD compared with the NC group ($p<0.05$) (figure 3b). This increase in numbers was also reflected in percentages, wherein they constituted 20% of the total MCs (figure 3c). However, there was no change in degranulating MCs in the subepithelium between all other groups compared with the NC group (figure 3b). In the alveolar septa, we observed significant increases in degranulated and non-degranulated MCs in all pathological groups compared with the NC group (figure 3d and e). Significant increases in degranulated MCs were observed in smokers and COPD-CS ($p<0.05$) compared with the NC group, while there was a substantial decline in COPD-ES ($p<0.05$) compared with COPD-CS (figure 3d). A similar significance was seen in the non-degranulated MC populations (figure 3e).

Total degranulated cells

In the small airway epithelium and subepithelium, the density of total degranulated cells remained unchanged and trended to be lower in COPD subjects than in the NC group (figure 4a and b). Interestingly, SAD had higher degranulated cells than COPD-CS and COPD-ES in the subepithelium ($p=0.013–0.02$) (figure 4b). Similar to our findings of degranulated MCs, we found significant increases in degranulated LAMP-1$^+$ cells in alveolar areas across pathological cohorts, suggesting active degranulation (figure 4c). COPD-CS had the most significant increases compared with the NC group ($p<0.0004$). In contrast, degranulated cells in COPD-ES ($p<0.01$) were substantially lower than COPD-CS, suggesting smoking cessation-related effects.

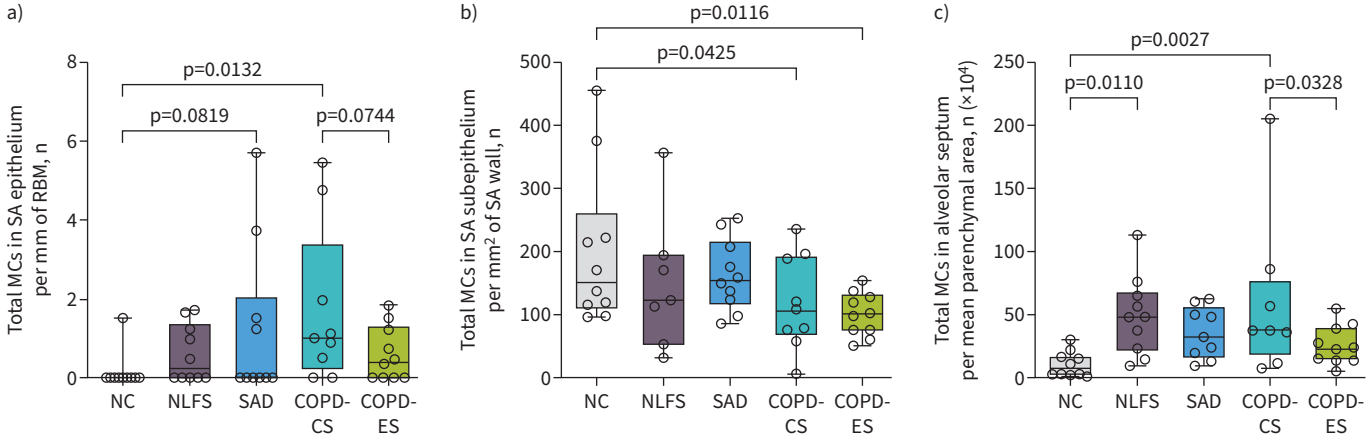

FIGURE 2 Data representative of the total mast cell (MC) counts in the small airway (SA) a) epithelium and b) subepithelium and c) in the alveolar septum of the parenchyma in non-smoking controls (NC), normal lung function smokers (NLFS), small airway disease (SAD), and mild-to-moderate COPD current smokers (COPD-CS) and ex-smokers (COPD-ES). RBM: reticular basement membrane.

