Eosinophil-derived IL-13 promotes emphysema

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This study suggests that eosinophils may contribute to the emphysema observed in a subset of patients with chronic airways disease http://ow.ly/mBsz30nvDaG

Cite this article as: Doyle AD, Mukherjee M, LeSuer WE, *et al*. Eosinophil-derived IL-13 promotes emphysema. *Eur Respir J* 2019; 53: 1801291 [https://doi.org/10.1183/13993003.01291-2018].

ABSTRACT The inflammatory responses in chronic airway diseases leading to emphysema are not fully defined. We hypothesised that lung eosinophilia contributes to airspace enlargement in a mouse model and to emphysema in patients with chronic obstructive pulmonary disease (COPD).

A transgenic mouse model of chronic type 2 pulmonary inflammation (I5/hE2) was used to examine eosinophil-dependent mechanisms leading to airspace enlargement. Human sputum samples were collected for translational studies examining eosinophilia and matrix metalloprotease (MMP)-12 levels in patients with chronic airways disease.

Airspace enlargement was identified in I5/hE2 mice and was dependent on eosinophils. Examination of I5/hE2 bronchoalveolar lavage identified elevated MMP-12, a mediator of emphysema. We showed, *in vitro*, that eosinophil-derived interleukin (IL)-13 promoted alveolar macrophage MMP-12 production. Airspace enlargement in I5/hE2 mice was dependent on MMP-12 and eosinophil-derived IL-4/13. Consistent with this, MMP-12 was elevated in patients with sputum eosinophilia and computed tomography evidence of emphysema, and also negatively correlated with forced expiratory volume in 1 s.

A mouse model of chronic type 2 pulmonary inflammation exhibited airspace enlargement dependent on MMP-12 and eosinophil-derived IL-4/13. In chronic airways disease patients, lung eosinophilia was associated with elevated MMP-12 levels, which was a predictor of emphysema. These findings suggest an underappreciated mechanism by which eosinophils contribute to the pathologies associated with asthma and COPD.

This article has supplementary material available from erj.ersjournals.com

Received: July 12 2018 | Accepted after revision: Jan 17 2019

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic respiratory condition associated with cigarette smoking and characterised by irreversible airflow obstruction. It is often accompanied by the presence of airway inflammation and emphysema. COPD is among the leading causes of death worldwide and is increasing in prevalence [1]. There is no cure for COPD, but a better understanding of disease subtypes has led to improved therapeutic options [2–4]. The lung inflammatory cell infiltrate in COPD is key to disease pathogenesis. Alveolar macrophages and infiltrating neutrophils mediate destructive activities on the architecture and function of the lung in part through the release of proteases and inflammatory cytokines [5]. Despite the canonical classification of COPD as a neutrophilic disease, blood and airway eosinophilia is found in roughly a third of COPD patients [6–8]. Clinical studies suggest an association between eosinophilia and increased risk of exacerbation [9, 10]. In an analysis of the SPIROMICS cohort, sputum eosinophil levels, in particular, correlated with increased exacerbations, worsening fixed airflow obstruction and poorer quality of life scores in COPD patients [11]. In the SPIROMICS cohort, significantly higher emphysema indices (as measured by quantitative computed tomography (CT)) were noted in patients stratified to the sputum eosinophil-high group [11].

Eosinophils produce a wide array of mediators, including toxic granule proteins (*e.g.* eosinophil peroxidase (EPX)), potent lipid derivatives (*e.g.* leukotrienes), and numerous cytokines and chemokines [12]. In mouse models of chronic lung inflammation, eosinophil mediators have been shown to contribute to pathologies observed in patients, including bronchoconstriction, fibrosis and mucus production [13, 14]. Eosinophil-derived interleukin (IL)-13 is of particular interest as it has been shown to contribute to all of these pathologies [15, 16]. IL-13 mediates these activities through numerous cell types, including alveolar macrophages that play a key role in the lung remodelling associated with severe asthma and COPD.

Alveolar macrophage production of proteases, including matrix metalloproteases (MMPs), contributes to alveolar destruction in COPD [17]. MMPs are extracellular matrix-degrading enzymes. MMP-12, in particular, has been shown to be a critical mediator of alveolar destruction in a mouse model of COPD [18] and evidence points to its importance in COPD patients [17]. IL-13 signalling can polarise macrophages to an M2 ("alternatively activated") phenotype, a feature found in chronic asthma and COPD [19], and induce MMP-12 production. Notably, IL-13-stimulated mouse alveolar macrophages produce MMP-12 *in vitro* [20], and overproduction of IL-13 in the lungs of transgenic mice was sufficient to induce MMP-12 production and destruction of alveolar spaces [21].

We hypothesised that eosinophils are an important source of IL-13 in chronic lung disease, thereby contributing to remodelling of the airways resulting in emphysema. To explore this, we utilised a mouse model of chronic T-helper (Th) type 2 pulmonary inflammation and also analysed sputum samples from patients with asthma or COPD. Identification of the pathways by which emphysema develops/progresses may present new therapeutic targets and strategies to prevent or stabilise this chronic respiratory disease feature.

Methods

Mice

Strains of mice employed include C57BL/6J wild-type (WT) (Jackson Laboratory, Bar Harbor, ME, USA), eoCRE [22], NJ.1638 [23], I5/hE2 [14], PHIL [13], MMP-12$^{-/-}$ (B6.129X-*Mmp12*^{tm1Sds}/J; Jackson Laboratory), floxed IL-4/13 (C.129P2(Cg)-*Il4/Il13*$^{tm1.1Lky}$/J; Jackson Laboratory) and IL-13$^{-/-}$ (gift from Andrew McKenzie, MRC Laboratory of Molecular Biology, Cambridge, UK [24]). Mice were analysed between 2 and 3 months of age. Mice were maintained in the Mayo Clinic Arizona Small Animal Facility (a specific pathogen-free facility) of the Mayo Clinic Arizona (Scottsdale, AZ, USA).

Studies involving mice were performed in accordance with National Institutes of Health and Mayo Clinic Institutional Animal Care and Use Committee guidelines.

Cell isolation and culture

See the supplementary material for full details.

In brief, eosinophils were isolated as described previously [25] from NJ.1638 or IL-13$^{-/-}$/NJ.1638 mice [23, 24]. IL-33 (Peprotech, Rocky Hill, NJ, USA) was added to activate the eosinophils to 50 ng·mL^{-1} (IL-33 was omitted from the resting eosinophil experimental group). Cells were incubated at 37°C and 5% CO_2, and 24 h later were washed 3 times to remove added cytokines. Macrophages were isolated from C57BL/6J or MMP-12$^{-/-}$ mice as described by Lasbury *et al.* [26]. Cells were plated at 150 000 cells per well in 24-well culture plates (Corning, Corning, NY, USA) and cultured for 48 h with the addition of either eosinophils (750 000 cells) or media alone in 500 µL total volume. To assess contact dependency, cultures were performed in transwell plates (0.4 µm pore) (Corning).

