Cigarette smoke-initiated autoimmunity facilitates sensitisation to elastin-induced COPD-like pathologies in mice

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MMP12-generated elastin fragments serve as a self-antigen and drive cigarette smoke-induced autoimmune processes in mice. These findings provide experimental evidence for cigarette smoke-induced autoimmunity and represent a novel mouse model of COPD. https://bit.ly/2XK9dC6

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ABSTRACT It is currently not understood whether cigarette smoke exposure facilitates sensitisation to self-antigens and whether ensuing auto-reactive T cells drive chronic obstructive pulmonary disease (COPD)-associated pathologies.

To address this question, mice were exposed to cigarette smoke for 2 weeks. Following a 2-week period of rest, mice were challenged intratracheally with elastin for 3 days or 1 month. *Rag1*$^{-/-}$, *Mmp12*$^{-/-}$, and *Il17a*$^{-/-}$ mice and neutralising antibodies against active elastin fragments were used for mechanistic investigations. Human GVAPGVGVAPGV/HLA-A*02:01 tetramer was synthesised to assess the presence of elastin-specific T cells in patients with COPD.

We observed that 2 weeks of cigarette smoke exposure induced an elastin-specific T cell response that led to neutrophilic airway inflammation and mucus hyperproduction following elastin recall challenge. Repeated elastin challenge for 1 month resulted in airway remodelling, lung function decline and airspace enlargement. Elastin-specific T cell recall responses were dose dependent and memory lasted for over 6 months. Adoptive T cell transfer and studies in T cells deficient *Rag1*$^{-/-}$mice conclusively implicated T cells in these processes. Mechanistically, cigarette smoke exposure-induced elastin-specific T cell responses were matrix metalloproteinase (MMP)12-dependent, while the ensuing immune inflammatory processes were interleukin 17A-driven. Anti-elastin antibodies and T cells specific for elastin peptides were increased in patients with COPD.

These data demonstrate that MMP12-generated elastin fragments serve as a self-antigen and drive the cigarette smoke-induced autoimmune processes in mice that result in a bronchitis-like phenotype and airspace enlargement. The study provides proof of concept of cigarette smoke-induced autoimmune processes and may serve as a novel mouse model of COPD.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a global public health concern. The disease encompasses two major clinical phenotypes, chronic bronchitis and emphysema. Cigarette smoking is the major risk factor of COPD, causing chronic airway inflammation, mucus hyperproduction, destruction of lung tissue and remodelling of small airways. These processes contribute to largely irreversible airflow obstruction as well as systemic comorbidities [1, 2]. The cellular and molecular mechanisms that contribute to cigarette smoke-induced COPD pathogenesis remain largely unknown. Similarly, it is not well understood why airway inflammation persists in patients with COPD following smoking cessation [3].

It is widely accepted that cigarette smoke exposure injures epithelial cells and activates alveolar macrophages, resulting in innate-driven inflammation. Moreover, cigarette smoke exposure activates dendritic cells to induce adaptive immune response, including $CD4^+$ T cells, cytolytic $CD8^+$ T cells, and B cells. Together, these immune inflammatory processes to emphysema formation [4, 5]. It has been shown that the anti-elastin antibodies were present in patients with COPD [6, 7], and elastin peptides could act as cognate antigens to stimulate T helper (Th)1- and Th17-polarised immune responses in subjects with emphysema [8]. These findings provide evidence that autoimmune processes may be implicated in the pathogenesis of COPD [9]. However, whether COPD indeed incorporates autoimmune disease processes remains controversial, as several groups did not observe increased anti-elastin antibodies in COPD [10, 11]. Moreover, cigarette smoke-induced autoimmune processes have not been shown in experimental COPD models.

The objective of the current study was to investigate whether elastin serves as a self-antigen to drive cigarette smoke-induced autoimmune processes. We show that cigarette smoke-exposure facilitated sensitisation to elastin through a matrix metalloproteinase (MMP)-12-dependent mechanism. Elastin challenge propagated T cell-dependent neutrophilic airway inflammation and induces COPD-like pathologies. Based on these findings, we propose that we established a rapid and novel autoimmune-driven mouse model that mimics most of the pathological features of human COPD.

Materials and methods

Additional details on methods are provided in the supplementary material.

Animals

6–8 weeks male C57BL/6 mice were purchased from the Animal Centre of Slaccas (Shanghai, China), *Rag1*$^{-/-}$ mice were purchased from Shanghai Model Organism (Shanghai, China), *Il17a*$^{-/-}$ mice were provided by the centre for Experimental Medicine and Systems Biology (Institute of Medical Science, University of Tokyo, Tokyo, Japan). *Mmp12*$^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained in the animal facility of the laboratory animal centre of Zhejiang University. Male mice aged 6–8 weeks were used for each experiment. All experimental protocols were approved by the Ethical Committee for Animal Studies at Zhejiang University.