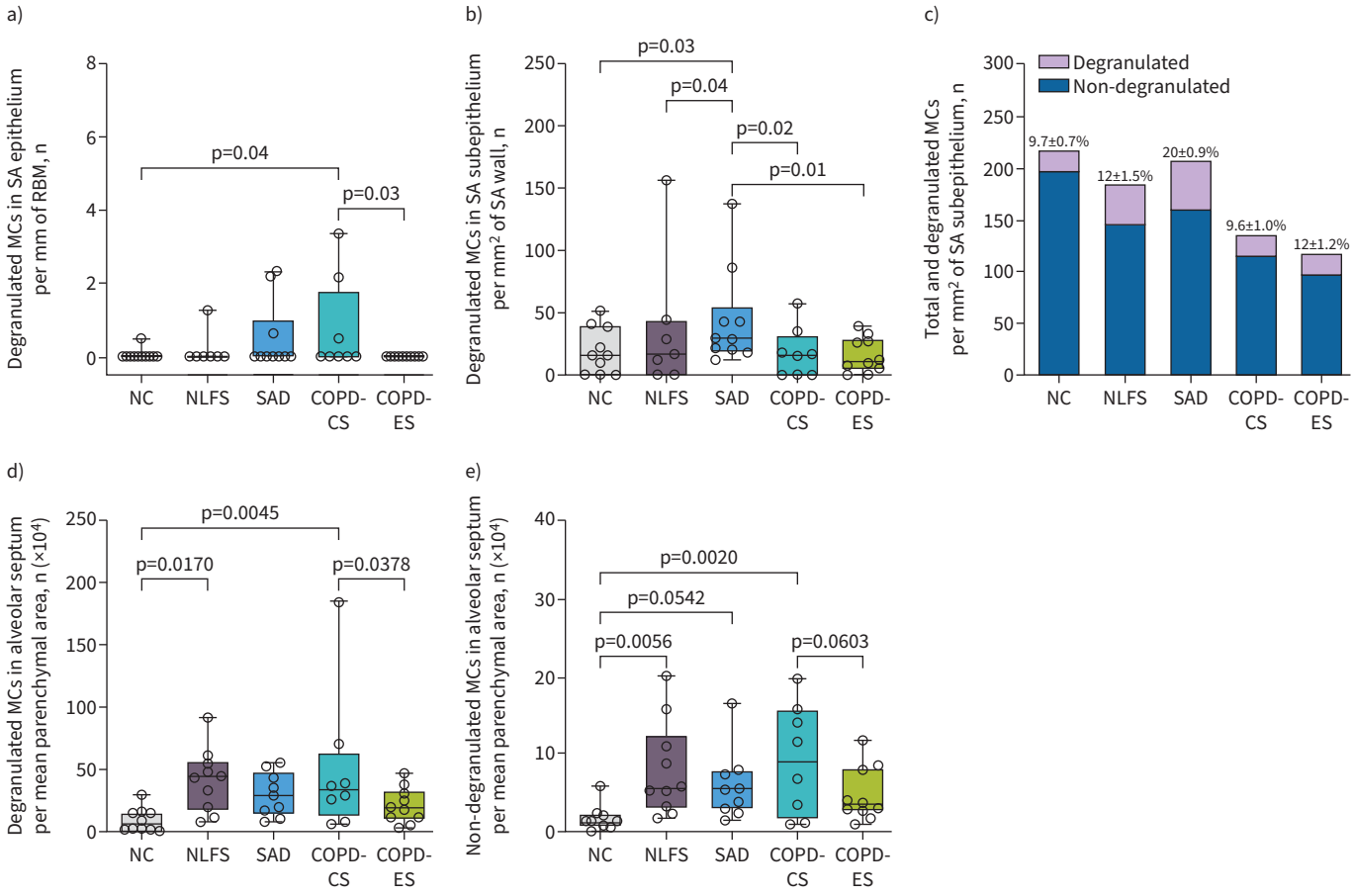

FIGURE 3 a, b) Data representative of the degranulated mast cell (MC) counts in the small airway (SA) a) epithelium and b) subepithelium in non-smoking controls (NC), normal lung function smokers (NLFS), small airway disease (SAD), and mild-to-moderate COPD current smokers (COPD-CS) and ex-smokers (COPD-ES). c) Percentage of degranulated MCs to the total MC population in the subepithelium. e) Total degranulated and f) non-degranulated MCs in the alveolar septum in the parenchymal areas for the NC and pathological groups.