Histology

Lung samples were processed for histological analysis as described previously [27]. Haematoxylin and eosin-stained sections were assessed for alveolar space characteristics as described previously [28].

ELISA and multiplex

MMP-12 was determined by mouse MMP-12 PicoKine ELISA (Boster Biological Technology, Pleasanton, CA, USA). EPX was measured as described previously [29]. Other mouse and human cytokines were assessed by bead-based multiplexing technology (Eve Technologies, Calgary, AB, Canada).

Bronchoalveolar lavage, lung homogenate, cytospins, cell counts and differentials

Bronchoalveolar lavage (BAL), lung collection and homogenate preparation were performed as described previously [14, 30]. Cytospins, cell counts and differentials were performed as described previously [31, 32].

Assessment of clinical sputa

We used sputum supernatants (noncellular fraction) from 43 patients with a diagnosis of either asthma or COPD that were previously stored based on observational protocols approved by the local hospital research ethics board in St Joseph's Hospital (Hamilton, ON, Canada). These sputum samples were induced and processed as described previously [33]. Samples from asthmatic subjects were randomly selected from patients who had clinically indicated sputum testing between January and November 2017. Asthma diagnosis was based on bronchodilator reversibility and/or provocative dose causing a 20% fall in forced expiratory volume in 1 s (FEV_1) <8 $mg{\cdot}mL^{-1}$ upon a methacholine challenge test. The sputa of patients with COPD were collected previously as part of a local observational study during a period of clinical stability (from November 2016 to November 2017). Inclusion criteria included fixed airflow obstruction (FEV_1/FVC <0.7) and/or evidence of emphysema on imaging with a ⩾10 pack-year history of smoking. Those with a previous diagnosis of asthma or a significant improvement in post-bronchodilator spirometry were excluded. COPD was assessed based on Global Initiative for Chronic Obstructive Lung Disease criteria [34]. CT scans of the chest were acquired during routine clinical work-up and as such had a variety of protocols applied. The most common CT scan available was of normal resolution without contrast. Emphysema grouping was confirmed by a three-step process: 1) extrapolation from the clinical radiologist's summary report, 2) applying a quantitative CT metric (low attenuation area <950 HU (%LAA −950 HU), using the open-source Chest Imaging Platform provided by 3D Slicer: https//: chestimagingplatform.org) and 3) confirmation of the presence of emphysema by a blinded radiologist. Eosinophilic patients were defined as those with presence of sputum eosinophils >3% and/or evidence of blood eosinophils >300 μL^{-1}, as well as those with historical evidence of eosinophils that were now suppressed due to high-dose inhaled or oral corticosteroid treatment.

Statistical analyses

Data were analysed with Prism version 7 (GraphPad, La Jolla, CA, USA). Statistical comparisons of mouse model and cell culture data were performed with the t-test. Results are represented as mean±SEM. Statistical comparisons between human subject groups were performed by ANOVA/Kruskal–Wallis nonparametric tests and associations were determined by Spearman's rank/Pearson correlation tests based on the distribution of the respective data sets (D'Agostino–Pearson omnibus normality tests). SPSS version 23.0 (IBM, Armonk, NY, USA) was used for multivariate regression analysis. p-values ⩽0.05 were considered significant.

Results

I5/hE2 mice exhibit eosinophil-dependent airspace enlargement

To explore the role of eosinophils in the chronically inflamed lung we employed the I5/hE2 mouse model of chronic type 2 pulmonary inflammation. The I5/hE2 mouse has been shown to develop marked airway remodelling dependent on lung eosinophilia [14, 27]. Examination of I5/hE2 *versus* WT haematoxylin and eosin-stained lung sections revealed airspace enlargement in the lung parenchyma of the I5/hE2 mice (figure 1a). We crossed the I5/hE2 mouse with the eosinophil-deficient PHIL mouse [13] to examine eosinophil-dependent changes in lung structure. The cross of I5/hE2 with PHIL revealed that these lesions were entirely eosinophil dependent. Quantification of the mean intercept length between alveolar septa showed >2-fold increase (~30 *versus* 70 um) in size in I5/hE2 mice compared with I5/hE2/PHIL mice (figure 1b).

Our previous investigations into eosinophil-dependent changes in gene expression in the I5/hE2 lung focused attention on MMP-12 as a candidate for mediating the airspace enlargement observed in I5/hE2. MMP-12 has been shown to cause the emphysema-associated breakdown of alveolar septa in a COPD model [18] and we found *MMP12* was the most upregulated eosinophil-dependent gene in the I5/hE2