In vivo *cigarette smoke exposures and treatments*

Mice were exposed to cigarette smoke in a stainless-steel chamber using a whole-body smoke exposure system (TE-10; Teague Enterprises (Woodland, CA, USA)) for approximately 2.5 h per day (100

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cigarettes), 5 days per week. Total particulate matter concentrations in the exposure chamber were between 150 and 180 $mg \cdot m^{-3}$. Serum cotinine levels measured immediately after cigarette smoke exposure were around 20 $ng \cdot mL^{-1}$. Control groups were exposed to filtered room air.

Mouse elastin (E6402-SPEC) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse elastin peptide (CB573) was purchased from Elastin Products Company (Owensville, MO, USA). 2 $mg \cdot mL^{-1}$ elastin/elastin peptide was suspended in sterile saline and sonicated. 100 µg elastin/elastin peptide in 50 µL saline was administered intratracheally. For elastin sensitisation, 100 µg elastin in 0.1 mL saline was mixed with 0.1 mL complete Freund's adjuvant (CFA) (F5881; Sigma-Aldrich) and injected intraperitoneally. BA4, a mouse monoclonal anti-elastin antibody (E4013; Sigma-Aldrich), 10 µg in 50 µL saline was instilled daily, 1 h before cigarette smoke exposure.

Lipopolysaccharide (1 µg in 50 µL saline) (LPS: L2630; Sigma-Aldrich) was delivered intratracheally using a micro-syringe.

Human study subjects

We enrolled healthy controls and hospitalised COPD patients for collecting peripheral blood and induced sputum. The diagnosis of COPD was made according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [12]. Exclusion criteria were as follows: any chronic cardiopulmonary disease other than COPD and an inability to give written informed consent or cooperate with the study investigators. Clinical characteristics are described in table 1. The study subjects were recruited from two medical centres. The study protocols were approved by the Ethics of Research Committee of the Second Affiliated Hospital, Zhejiang University. Written informed consent was obtained from all participants.

Results

Exposure to cigarette smoke sensitises to elastin and elicits IL17A-predominant immune responses and bronchitis-like airway inflammation

To investigate whether cigarette smoke exposure sensitises to elastin, mice were exposed to cigarette smoke for 2 weeks followed by 2 weeks of rest prior to challenge with elastin on three consecutive days (figure 1a). Elastin challenge in cigarette smoke-exposed mice induced significant neutrophilic airway inflammation, reaching up to 60% neutrophils in the bronchoalveolar lavage fluid (BALF) (figure 1b). Histological assessment showed neutrophilic airway inflammation, corroborating the observations in the

TABLE 1 Clinical characteristics of healthy controls and hospitalised chronic obstructive pulmonary disease (COPD) patients

Parameter	Healthy controls (n=20)	Smokers with COPD (n=17)	p-value
Age years	59.15±1.39	60.53±1.47	0.501
Male	16 (80)	14 (82)	0.8604
Smoking pack-years	NA	32.82±7.79	NA
BMI $kg \cdot m^{-2}$	24.5±0.6	22.98±0.7	0.121
FEV_1 L	2.67±0.14	1.47±0.15	<0.001
FEV_1 % predicted	96.20±3.75	53.34±4.63	<0.001
FEV_1/FVC %	83.60±2.02	52.05±2.65	<0.001
GOLD stage,			
I-II/III-IV	NA	8/9 (47/53)	NA
Sputum cell counts			
Eosinophils %	0.12±0.05	0.38±0.18	0.040
Neutrophils %	27.65±2.42	41.94±1.97	<0.001
Macrophages %	72.03±2.40	57.65±2.28	<0.001
Lymphocytes %	0.20±0.08	0.68±0.25	0.059
Treatment regimens			
ICS/LABA	NA	15 (88)	NA
LAMA	NA	4 (24)	NA
SABA	NA	10 (59)	NA
Theophylline	NA	12 (71)	NA

Data are presented as mean±SEM or n (%), unless otherwise stated. BMI: body mass index; FEV_1: forced expiratory volume in the first second; FVC: forced vital capacity; GOLD: global initiative for chronic obstructive lung disease; ICS: inhaled corticosteroid; LABA: long-acting β-agonist; LAMA: long-acting muscarinic agonist; SABA: short-acting β_2-agonist; NA: not applicable.