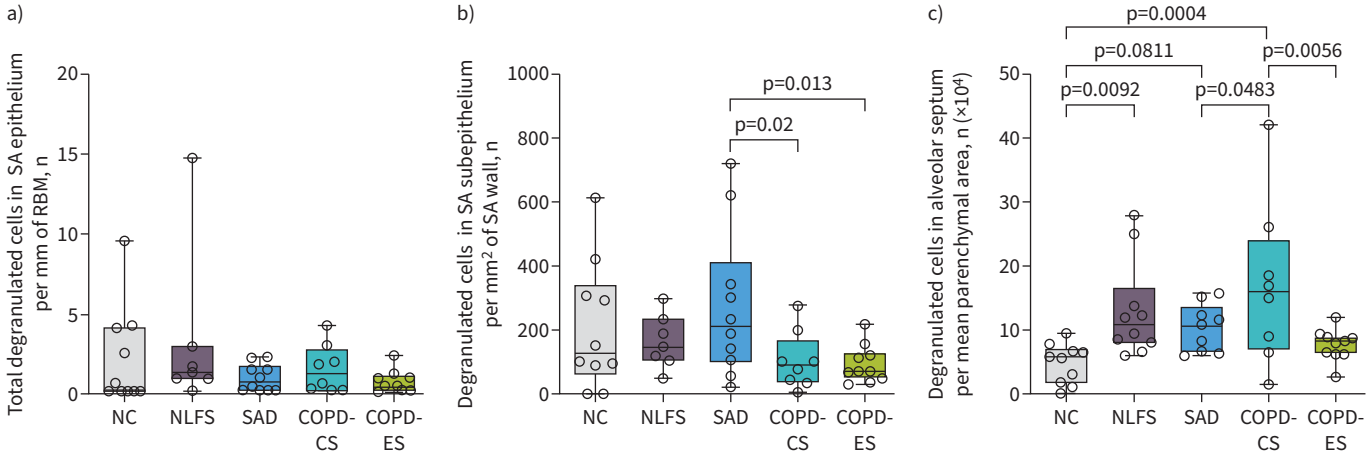

FIGURE 4 Graphical representation of total lysosome-associated membrane protein (LAMP)-1$^+$ degranulated cells as examined in the small airway (SA) wall and the percentage contribution of degranulating mast cells to the total LAMP-1$^+$ cell population in non-smoking controls (NC), normal lung function smokers (NLFS), small airway disease (SAD), and mild-to-moderate COPD current smokers (COPD-CS) and ex-smokers (COPD-ES): a) epithelium, b) subepithelium and c) alveolar septum of the parenchymal areas. RBM: reticular basement membrane.

Small airway wall MCs and degranulated cells correlate with smoking history and lung physiological parameters

An increase in smoking history (pack-years) correlated directly with the decrease in total MCs and degranulated cells in the small airway subepithelium in COPD-CS but not in NLFS subjects (figure 5a and b), suggesting a COPD-specific effect. In addition, there was a positive correlation between total subepithelium