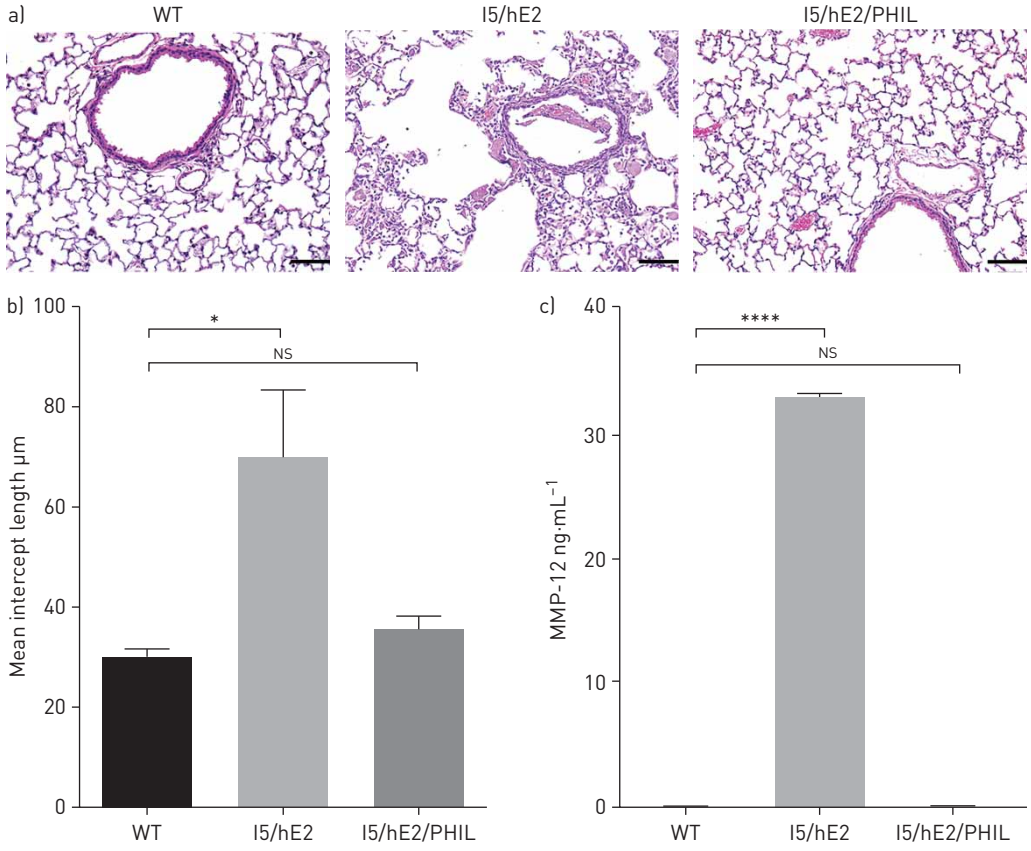

FIGURE 1 Eosinophil-dependent alveolar destruction and modulation of matrix metalloprotease (MMP)-12. a) Haematoxylin and eosin-stained lung sections demonstrate increased alveolar spaces in I5/hE2 lungs relative to wild-type (WT) and their eosinophil-deficient counterparts I5/hE2/PHIL. Lung parenchyma; scale bar: 50 µm. b) Quantification of average alveolar space revealed a 2-fold increase in mean intercept length in I5/hE2 lungs. n=5 mice. c) Bronchoalveolar lavage fluid was collected and assessed by ELISA for MMP-12, a lung remodelling mediator with dramatically increased expression in I5/hE2 lungs. n=6 mice. *: p<0.05; ****: p<0.0001; NS: nonsignificant.

lung [27]. Consistent with this observation, MMP-12 protein levels in the BAL fluid were elevated in I5/hE2 mice and nearly undetectable in I5/hE2/PHIL mice (figure 1c). Taken together, these results suggest that eosinophils promote MMP-12 production and airspace enlargement.

MMP-12 is a mediator of alveolar destruction in I5/hE2 mice

To explore the significance of MMP-12 to the airspace enlargement observed in I5/hE2 we compared I5/hE2 mice with their MMP-12-deficient counterparts I5/hE2/MMP-12$^{-/-}$. Histological examination of the lungs revealed reduced lesions in the absence of MMP-12 (figure 2a). Quantification of the mean intercept length between alveolar septa showed a significant reduction in I5/hE2/MMP-12$^{-/-}$ mice (figure 2b). However, I5/hE2/MMP-12$^{-/-}$ mice had an increased mean intercept length relative to WT mice, suggesting other eosinophil-dependent mediators are contributing to these lesions. For example, other MMPs (MMP-2 and -9) have been linked with emphysema [35, 36] and we found these were also elevated in I5/hE2 lungs (supplementary figure S1). BAL cellularity (I5/hE2 (46.85±6.45×10^5 cells·mL^{-1}) and I5/hE2/MMP-12$^{-/-}$ (35.23±4.25×10^5 cells·mL^{-1})) and eosinophil numbers (I5/hE2 (43.79±5.53×10^5 cells·mL^{-1}) and I5/hE2/MMP-12$^{-/-}$ (34.6±4.85×10^5 cells·mL^{-1})) were not affected by the loss of MMP-12 in this model.

Eosinophil-derived IL-13 promotes alveolar macrophage MMP-12 production in vitro

To investigate the relationship between eosinophils and MMP-12 production we performed *in vitro* experiments with eosinophils and known airway MMP-12-producing cells, alveolar macrophages [37, 38]. *In vitro* experiments revealed that MMP-12 was not detectable in eosinophil culture supernatants, whereas MMP-12 could be detected in alveolar macrophage culture supernatants. Eosinophil and macrophage co-culture resulted in significantly increased levels of MMP-12 compared with macrophage culture

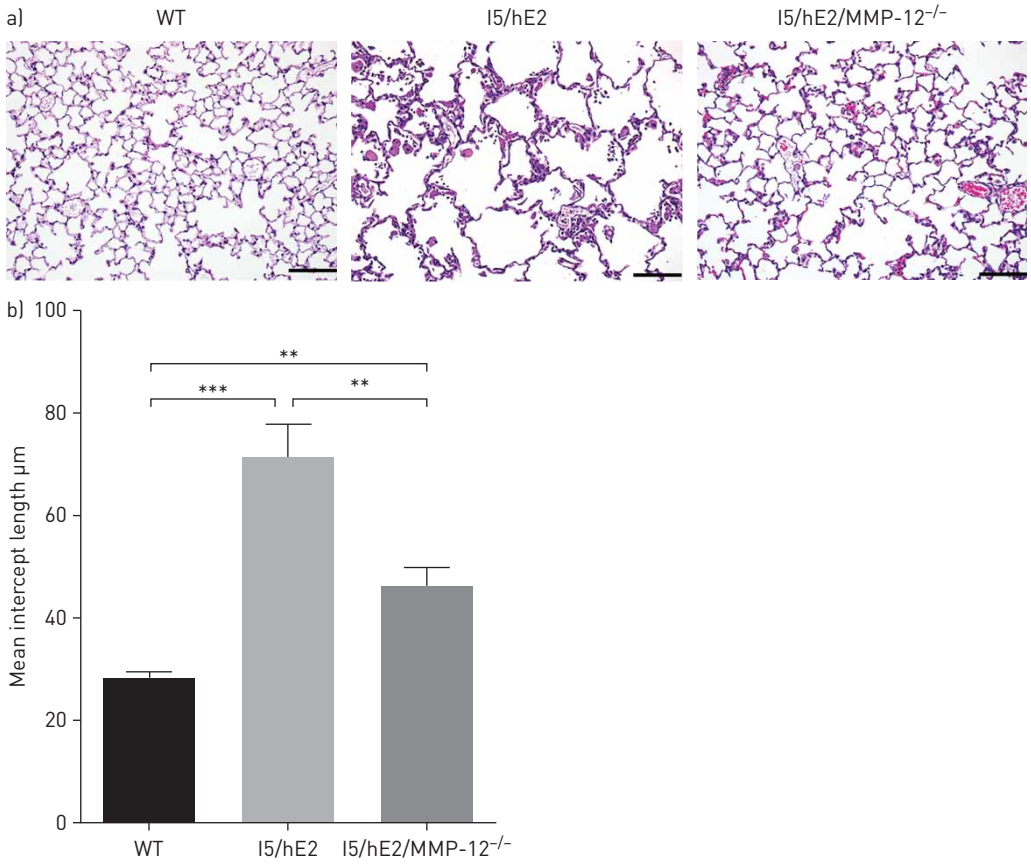

FIGURE 2 Matrix metalloprotease (MMP)-12 mediates alveolar destruction in the I5/hE2 model. a) Haematoxylin and eosin-stained lung sections demonstrate enlarged airspaces in I5/hE2 mice relative to wild-type (WT) and MMP-12$^{-/-}$ mice. Lung parenchyma; scale bar: 50 µm. b) Haematoxylin and eosin-stained lung sections assessed for mean intercept length demonstrate increased alveolar spaces in I5/hE2 mice relative to their MMP-12-deficient counterparts I5/hE2/MMP-12$^{-/-}$. n⩾3 mice. **: p<0.01; ***: p<0.001.

alone (figure 3a). Co-culture of WT eosinophils with MMP-12$^{-/-}$ alveolar macrophages showed that the MMP-12 in these culture experiments is derived from macrophages (figure 3a).