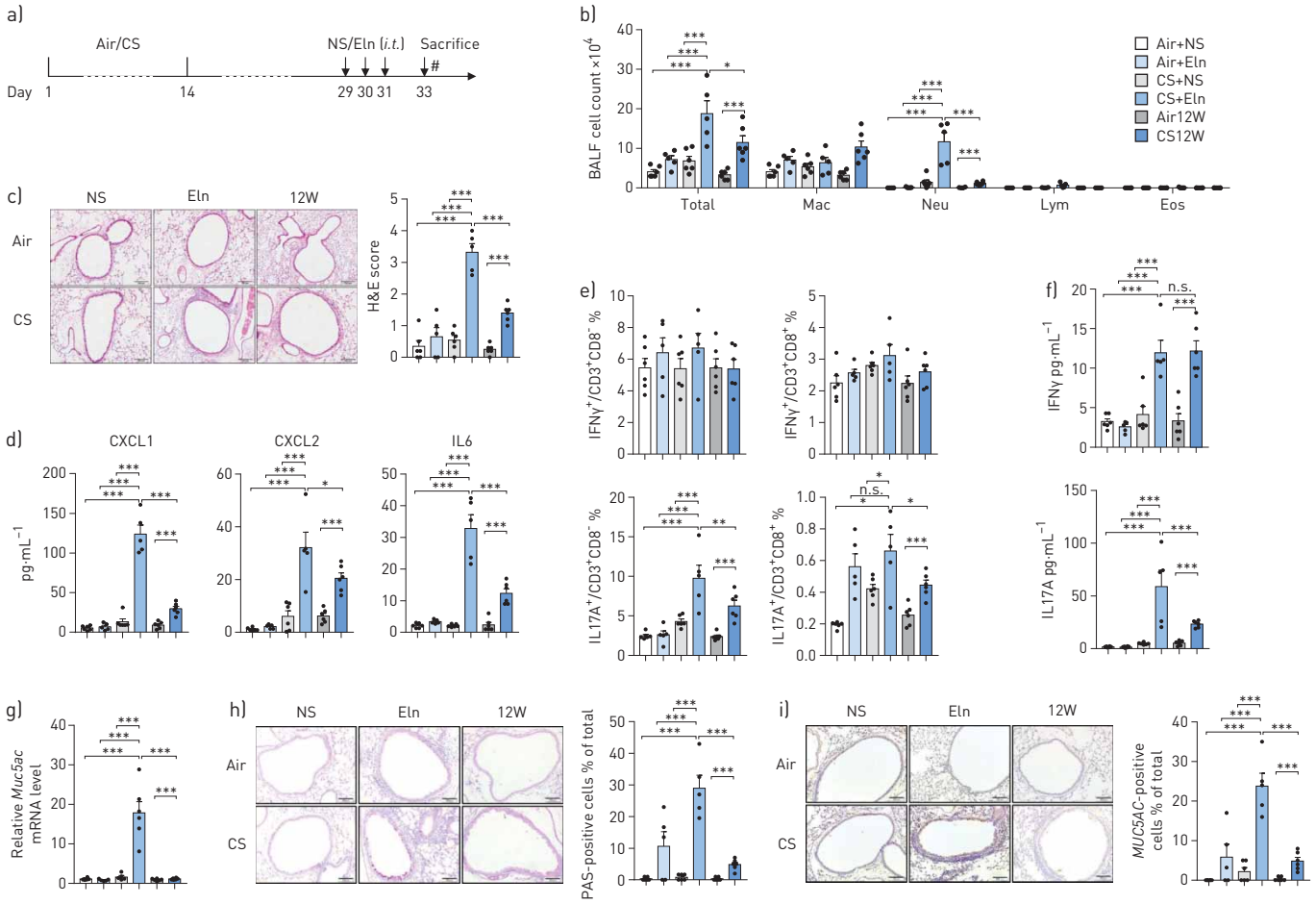

FIGURE 1 Exposure to cigarette smoke sensitises to elastin and elicits interleukin (IL)17A-predominant immune responses and bronchitis-like airway inflammation. a) Experimental outline. Mice were exposed to cigarette smoke (CS) or room air for 2 weeks, and were hosted at room air for another 2 weeks. Mice were then challenged with elastin (Eln, 100 µg) or normal saline (NS) intratracheally (*i.t.*), three times at day 29, 30 and 31, and were sacrificed 48 h after the last elastin challenge. For the traditional cigarette smoke model, mice were exposed to cigarette smoke for 3 months, as detailed in the materials and methods section. b) Inflammatory cell counts in the bronchoalveolar lavage fluid (BALF). c) Representative images and semi-quantified scoring of haematoxylin and eosin (H&E) staining of mouse lung sections. d) The concentrations of CXCL1, CXCL2, and IL6 in BALF. e) Levels of Th1, Tc1, Th17, and Tc17 in mouse lungs. f) Levels of interferon (IFN)γ and IL17A in mouse lung homogenates. g) Expression of *Muc5ac* mRNA transcripts in mouse lungs. h, i) Representative images and the semi-quantified scorings of periodic acid–Schiff (PAS) (h) or MUC5AC (i) staining in mouse lung sections. Labelling for all the columns throughout is shown in (b). Mac: macrophages; Neu: neutrophils; Lym: lymphocytes; Eos: eosinophils. Data are representative of 5–6 mice and were replicated in at least three independent experiments. Data are presented as mean±SEM. *: p<0.05; **: p<0.01; ***: p<0.001 by one-way ANOVA. n.s.: not significant.