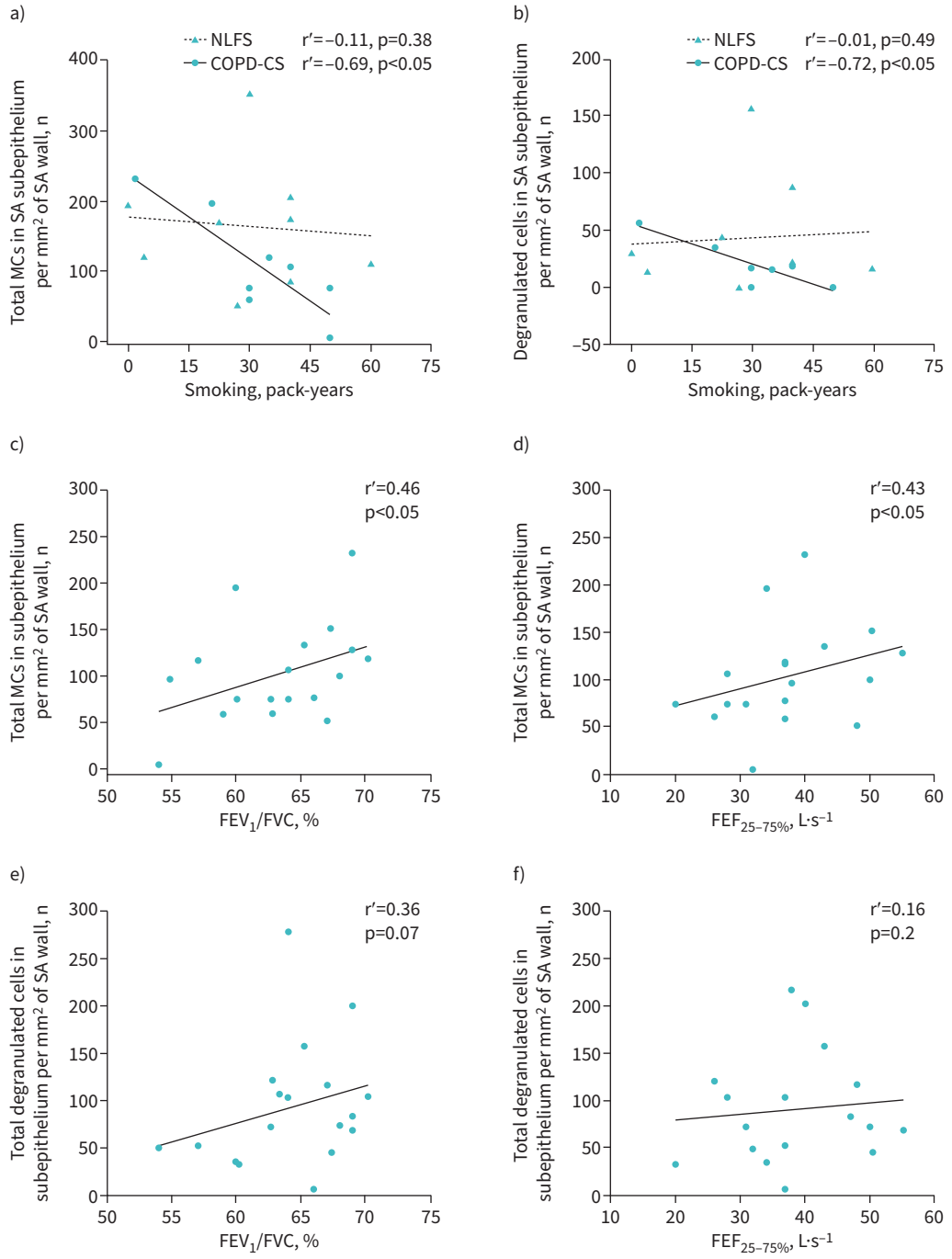

FIGURE 5 a, b) Correlation between smoking history (pack-years) and a) total mast cells (MCs) and b) degranulated lysosome-associated membrane protein (LAMP)-1$^+$ cells in normal lung function smokers (NLFS) and COPD current smokers (COPD-CS). c–f) Correlation of total MCs and degranulated LAMP-1$^+$ cells with lung physiology parameters in the COPD groups: c, e) forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) and d, f) forced expiratory flow at 25–75% of FVC ($FEF_{25–75\%}$). SA: small airway.

MC numbers in COPD *versus* large and small airway obstruction (FEV_1/FVC (r′=0.46, p<0.05) and $FEF_{25–75\%}$ (r′=0.43, p<0.05), respectively) (figure 5c and d). There was also a positive but lower correlation between total degranulating cells in these groups and lung function (figure 5e and f).

MCs in the small airways and relation to airway remodelling

For COPD groups, there was a strong relationship between decreasing total numbers of subepithelial MCs and indices of small airway remodelling: increased thickening of the small airway wall lamina propria (figure 6a–c). In contrast, however, the relationship with the smooth muscle layer was positive, *i.e.* the more MCs, the thicker the muscle layer (figure 6d).

Correlation of parenchymal alveolar total and degranulated MCs, and degranulated and non-degranulated cells, with lung physiology

Total and degranulated MC numbers were increased in the COPD groups and negatively correlated with both small airway/peripheral obstruction parameter $FEF_{25–75\%}$ (p=0.05 and p=0.041, respectively) and lung function FEV_1/FVC (figure 7). Conversely, we found that non-degranulated MCs did not correlate with both parameters (figure 8a and b). In addition, significant negative correlations with lung parameters were also observed with total degranulated cells (figure 8c and d).

Discussion

MCs are critical immune cells of myeloid origin with crucial physiological roles in maintaining tissue structural homeostasis, regulating innate and adaptive immune functions, and controlling infections [10, 26]. In this study, we demonstrate the variable existence of MCs, degranulating MCs and other degranulating cells in the small airways and the parenchyma of mild-to-moderate COPD, NLFS and SAD groups when compared with normal non-smoking controls (NC group). A general reduction in total MC