To explore the mechanism of eosinophil-mediated macrophage MMP-12 production we first assessed the role of eosinophil activation with IL-33, a cytokine associated with chronic lung disease and elevated in the I5/hE2 mouse lung [27, 39]. Eosinophil activation by IL-33 has been demonstrated to increase release of soluble mediators that may influence the production of MMP-12 from macrophages [40, 41]. Activation of the eosinophils with IL-33 prior to co-culture with alveolar macrophages enhanced macrophage MMP-12 production relative to co-culture with nonactivated eosinophils (figure 3b). We next examined contact dependency by transwell co-culture of eosinophils with alveolar macrophages. We showed that the increased MMP-12 from macrophages is not contact dependent (figure 3c), suggesting that macrophages produce MMP-12 in response to an eosinophil-secreted mediator.

Previous studies have demonstrated IL-13 promotes MMP-12 production by macrophages [20, 36] and IL-33 is known to induce IL-13 from eosinophils [15, 42]. We assessed the role of eosinophil-derived IL-13 by co-cultures of WT alveolar macrophages with eosinophils sufficient or deficient for IL-13. We found that eosinophil-derived IL-13 promoted alveolar macrophage MMP-12 production (figure 3d).

I5/hE2 mice exhibit airspace enlargement dependent on eosinophil-derived IL-13 and MMP-12

To examine if the lung eosinophilia in our mouse model of chronic type 2 pulmonary inflammation induces alveolar destruction *via* IL-13-mediated MMP-12 production we generated conditional knockout mice [43] by crossing the eosinophil-specific Cre recombinase mouse, eoCRE, with the available floxed IL-4/13 mouse [44], resulting in eosinophil-specific loss of IL-13 expression (as well as IL-4) (supplementary figure S2). We then crossed these mice with I5/hE2 to create I5/hE2/eoCRE/4/13$^{fl/fl}$, which

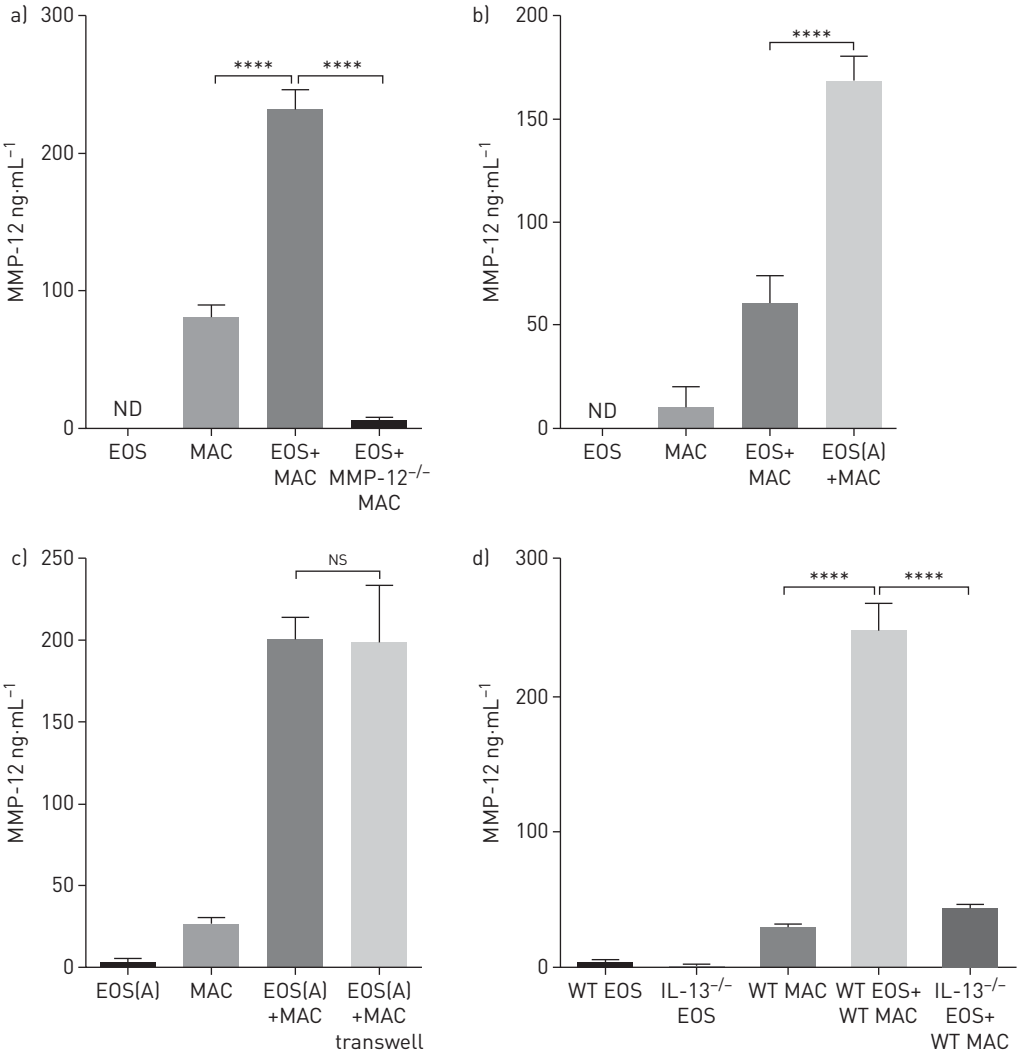

FIGURE 3 Eosinophils directly modulate alveolar macrophage production of matrix metalloprotease (MMP)-12 through interleukin (IL)-13-dependent mechanisms: ELISA measurement of MMP-12 in cell culture supernatant from *in vitro* 48 h cultures of eosinophils and/or alveolar macrophages. ND: not detected; EOS: eosinophils; MAC: macrophages; EOS(A): activated eosinophils; WT: wild-type. a) Eosinophils cultured alone, macrophages cultured alone and co-culture (eosinophils+macrophages). WT eosinophils were activated (IL-33 for 24 h then washed) and cultured with WT macrophages or MMP-12$^{-/-}$ macrophages showing the MMP-12 is from macrophages. b) Assessment of co-culture supernatants from resting eosinophils *versus* activated eosinophils with macrophages demonstrated that pre-activation of eosinophils (IL-33 for 24 h then washed) enhances macrophage MMP-12 production. c) Transwell co-culture of activated eosinophils with macrophages shows that contact is not required for the enhanced macrophage MMP-12 production. d) ELISA measurements from co-culture supernatants of IL-13$^{-/-}$ *versus* WT eosinophils with WT macrophages demonstrates that eosinophil-derived IL-13 is promoting macrophage MMP-12 production. ****: p<0.0001; NS: nonsignificant.