BALF (figure 1c). Neutrophilic inflammation was associated with increased expression of IL6, CXCL1, and CXCL2 in BALF (figure 1d). Neutrophilic inflammation elicited by elastin challenge in cigarette smoke-exposed mice was dose dependent (supplementary figure S1). While decreased, we observed significant neutrophilic airway inflammation and increased expression of CXCL1, CXCL2 and IL-6 following challenge with lower concentration of elastin. Of note, no inflammation was observed in mice exposed to cigarette smoke and challenged with vehicle (normal saline) or mice challenged with elastin without prior exposure to cigarette smoke (figure 1b–d). These findings indicate that inflammation was related to the combination of cigarette smoke exposure and elastin administration, rather than inflammatory processes driven by elastin instillation alone or residual inflammation following 2 weeks of smoking cessation.

Flow cytometric analysis of lung T cells (supplementary figure S2) showed that cigarette smoke exposure followed by elastin challenge induced an IL17A-predominant T cell response (figure 1e), while no increase was observed in interferon (IFN)γ-positive T cells. Contrasting these observations, IFNγ and IL17A were both significantly elevated in lung tissues of cigarette smoke exposed and elastin-challenged mice (figure 1f). Cigarette smoke exposure and elastin challenge also induced a remarkable mucus hyperproduction, as evidenced by enhanced *Muc5ac* (mucin 5AC, oligomeric mucus/gel-forming) mRNA transcripts, periodic acid–Schiff (PAS)-positive staining, and MUC5AC expression in the airway epithelium (figure 1g–i).

As a direct comparison, inflammatory parameters in the cigarette smoke-elastin model were compared with the chronic cigarette smoke exposure. Cigarette smoke exposure for 3 months induced airway inflammation, while all inflammatory markers were lower than those observed in the cigarette smoke-elastin model (figure 1b–i). Collectively, these data demonstrate that cigarette smoke exposure and elastin challenge induces a bronchitis-like phenotype that appears more robust than chronic cigarette smoke exposure alone.

Sensitisation and short-term challenge with mouse elastin induce Th1 immune responses and a bronchitis-like airway inflammation in mice

Elastin is a self-antigen; hence, it is plausible that a state of tolerance exists towards this antigen. To explore whether mice can be sensitised to elastin, mice were injected intraperitoneally with elastin and Freund's adjuvant followed by intratracheal challenge with elastin (supplementary figure S3a). Elastin sensitisation and challenge induced a bronchitis-like phenotype comparable to the cigarette smoke–elastin model (supplementary figure S3b–i) with the exception that the resulting T cell response was Th1-predominant (supplementary figure S3e). Altogether, these data provide evidence that the cigarette smoke-induced sensitisation was likely targeted against elastin.

Cigarette smoke-sensitised T cell immunity facilitates mice to elastin-induced airway inflammation

Next, we investigated whether cigarette smoke-induced sensitisation to elastin persists for a prolonged period. To this end, mice were exposed to cigarette smoke and rested for 4, 12 and 24 weeks prior to intratracheal elastin challenge. Elastin challenge induced a significant neutrophilic airway inflammation at all time points, although levels of inflammation declined in a time-dependent manner (figure 2a).