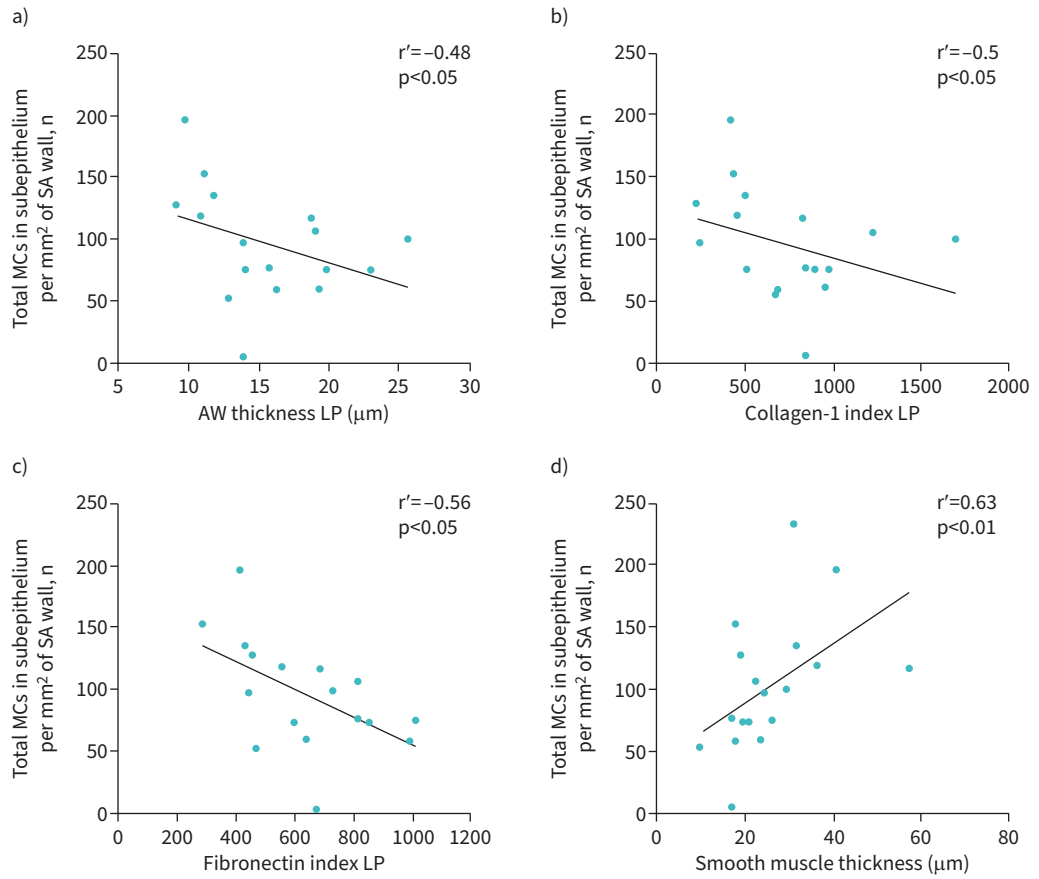

FIGURE 6 Correlation analysis between total mast cell (MC) numbers in the small airway (SA) wall and a) airway wall (AW) thickness, b) collagen-1 index, c) fibronectin index and d) smooth muscle thickness in the COPD groups. LP: lamina propria.

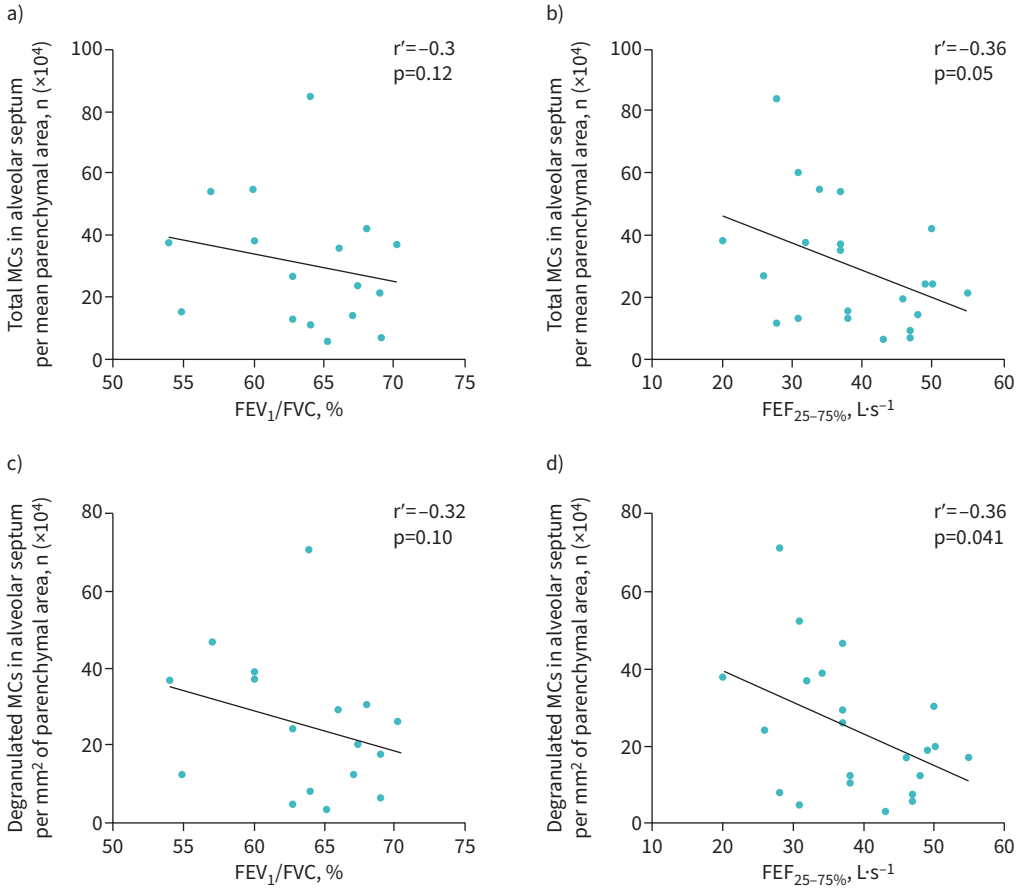

FIGURE 7 Correlation analysis between total and degranulated mast cells (MCs) with a, c) forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) and b, d) forced expiratory flow at 25–75% of FVC ($FEF_{25–75\%}$) in the COPD groups.