was compared with I5/hE2/eoCRE as a control. Histological examination of the lungs again showed airspace enlargement in I5/hE2/eoCRE that was dependent upon eosinophil-derived IL-4/13 (figure 4a). Notably, alveolar spaces in I5/hE2/eoCRE/4/13$^{fl/fl}$ were equivalent to those of WT mice, suggesting that the enlarged alveolar spaces in I5/hE2 mice were entirely dependent on eosinophil-derived IL-4/13 (figure 4b). BAL cellularity (I5/hE2/eoCRE ($39.1\pm8.74\times10^5$ cells·mL^{-1}) and I5/hE2/eoCRE/4/13$^{fl/fl}$ ($22.98\pm4.31\times10^5$ cells·mL^{-1})) and eosinophil numbers (I5/hE2/eoCRE ($26.1\pm4.04\times10^5$ cells·mL^{-1}) and I5/hE2/eoCRE/4/13$^{fl/fl}$ ($41.53\pm8.28\times10^5$ cells·mL^{-1})) were not affected by the loss of eosinophil-derived IL-4/13 in this model. Significantly, BAL MMP-12 levels were entirely dependent on eosinophil-derived IL-4/13 (figure 4c). Taken together, our data suggest eosinophil-derived IL-13 is a key mediator of alveolar macrophage MMP-12 production and alveolar destruction in this chronic pulmonary inflammation model.

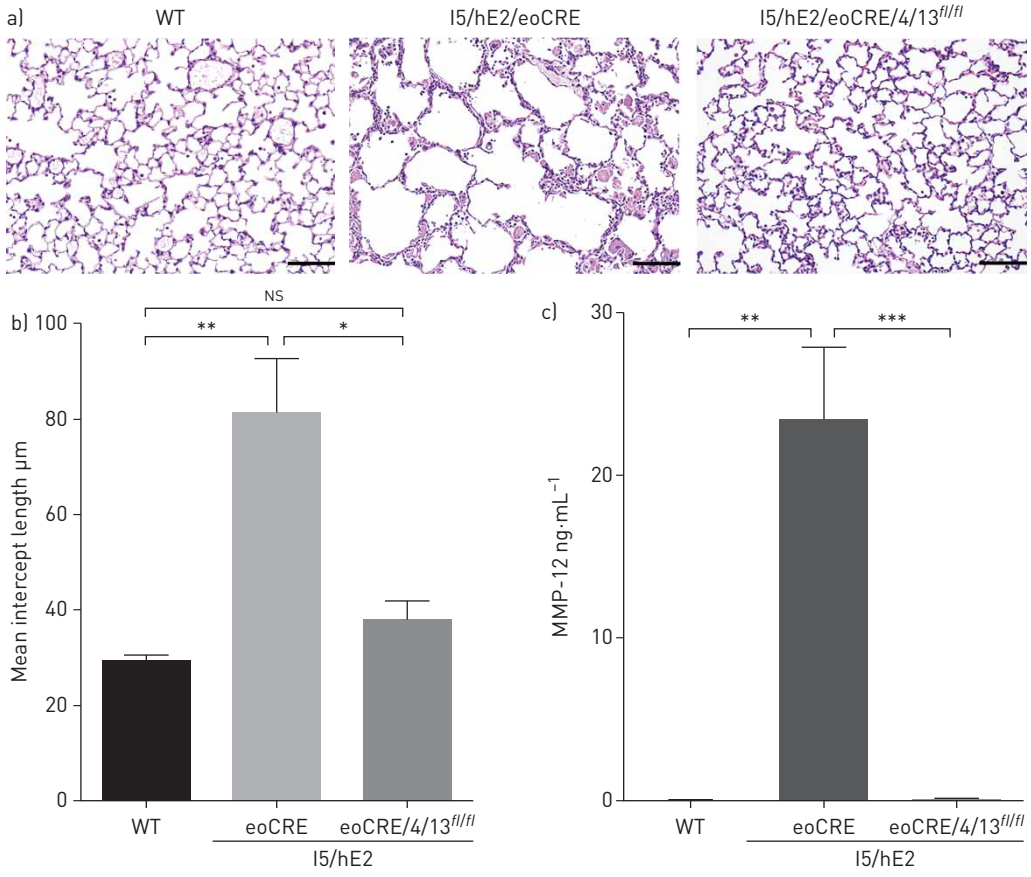

FIGURE 4 Eosinophil-derived interleukin (IL)-4/13 induces matrix metalloprotease (MMP)-12 production and alveolar destruction in I5/hE2 mice. WT: wild-type. a) A genetic cross of I5/hE2 with the eosinophil-specific Cre mouse (eoCRE) and then the available floxed IL-4/13 mouse (I5/hE2/eoCRE/4/13$^{fl/fl}$) revealed that enlarged alveolar spaces in I5/hE2 mice are dependent on eosinophil-derived IL-4/13. Lung parenchyma; scale bar: 50 µm. b) Haematoxylin and eosin-stained lung sections demonstrate enlarged airspaces in I5/hE2 mice relative to eosinophil-derived IL-4/13-deficient mice. c) ELISA measurement of MMP-12 in bronchoalveolar lavage showed MMP-12 in I5/hE2 mice is dependent on eosinophil-derived IL-4/13. n⩾3 mice. *: p<0.05; **: p<0.01; ***: p<0.001; NS: nonsignificant.

Elevated MMP-12 is associated with eosinophilia, emphysema and decreased lung function in human subjects

Next, we investigated the relationship between MMP-12, eosinophilia, emphysema and lung function in humans. Out of the 44 patient sputa randomly selected, five were rejected (three rejected for comorbidities/other inflammatory abnormalities, one rejected for not meeting criteria for asthma or COPD and one rejected for age <18 years). Of the remaining subjects, 16 had asthma and 23 had COPD; their clinical characteristics are represented in tables 1 and 2. Sputum EPX levels were higher in the eosinophilic group (p=0.002) (figure 5a) and correlated significantly with sputum IL-13 levels (r=0.536; p=0.0004, Spearman rank). Irrespective of diagnosis, the MMP-12 levels detected in the sputa were higher in the eosinophilic group (p=0.01) (figure 5b) and modestly correlated with sputum IL-13 levels (r=0.35; p=0.03) (figure 5c). The significant correlation of MMP-12 with airway IL-13 levels and not EPX (r=0.05; p=0.7) suggested the possibility that MMP-12 was not directly associated with eosinophilia *per se*, but instead associated with eosinophil-derived, and potentially other sources of, IL-13 inflammation.