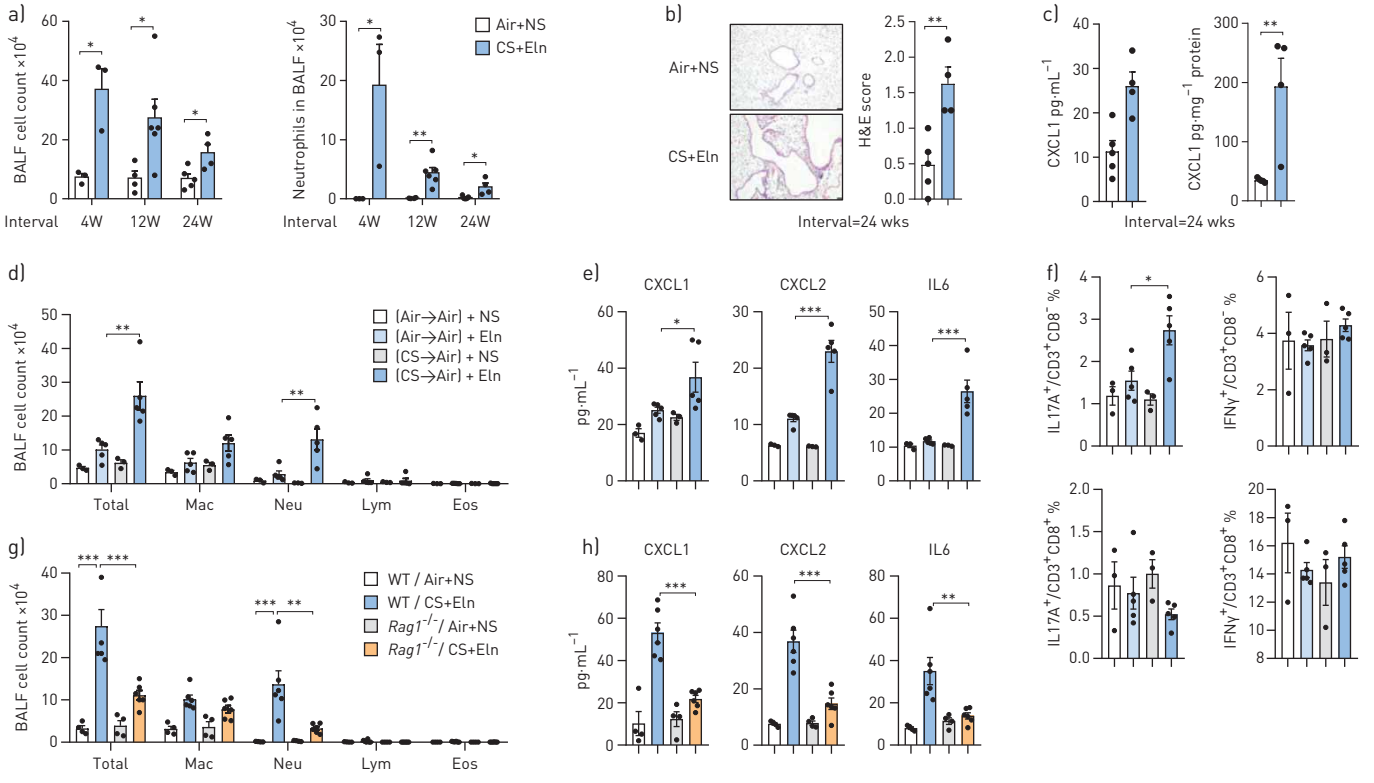

FIGURE 2 Cigarette smoke-sensitised T cell immunity facilitates mice to elastin-induced airway inflammation. a–c) Mice were exposed to cigarette smoke for 2 weeks, and seated at room air for 4, 12, or 24 weeks. Mice were then challenged with elastin (Eln, 100 µg) intratracheally (*i.t.*) once a day for 3 days and were sacrificed 48 h after the last elastin challenge. a) Total inflammatory cell and neutrophil counts in the bronchoalveolar lavage fluid (BALF). b) Representative images of haemotoxylin and eosin (H&E) staining and the semi-quantified scoring of mouse lung sections. c) Concentrations of CXCL1 in BALF (left panel) and lung tissues (right panel). d–f) $CD3^+$ T cells from mediastinal lymph nodes of air controls or cigarette smoke-sensitised mice were transferred to naïve recipients and were challenged with elastin, and the inflammatory cell counts (d), inflammatory factors of CXCL1, CXCL2 and interleukin (IL)6 in BALF (e) and T cell responses in lung (f) were detected. g–h) *Rag1*$^{-/-}$ attenuated the airway inflammation induced by cigarette smoke-sensitisation and elastin challenge. g) Inflammatory cells in BALF. h) The concentrations of CXCL1, CXCL2, and IL6 in BALF. Mac: macrophages; Neu: neutrophils; Lym: lymphocytes; Eos: eosinophils. Data are representative of 3–6 mice. Data are presented as mean±SEM. *: p<0.05; **: p<0.01; ***: p<0.001 by one-way ANOVA.

Assessment of lung histology and expression of CXCL1 showed increased inflammation at the 24-week time point (figure 2b and c). These data suggest that there might be T cell-mediated memory in cigarette smoke-sensitised mice.

To show conclusively that inflammatory processes were T cell-dependent, we performed the following experiments. First, we transferred $CD3^+$ T cells from room air and cigarette smoke-exposed mice into naïve recipients. Subsequently, recipient mice were challenged with elastin daily for 3 days. We observed a significant increase in neutrophilic airway inflammation and inflammatory mediators in mice that received T cells from cigarette smoke-exposed mice (figure 2d and e). Of note, levels of Th17, rather than Tc17, Th1 or Tc1, were markedly elevated in mice adoptively transferred with T cells from cigarette smoke-exposed mice (figure 2f). Next, we exposed *Rag1*$^{-/-}$ mice to cigarette smoke followed by elastin challenge. Supplementary figure S4 shows that *Rag1*$^{-/-}$ are largely deficient of T cells. Of note, airway inflammation in the cigarette smoke-elastin model was significantly attenuated in *Rag1*$^{-/-}$ mice (figure 2g and h). We also isolated lung single-cell suspensions from air control or cigarette smoke-sensitised mice (with repeated cigarette smoke exposure/rest cycle to intentionally enhance the T cell memory), and stimulated with elastin for 72 h. Although the levels of T cell response were very low (data not shown), while interestingly, the levels IL17A and IFNγ in culture medium were significantly increased in the repeated cigarette smoke exposure group (supplementary figure S5). Together, these results suggested that the cigarette smoke-exposure induced elastin-specific T cells that have the capability to drive autoimmune inflammatory processes.