numbers in the small airways in COPD patients' subepithelial regions was confirmed, although degranulating MCs remained unchanged in their subepithelium. Interestingly, the only significant increase of degranulating MCs was observed in the SAD group, possibly pointing to a smoking-specific effect. However, in contrast, in the parenchyma, we demonstrate that MCs, regardless of their degranulating status, were significantly higher in the pathological groups of NLFS, COPD-CS, COPD-ES and SAD compared with the NC group. We also demonstrate for the first time that these changes in MC numbers, specific to their region, significantly impact the functional parameters and airway remodelling changes. The current data on small airways also contrasted with our previous observations of MCs in the large airway wall, wherein there was an increase in MC numbers in COPD bronchial biopsy tissue [7].

Our observation of reduced MCs in the subepithelial of COPD patients is comparable to reports by Andersson *et al*. [27]. They found a similar decrease in MCs in the subepithelial region of the small airways of COPD patients across GOLD stage I–IV subjects, which negatively correlated with the severity of disease. Our current study provides additional information on COPD ex-smokers where the levels of MCs remained low even after the donors had ceased smoking, suggesting that the effect observed is mainly smoking driven. Moreover, when correlated with smoking history in the COPD-CS and NLFS groups, the suppression of total and degranulating MCs was more significant in the COPD-CS group, again suggesting that decreases in these cells are linked to airflow obstruction and smoking. Our observation of increases in epithelial MC numbers in COPD is similar to that of Aktas *et al*. [24], who also observed a rise in MCs in the small airway epithelium of COPD patients. However, in contrast, they found no change in the subepithelial lamina propria, but had no data on MC activation. The active migration of MCs could explain the increase in epithelial numbers from the wall into the airway lumen. Fate-mapping studies with MCs in an animal model of COPD would be valuable in elucidating this.