In the subgroup analysis, differential expression of MMP-12 in the asthma subset with respect to eosinophilia was unremarkable (p>0.99) (figure 5d); however, increased detection of MMP-12 was evident in the eosinophilic COPD airways (p=0.05) (figure 5d). In fact, MMP-12 levels in COPD patients were higher in those with concomitant eosinophilia and emphysema compared with those who did not exhibit either (p=0.02) (figure 5e). Comparing %LAA −950 HU by eosinophilic status revealed a higher value in those with eosinophilia (median 1.4% *versus* 0.08%; p=0.04, Mann–Whitney test). Emphysema grouping was confirmed by the three steps described in the Methods section. The accuracy of grouping (by clinical radiologist report) was supported by a significantly higher %LAA −950 HU in the emphysema group

TABLE 1 Demographics of recruited patients: asthma and chronic obstructive pulmonary disease (COPD)

	Asthma	COPD
Patients	16	23
Age years	62±10	64±11
Female	9 (56)	10 (43)
FEV_1 % pred	65±17*	49±17*
FEV_1/FVC	0.62±0.13*	0.49±0.13*
D_{LCO} % pred	99±33*,#	61±18*,¶
Current smoker	1 (6)*	12 (52)*
Ex-smoker	0 (0)	7 (30)
Smoking history pack-years	NA	46±24
Emphysema	1 (6)*	15 (65)*
Centrilobular	1	7
Paraseptal	0	1
Both centrilobular and paraseptal	0	7
Eosinophilia	11 (69)	11 (48)
Sputum eosinophils %	7.0±9.1	5.9±15
Sputum EPX ng·mL^{-1}·g^{-1}	375±587*	91.4±144*
ICS dose μg·day^{-1} fluticasone equivalent	750 (2000–0)	750 (3500–0)
ICS use	15 (94)	20 (87)
Maintenance OCS use	5 (31)	2 (9)
LABA use	15 (94)	18 (83)
LAMA use	0 (0)*	12 (52)*

Data are presented as n, mean±SD, n (%) or median (minimum–maximum). All COPD patients were current smokers or ex-smokers with ⩾10 pack-year history. FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide; NA: not available; EPX: eosinophil peroxidase; ICS: inhaled corticosteroid; OCS: oral corticosteroid; LABA: long-acting β-agonist; LAMA: long-acting muscarinic antagonist. #: based on n=3; ¶: based on n=9. *: statistically significant difference between groups ($p \leqslant 0.05$).

TABLE 2 Demographics of recruited patients: eosinophilic and noneosinophilic

	Eosinophilic	Noneosinophilic
Patients	22	17
Asthma	11 (50)	5 (29)
Age years	63±7.5	64±14
Female	10 (46)	9 (53)
FEV_1 % pred	52±18	60±19
FEV_1/FVC	0.53±0.15	0.56±0.14
D_{LCO} % pred	71±32#	NA
Current smoker	5 (23)	9 (53)
Ex-smoker	5 (23)	2 (12)
Smoking history pack-years	25±32	42±24
Emphysema	8 (36)	8 (47)
Centrilobular	5	3
Paraseptal	1	0
Both centrilobular and paraseptal	2	5
Sputum eosinophils %	11±15*	0.25±0.40*
Sputum EPX ng·mL^{-1}·g^{-1}	293±506*	81±118*
ICS dose μg·day^{-1} fluticasone equivalent	750 (2000–0)	500 (3500–0)
ICS use	19 (86)	16 (94)
Maintenance OCS use	5 (23)	2 (12)
LABA use	20 (91)	14 (82)
LAMA use	7 (32)	5 (29)

Data are presented as n, n (%), mean±SD or median (minimum–maximum). FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide; NA: not available; EPX: eosinophil peroxidase; ICS: inhaled corticosteroid; OCS: oral corticosteroid; LABA: long-acting β-agonist; LAMA: long-acting muscarinic antagonist. #: based on n=7. *: statistically significant difference between groups ($p \leqslant 0.05$).