IL17A mediates the airway inflammation induced by cigarette smoke sensitisation and elastin challenge

Since cigarette smoke sensitisation and elastin challenge induced IL17A-predominant T cell responses, we next investigated the role of IL17A in this model. All the bronchitis-like features induced by cigarette smoke exposure and elastin challenge were significantly decreased in the *Il17a*$^{-/-}$ mice (figure 3a–e). Similarly, administration of an IL17A neutralising monoclonal antibody during elastin challenge significantly attenuated neutrophilic airway inflammation in this model (figure 3f).

Taken together, these findings show that cigarette smoke exposure sensitises to elastin and that the inflammatory processes following exposure to elastin were IL17A-dependent.

MMP12 is critically required for cigarette smoke-induced sensitisation to elastin

To assess the cigarette smoke-induced elastin specificity, cigarette smoke-exposed mice were challenged with ovalbumin (OVA), an allergen commonly used for mouse model of asthma (figure 4a). Interestingly, OVA failed to induce airway inflammation in cigarette smoke-exposed mice (figure 4b), suggesting that cigarette smoke-induced sensitisation was more specific to elastin.

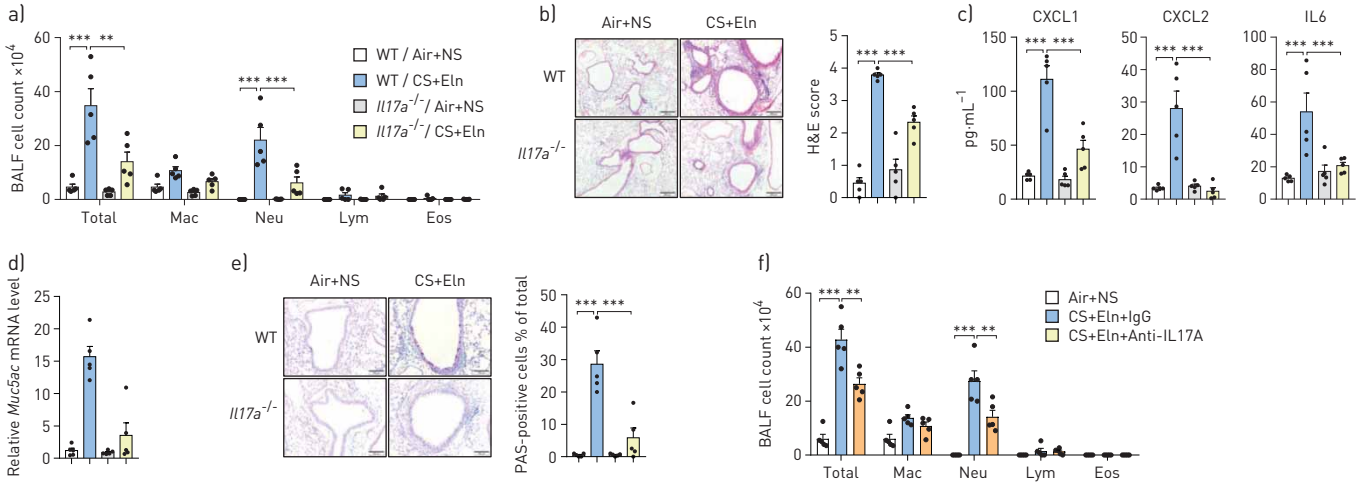

FIGURE 3 Interleukin (IL)17A mediates the airway inflammation induced by cigarette smoke sensitisation and elastin challenge. *Il17a*$^{-/-}$ mice and wildtype controls (WT) were sensitised with cigarette smoke and challenged with elastin, and the inflammatory cell counts in bronchoalveolar lavage fluid (BALF) (a), haemotoxylin and eosin (H&E) staining and the semi-quantified inflammatory scoring (b), the concentrations of CXCL1, CXCL2, and IL6 in BALF (c), the expression of *Muc5ac* in lung tissues (d), and the mucus hyperproduction in airways (e) were detected. f) IL17A neutralisation attenuates the inflammatory cell counts in BALF. IL17A neutralisation antibody (5 µg) was administrated intratracheally together with elastin challenge. Mac: macrophages; Neu: neutrophils; Lym: lymphocytes; Eos: eosinophils. Data are representative of 3–6 mice. Data are presented as mean±SEM. **: p<0.01; ***: p<0.001 by one-way ANOVA.