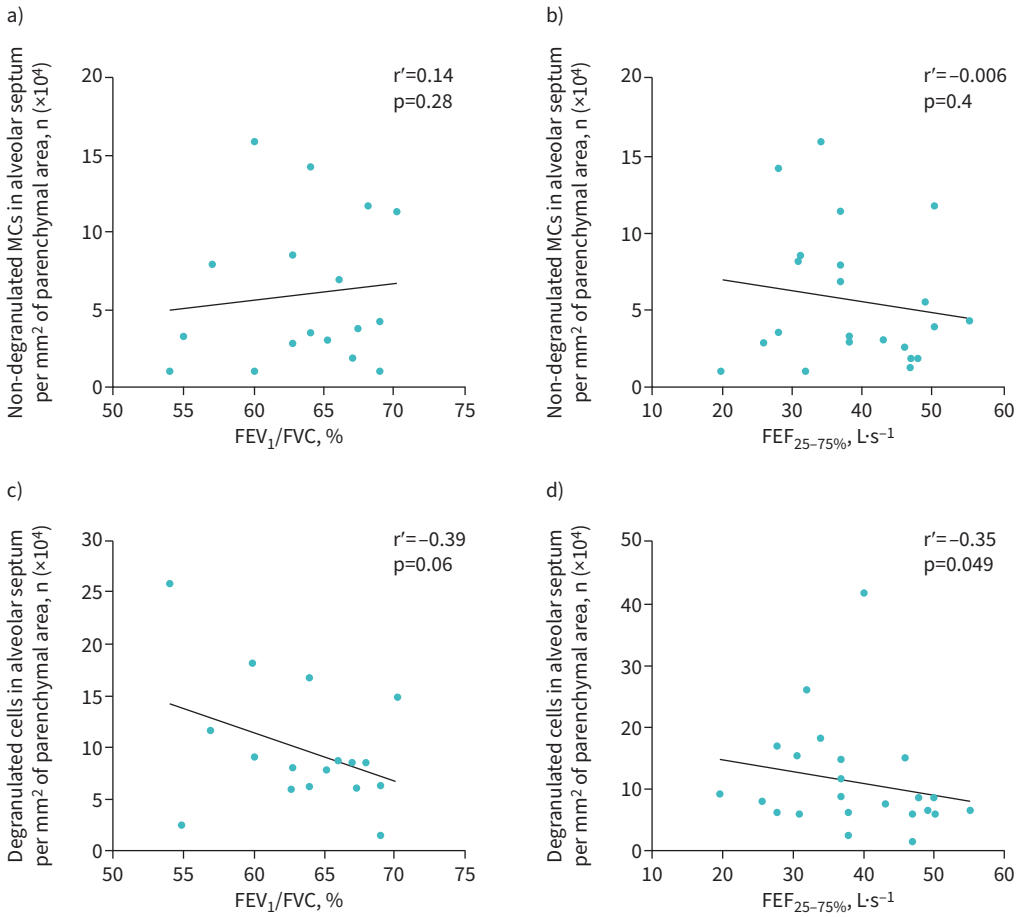

FIGURE 8 Correlation analysis between a, b) non-degranulated mast cells (MCs) and c, d) degranulated cells with a, c) forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) and b, d) forced expiratory flow at 25–75% of FVC ($FEF_{25–75\%}$) in the COPD groups.

Similar to Anderson *et al*. [27], our findings also showed that the decrease in MCs was negatively associated with lung function and decreased small airway calibre measured by $FEF_{25–75\%}$, in the COPD group. It is difficult to rationalise this relationship of reduced MCs with airflow obstruction. It is unlikely to be causative since we previously showed the deleterious effects of MC tryptases and chymase-1 in pathogenesis [12–14], but more likely that both features are related to other more fundamental processes. In certain circumstances, MCs can limit inflammation and the degree of tissue injury [28, 29]. We also, however, identified an opposite relation between lung function and parenchymal MCs. Interestingly, this relationship was more related to degranulated than non-degranulated MCs, matching our previous data with tryptases and chymase-1. Thus, region-specific degranulation of MCs may have differential outcomes.

Evidence suggests that both tryptase and chymase are reliable markers for MCs. However, in the current study, we used tryptase only as the MC marker, as these cells are more specifically located in the airway mucosa than chymase-expressing MCs present more in the adventitial connective tissue [11, 14, 30]. Consistent with our findings, Gosman *et al*. [17] found that MCs are concentrated in the small peripheral airways compared with central airways in both COPD and control groups, and suggested that this could be crucial in small airway remodelling in COPD. Interestingly, and perhaps paradoxical to this suggestion, in our current study, the reduction in $tryptase^+$ MCs in the subepithelium of COPD patients strongly negatively correlated with airway wall thickening and our indices of ECM remodelling changes in collagen-1 and fibronectin. Again, another more fundamental process may be driving both phenomena. However, the current findings may complement earlier observations in other diseases, demonstrating the importance of MC tryptase in specific locations in maintaining tissue homeostasis through the degradation of excessive ECM collagen deposition indirectly by degrading the production of increased matrix metalloprotease (MMP)-9 activity [31, 32].

MC tryptase is also known to activate pro-MMP-3 and pro-MMP-13, which are crucial for the digestion of ECM proteins such as collagen and fibronectin [33]. Thus, the observed suppression of tryptase$^+$ MCs in the airway wall in COPD could be related to the aberrant accumulation of ECM protein and airway remodelling. Interestingly, there was a positive relationship between MC numbers in the lamina propria and the thickness of the smooth muscle layer, suggesting that there may be some (perhaps mutual) trophic relationship between them.

MCs contain granules (secretory lysosomes) that hold an array of lysosomal proteins such as acid hydrolases (*e.g.* β-hexosaminidase) [32, 34] and biogenic amines such as histamine and serotonin. In addition, they also store and secrete extracellularly mature forms of serine and other proteases, which include tryptase, chymase-1, cathepsin G, granzyme B and carboxypeptidase, among others [14, 35]. Based on their content, MC secretory lysosomes are characterised into three subtypes. Specifically, LAMP-1 marks the more mature or active type 1 and type 2 secretory lysosomes ready to be exocytosed and containing one or more of these fully functional mediators. Unfortunately, there are few studies on degranulating markers in COPD research, especially with LAMP-1 [36]. Previous estimates of MC degranulation by Gosman *et al*. [17] used morphological and cellular conformational analysis based on the secretion pattern of tryptase MCs. However, the limitation of this approach is in differentiating pre-formed MC tryptase from its mature form that is stored in secretory lysosome granules ready for release. Our current study provides specific evidence on degranulating activity in MCs by dual staining them for LAMP-1 and tryptase. The degranulating LAMP-1$^+$ MCs in the lamina propria constituted ~15–20% of the total MC population. However, this was not different overall from normal despite some smokers having relatively large numbers. This is in contrast to the epithelium, where the COPD-CS group had significant increases in degranulating MCs although not in percentage terms of total MC numbers in the small airway epithelium. Currently, no firm conclusion can be drawn about the role of LAMP-1 or MC lysosomal exocytosis in COPD, and this is a complex process that may involve multiple types of lysosomes [35].