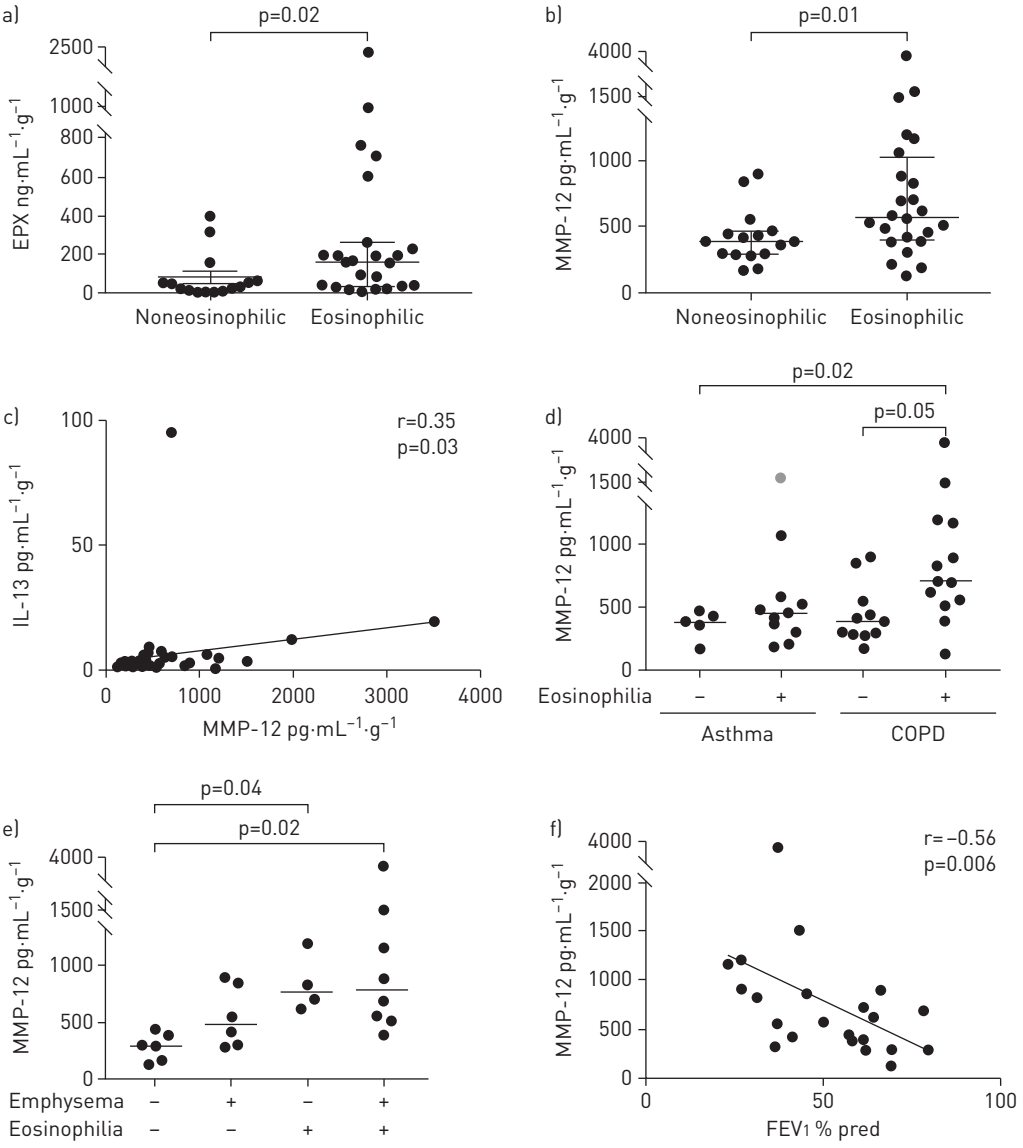

FIGURE 5 Matrix metalloprotease (MMP)-12 expression in eosinophilic emphysematous airways. EPX: eosinophil peroxidase; IL: interleukin; FEV_1: forced expiratory volume in 1 s; COPD: chronic obstructive pulmonary disease. a) EPX and b) MMP-12 expression in airways of n=40 patients with chronic airways disease (diagnosis of either asthma or COPD) classified based on eosinophilia. Mann–Whitney test. c) Correlation of sputum MMP-12 with IL-13 (n=40). Spearman rank. d, e) MMP-12 expression in airways of d) both asthma (n=16) and COPD (n=24) subjects, classified based on eosinophilia, and e) COPD subjects only, classified with respect to eosinophilia and emphysema. Kruskal–Wallis with Dunn's multiple correction. f) Correlation of sputum MMP-12 with FEV_1 % pred in COPD patients (n=24). Spearman rank. Each symbol represents individual patient data and, where appropriate, the median is indicated for each subset. The grey symbol in (d) indicates an asthmatic patient with evidence of airspace enlargement. $p \leqslant 0.05$ considered as significant.

(median 1.97% *versus* 0.18%; p=0.0073, Mann–Whitney test) and confirmation of emphysema by a blinded radiologist for each subject. Of interest, the patient in the eosinophilic asthma group who had the highest level of MMP-12 (marked as the grey symbol in figure 5d) had CT evidence of air space enlargement. If this data-point is removed, MMP-12 levels were significantly higher in the eosinophilic COPD patient sputa compared with eosinophilic asthmatic subjects (p=0.01, Mann–Whitney), which were otherwise comparable (p=0.43) (figure 5d). In a multivariate regression model, MMP-12 was computed to be a predictor (β=0.361, R^2=0.13, standard error of estimate 0.47; p=0.02) for the presence of emphysema (CT evidence) and not eosinophilia, EPX or IL-13 (supplementary table S1). Finally, MMP-12 levels were negatively associated with FEV_1 % pred in patients with COPD (r=−0.56; p=0.006) (figure 5f) and this association remained significant with the inclusion of asthmatic participants (r=−0.45; p=0.005).

Discussion

Although type 2 inflammation is more frequently associated with asthma than COPD, it can be present or absent in either condition. In this study, we used a mouse model of chronic type 2 pulmonary inflammation (I5/hE2) to explore the link between eosinophils and alveolar destruction, without any connotation regarding the physiological features required for a diagnosis of asthma or COPD, or involvement of smoking-induced pathways. These mice develop eosinophil-dependent lung pathologies that are absent when crossed with eosinophil-deficient mice (I5/hE2/PHIL), but may be restored with eosinophil-sufficient bone marrow engraftment [14]. We found that I5/hE2 mice develop eosinophil-dependent airspace enlargement. Significantly, MMP-12, a protease known to be a critical mediator of alveolar destruction, was elevated in I5/hE2 lungs and this was entirely dependent upon the presence of eosinophils. We demonstrated *ex vivo* that eosinophil-derived IL-13 stimulated alveolar macrophages to produce MMP-12. To confirm these pathways *in vivo* we crossed I5/hE2 with various genetically engineered mice and showed that eosinophils promote airspace enlargement dependent on IL-13 (and possibly IL-4, as we utilised the available floxed IL-4/13 mouse) and MMP-12. Consistent with these observations, it has been shown previously that MMP-12 is induced by IL-13 in the lung [20, 36] and eosinophils are a known source of IL-13 [15, 42]. Furthermore, overexpression of IL-13 in the mouse lung resulted in increased MMP-12 levels and airspace enlargement [36]. Taken together, these data reveal a clear path by which eosinophils may contribute to the destruction of airspaces in chronic respiratory diseases.

Eosinophil-derived IL-13 has been demonstrated to promote M2 macrophage differentiation as well as accumulation in a mouse model of allergic pulmonary inflammation [15]. M2 alveolar macrophages have been identified as MMP-12-producing cells (reviewed in [38]). Notably, we found that other proteases associated with alveolar destruction, *i.e.* MMP-2 and -9, are also elevated in the lungs of I5/hE2 mice dependent on eosinophil-derived IL-4/13. Eosinophils have been shown to express MMP-9 in asthmatic airways [45] and it is likely that eosinophils are indirectly linked, through interactions with other cell types such as macrophages, to the production of numerous remodelling agents, including MMP-2 and -9. Indeed, we showed that removal of MMP-12 only partially reduced the alveolar destruction in I5/hE2 lungs and observed that in older MMP-12-deficient I5/hE2 mice the extent of alveolar destruction approached that of I5/hE2 (data not shown), suggesting a role for other mediators. Key contributors likely include other MMPs and cathepsins previously observed to contribute to these changes in a transgenic mouse with overexpression of IL-13 in the lung [21]. Future studies are needed to determine other key eosinophil-associated mediator(s) of alveolar destruction in the I5/hE2 lung. Taken together, these interactions highlight the role of eosinophils as regulators of local immune responses and remodelling events [46].

The I5/hE2 model enabled us to isolate the contribution of eosinophils to airspace enlargement. It is likely that other cell types can participate in the pathways explored in this work. In particular, group 2 innate lymphoid cells (ILC2s) and Th2 T-cells produce IL-13 in the lung (reviewed in [47, 48]); moreover, the airway epithelium and smooth muscle have been identified as producers of MMP-12 [49, 50]. Nevertheless, our finding that eosinophils can mediate these activities is intriguing. While IL-13 is a secreted factor, it is possible that eosinophils are a particularly potent source for these interactions. Clearly, the interactions between eosinophils and alveolar macrophages deserve further investigation as it may be possible that a spatial, temporal and/or multifactor association (*e.g.* IL-13 in combination with other signals) is important for these activities.

A pathogenic role of eosinophils in COPD has long been considered. Despite their potential importance in the pathophysiology of COPD, efforts to target eosinophils in clinical trials of COPD have met with limited success. A very modest clinical benefit has been noted in patients with blood eosinophilia post-treatment with subcutaneous mepolizumab (an anti-IL-5 antibody that results in eosinophil depletion) (METREX trial) [51]. In the parallel study evaluating two doses of mepolizumab as an add-on treatment for frequently exacerbating COPD patients characterised by eosinophil level (METREO trial), the annual rate of exacerbations compared with the placebo arm was deemed insignificant [51]. These findings corroborated those reported in a smaller study where the drug reduced both sputum and blood eosinophils, and yet failed to show any significant improvement in the rate of exacerbations, lung function or radiological evidence of disease severity [52]. Similarly, targeting eosinophils in COPD using benralizumab (an anti-IL-5 receptor α antibody that results in eosinophil depletion) showed no clinical improvement in the rate of exacerbations compared with placebo. However, a modest improvement in FEV_1 was reported in the drug arm, indicating eosinophils or, by extension, eosinophilic factors might be affecting lung function [53].