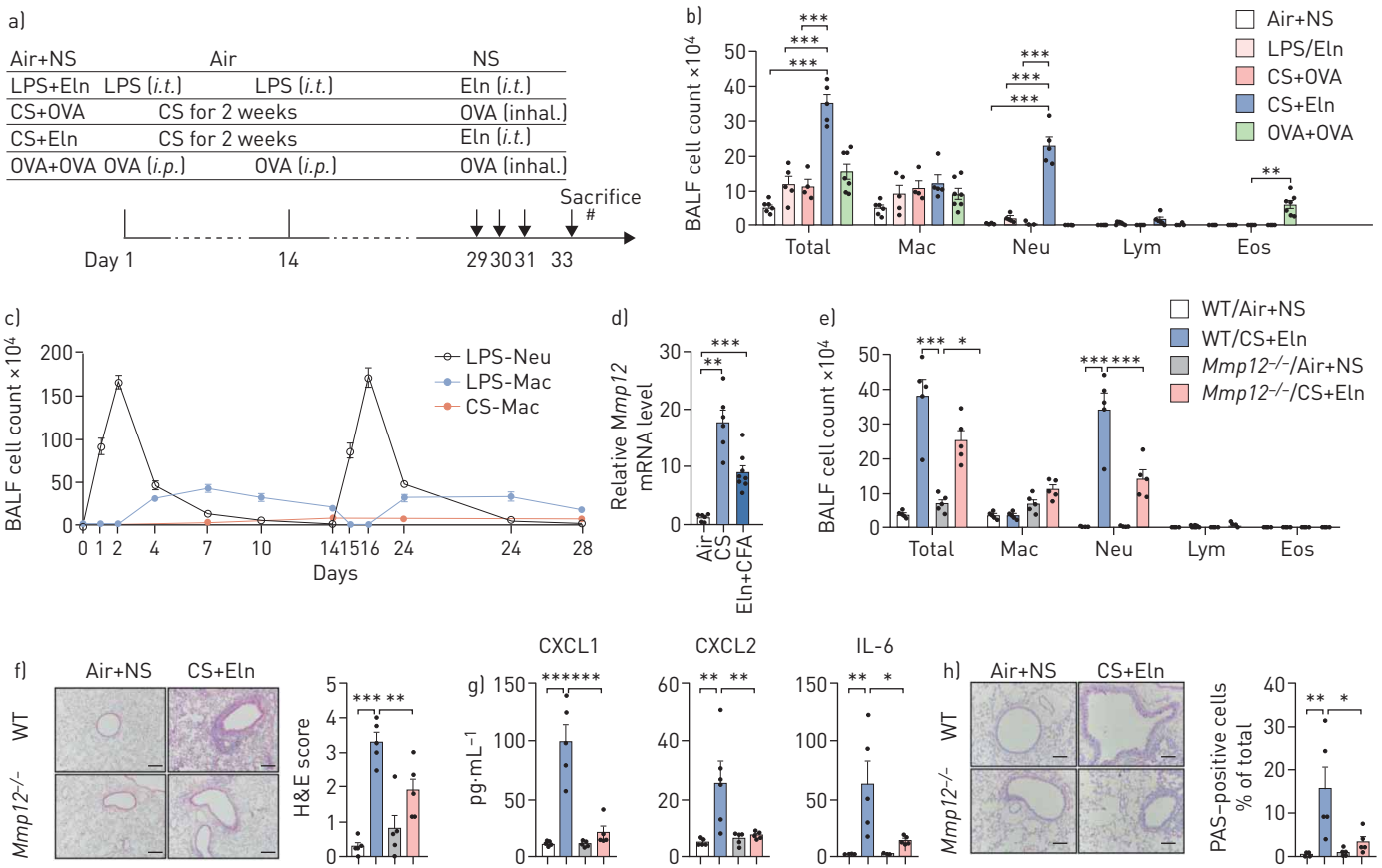

FIGURE 4 Matrix metalloprotease (MMP)12 is critically required for cigarette smoke-induced sensitisation to elastin. a) Experimental outline. Mice were sensitised with intratracheal instillation of lipopolysaccharide (LPS) (1 µg per mouse) at day 0 and 14, or 2 weeks of cigarette smoke exposure. Mice were then challenged with elastin or ovalbumin (OVA) three times at indicated time points. b) Inflammatory cell counts in the bronchoalveolar lavage fluid (BALF). c) Time course of the amounts of neutrophils or macrophages in BALF intratracheally treated with LPS (1 µg for twice) or exposed to cigarette smoke for 2 weeks. d) The levels of *Mmp12* mRNA transcripts in BALF cells from mice sensitised with cigarette smoke for 2 weeks, or elastin plus complete Freund's adjuvant (CFA). e–h) *Mmp12*$^{-/-}$ mice and wildtype controls (WT) were sensitised with cigarette smoke and challenged with elastin, and the inflammatory cell counts in BALF (e), haemotoxylin and eosin (H&E) staining and the semi-quantified inflammatory scoring (f), the concentrations of CXCL1, CXCL2, and interleukin (IL)6 in BALF (g), and the mucus hyperproduction in airways (h) were detected. Mac: macrophages; Neu: neutrophils; Lym: lymphocytes; Eos: eosinophils. Data are representative of 5–6 mice and were replicated in at least three independent experiments. Data are presented as mean±SEM. *: p<0.05; **: p<0.01; ***: p<0.001 by student's t-test ((a–c), for comparisons of two groups) or by one-way ANOVA ((d, f and g), for comparisons of multiple groups).