The decrease in LAMP-1 expression in the COPD airway seemed related to an overall cellular dysfunction in potentially degranulating cells and was not limited to MCs. McKendry *et al.* [25] also reported that CD8$^+$ cells from COPD patients had reduced degranulating capacity, using LAMP-1 expression when *ex vivo* lung slices were exposed to influenza A virus. Indeed, an advantage of using LAMP-1 is that it is a general degranulation marker that can be used to assess the total degranulating cell population in tissues such as the small airways [23].

There are some limitations to our observations. First, as always, more subject tissue sample numbers could be assessed per clinical group, although our findings are statistically robust and consistent, without likely type 1 or type 2 errors. Also, LAMP-1 alone may not be sufficient to determine MC granulation capacity or activity. Thus, more lysosomal markers should be tested as this work progresses. In addition, our current study made inferences about non-MCs. Thus, further studies need to perform double staining for LAMP-1

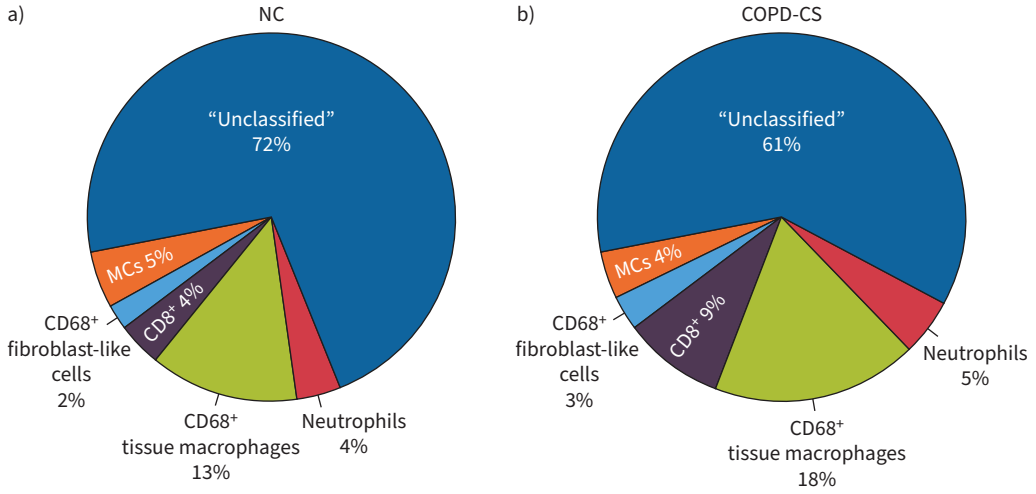

FIGURE 9 Pie chart representing the percent inflammatory cell populations in small airways of a) normal controls (NC) compared with b) COPD current smokers (COPD-CS). MCs: mast cells.

and other specific cell types such as CD8, natural killer cells and macrophages, all granular cells capable of degranulation on activation. Finally, the current data were mainly obtained using small airways from surgically resected tissue derived from cancer patients. It is conceivable that the presence of cancer in the lungs of these patients affected the MC populations and their activity, although all cell assessments were conducted on airways well away from the cancerous region, as we have previously reported [37, 38].

Finally, we wish to also summarise our findings on the novel contribution of the classic inflammatory cell types from our previous published studies on airway immune cells [2] and now from our current study on MCs. Of the total cell counts, the key immune cell population constituted ~30–40% of the overall cellularity in the small airway, leaving the basis for further investigation on the role of other cell types in the airway wall, which we believe are largely unclassified cells (figure 9).

Conclusions

We show here that MC numbers are reduced in the small airway lamina propria, which is congruent with our findings of more general hypocellularity. In contrast, increased MCs in alveolar septa showed the propensity to affect lung function in these patients. Furthermore, in the small airway mucosa, the overall percentage change of MCs to overall cellularity remained unchanged between the COPD-CS and NC groups, as did the number and percentage of active MCs, as indicated by LAMP-1 positivity indicating degranulation. These region-specific differences in total and degranulated MCs and total degranulated cells in smokers/COPD may be directly associated with airway wall changes and impaired lung function, pointing to an active role in maintaining normal tissue homeostasis and, indirectly, in airway architecture.

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Conflict of interest: M.S. Eapen is currently a full-time employee in Mucpharm Pty Ltd; the work presented was not carried out as part of his employment with Mucpharm Pty Ltd. S.S. Sohal has served on the Small Airway Advisory Board for Chiesi Australia and has received the honorarium, outside the submitted work; reports travel support from Chiesi, GSK and AstraZeneca, outside the submitted work; reports a lecture honorarium from Chiesi, outside the submitted work; and has received a research grant from Lung Therapeutics, outside the submitted work. All the other authors do not have any conflict of interest to declare.

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