The limited improvement in clinical trials examining anti-eosinophil agents in COPD may be due to irreversible airspace enlargement. Our experiments suggest a role of eosinophils in the initiation/development of airspace enlargement, albeit indirectly through stimulation of MMP-12 (and possibly other

protease) production by alveolar macrophages. Indeed, in COPD patients with evidence of eosinophilia there was a detectable increase in MMP-12, which was computed to be a predictor for the presence of CT evidence of emphysema. These findings suggest strategies targeting eosinophils in eosinophilic COPD may be beneficial, but that long-term studies with lung function as the primary outcome may be needed to appreciate this benefit. Alternative strategies to IL-5 blockade that target eosinophils and other cells (*e.g.* ILC2s) could potentially reduce exacerbations, and should be considered for future studies. One such strategy suggested by previous observations and the findings from this study is a combination of IL-33 and IL-13 blockade.

The concept of eosinophil-driven emphysema has been studied previously within COPD cohorts, which identified associations between blood eosinophils and markers of connective tissue destruction [54], and between sputum eosinophils and emphysema indices on quantitative CT [11]. Furthermore, a network analysis incorporating measures of the blood, bone marrow, as well as clinical variables, demonstrated that the capacity for immune-mediated repair was impaired in the presence of elevated blood eosinophils and/or emphysema [55]. The current study provides mechanistic, mouse-model evidence and corroborating data from a "real-life" COPD cohort that lend support to the previous findings.

In summary, our findings reveal a role for eosinophil-derived IL-13 in alveolar destruction through induction of MMP-12. Asthma and COPD are diagnosed based on physiological criteria, with the former demonstrating reversible airflow obstruction or airway hyperresponsiveness, and the latter demonstrating fixed airflow obstruction. We translated our findings to patients with eosinophilic COPD, suggesting a contribution of eosinophil-mediated induction of MMP-12 in the development of emphysema. Sputa was tested in patients with asthma or COPD, given that eosinophilic inflammation can be a shared component between these diseases, but emphysema is not classically associated with asthma. It is interesting to speculate that our findings may extend to other chronic inflammatory respiratory diseases. Notably, asthma disease progression and severity is associated with eosinophilia and may develop into/overlap with COPD. Indeed, the asthmatic patient with highest MMP-12 detection (levels comparable to the COPD subset) had persistent eosinophilia as well as CT evidence of emphysema. A recent report from Gelb *et al.* [56] builds on evidence of asthma subjects with a type 2 phenotype developing mild emphysema and loss of lung elasticity. In addition, a case report of a nonsmoking eosinophilic bronchitis patient developing emphysema highlights this possibility [57]. Taken together, these findings suggest that a focus on therapeutic management of lung eosinophilia, IL-13 and proteases including MMP-12 may provide effective strategies for slowing the progression of emphysema in certain patient populations.

Acknowledgements: We thank all members of the Lee laboratory group (Mayo Clinic Arizona, Scottsdale, AZ, USA) for their support, and we recognise the excellent administrative support provided by Stefanie Brendle, Linda Mardel and Shirley "Charlie" Kern (Mayo Clinic Arizona). We also recognise Melanie Kjarsgaard, Nicola LaVigne, Katherine Radford and Colm Boylan (McMaster University and St Joseph's Healthcare, Hamilton, ON, Canada) for their expert technical assistance. This work is dedicated to the memory of our dear friend and colleague James "Jamie" Lee whose larger than life character was matched only by his scientific genius. We miss him dearly.

Author contributions: A.D. Doyle, J.J. Frere and J.J. Lee designed the research study. A.D. Doyle, W.E. LeSuer, T.B. Bittner, S.M. Pasha, J.J. Frere, J.L. Neely, J.A. Kloeber, K.P. Shim, S.I. Ochkur, T. Ho and M. Mukherjee performed the research. A.D. Doyle, W.E. LeSuer, T.B. Bittner, S.M. Pasha, J.J. Frere, S.I. Ochkur, B.L. Wright, M.A. Rank, M. Mukherjee, P. Nair, J.J. Lee and E.A. Jacobsen analysed the data. A.D. Doyle wrote the initial draft of the manuscript. P. Nair, B.L. Wright, M.A. Rank, M. Mukherjee and E.A. Jacobsen provided critical assessments during the revision process leading to the final submitted manuscript. All authors have reviewed and approved the final version of this manuscript.

Conflict of interest: A.D. Doyle reports grants from NIH (F31HL124959), donor money paid to the Mayo Clinic from Donald R. Levin Family Foundation, and a scholarship at the Mayo Clinic from Mayo Clinic Sidney Luckman Family Predoctoral Fellowship, during the conduct of the study. M. Mukherjee has nothing to disclose. W.E. LeSuer has nothing to disclose. T.B. Bittner has nothing to disclose. S.M. Pasha has nothing to disclose. J.J. Frere has nothing to disclose. J.L. Neely has nothing to disclose. J.A. Kloeber has nothing to disclose. K.P. Shim has nothing to disclose. S.I. Ochkur has nothing to disclose. T. Ho has nothing to disclose. S. Svenningsen has nothing to disclose. B.L. Wright reports donor money paid to the Mayo Clinic from Donald R. Levin Family Foundation, during the conduct of the study. M.A. Rank has nothing to disclose. J.J. Lee received grants from NIH (HL058723), NIH NCRR (K26 RR0109709) and the Mayo Foundation for Medical Education and Research, during the conduct of this study; J.J. Lee is deceased and this statement was made on behalf of the author by the corresponding author. P. Nair reports grants and personal fees for consultancy and lecturing from AstraZeneca and Teva, grants from Boehringer Ingelheim, grants and personal fees for lecturing from Novartis, grants and personal fees for consultancy from Sanofi and GSK, and personal fees for consultancy from Theravance and Knopp, outside the submitted work. E.A. Jacobsen reports grants from NIH (HL065228), and donor money paid to the Mayo Clinic from Donald R. Levin Family Foundation, during the conduct of the study.

Support statement: This work was supported by Mayo Foundation for Medical Education and Research, NIH NHLBI R01 HL058723 and HL065228, and NIH NCRR K26 RR0109709. A.D. Doyle was supported by the Mayo Graduate School, the Mayo Clinic Sidney Luckman Family Predoctoral Fellowship, the Donald R. Levin Family Foundation and

NIH NHLBI F31HL124959. P. Nair is supported by the Frederick E. Hargreave Teva Innovation Chair in Airway Diseases. Funding information for this article has been deposited with the Crossref Funder Registry.

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