We next aimed to explore the mechanisms that contribute to elastin sensitisation in cigarette smoke-exposed mice. The generation of elastin fragments in the lungs is generally attributed to neutrophil elastase and MMPs [13, 14], specifically, the macrophage metalloproteinase MMP12 [15, 16]. To test the function of neutrophil elastase, we exposed mice to LPS (figure 4a), a well-known model of neutrophilic lung inflammation. Although LPS instillation induced a marked airway neutrophilia (figure 4c), elastin challenge following resolution of the initial inflammatory response did not induce neutrophilic airway inflammation in mice exposed to low-dose LPS (figure 4b). These data suggest that LPS exposure is less effective at eliciting elastin-specific immune responses compared with cigarette smoke exposure.

To assess the function of MMP-12, we exposed *Mmp12*$^{-/-}$ mice to cigarette smoke followed by elastin challenge. In wild type mice, *Mmp12* mRNA expression was markedly increased in BALF cells of mice exposed to cigarette smoke (figure 4d). Importantly, *Mmp12*$^{-/-}$ mice displayed markedly reduced neutrophilic inflammation in BALF and airways, decreased production of inflammatory cytokines, and diminished mucus production (figure 4e–h).

These data suggest that induction of MMP12 is critical for cigarette smoke-induced sensitisation to elastin, while neutrophils and neutrophil elastase play a less important role.

Cigarette smoke-induced elastin fragments drive the subsequent airway inflammation and mucus production

One of the pathologic mechanisms for MMP12 in COPD is due to the production of elastin fragments containing GXXPG or XGXPG conformational motives (where X is a hydrophobic amino acid) which exert a high monocyte chemotactic activity [17]. To investigate whether these motives contribute to inflammatory processes in the cigarette smoke–elastin model, cigarette smoke-exposed mice were challenged with peptides containing repeating VGVAPG sequences, a common and repeated motif in human elastin. VGVAPG peptides elicited a bronchitis-like phenotypes in cigarette smoke-exposed mice (figure 5a–e).

We next treated mice with BA4 antibody during cigarette smoke sensitisation (figure 5f). This antibody has been shown to block the activity of endogenous elastin peptides, including the GXXPG and XGXPG motifs, and reduce the cigarette smoke-induced airway inflammation in mice [17]. Neutrophilic airway inflammation following elastin challenge in cigarette smoke-exposed mice was significantly attenuated by BA4 antibody treatment (figure 5g–i). Mucus hyperproduction induced by cigarette smoke exposure and elastin challenge was also markedly reduced by BA4 (figure 5j).

These data suggest that the elastin fragments induced by cigarette smoke exposure, most likely the GXXPG and XGXPG motifs, facilitate sensitisation to elastin and orchestrate inflammatory responses and mucus hyperproduction in the cigarette smoke–elastin model.

Cigarette smoke sensitisation and sub-chronic elastin challenge induce an emphysema-like phenotype in mice

Next, we asked whether repeated elastin challenge in cigarette smoke-exposed mice would elicit airway remodelling and airspace enlargement in mice, mimicking human emphysema. Mice were exposed to cigarette smoke and then challenged with elastin every other day for 30 consecutive days (figure 6a). Repeated elastin challenge decreased the body weight (figure 6b) and induced a sustained neutrophilic airway inflammation (figure 6c). Persistent inflammation was associated with a marked decline in forced expiratory volume in 20 s (FEV_{20}) and the FEV_{20}/forced vital capacity (FVC) (figure 6d), increased the collagen deposition around airways (figure 6e) and evidence of mucus hyperproduction (figure 6f). Moreover, repeated elastin challenge led to a significant increase in lung airspace, as determined by comparative histologic examination and mean linear intercept (MLI) measurements (figure 6g). These data suggest that cigarette smoke exposure and chronic elastin challenge induced a COPD-like phenotype similar to what is observed in mice exposed to cigarette smoke for 6 months (supplementary figure S6). It should be noted that in the chronic cigarette smoke model, mice were generally 4 months older than those in the cigarette smoke–elastin model. Though the levels of collagen deposition and the airspace enlargement were comparable between the two models, airway inflammation and mucus production induced by 6 months cigarette smoke exposure appeared to be less severe compared to the cigarette smoke–elastin model (supplementary figure S6 *versus* figure 6).

Elastin-mediated autoimmunity is elevated in COPD patients

Finally, we examined elastin-mediated immune responses in COPD patients (table 1). In agreement with previous reports [6, 7], the levels of elastin antibodies in plasma were elevated in COPD patients (figure 7a). Moreover, levels of Th1, Th17, and Tc17 in peripheral blood were significantly increased in COPD patients relative to healthy controls (figure 7b). In induced sputum, protein levels of IL17A were increased (figure 7c). To address whether the pathogenic T cells in COPD could interact with elastin fragments, we

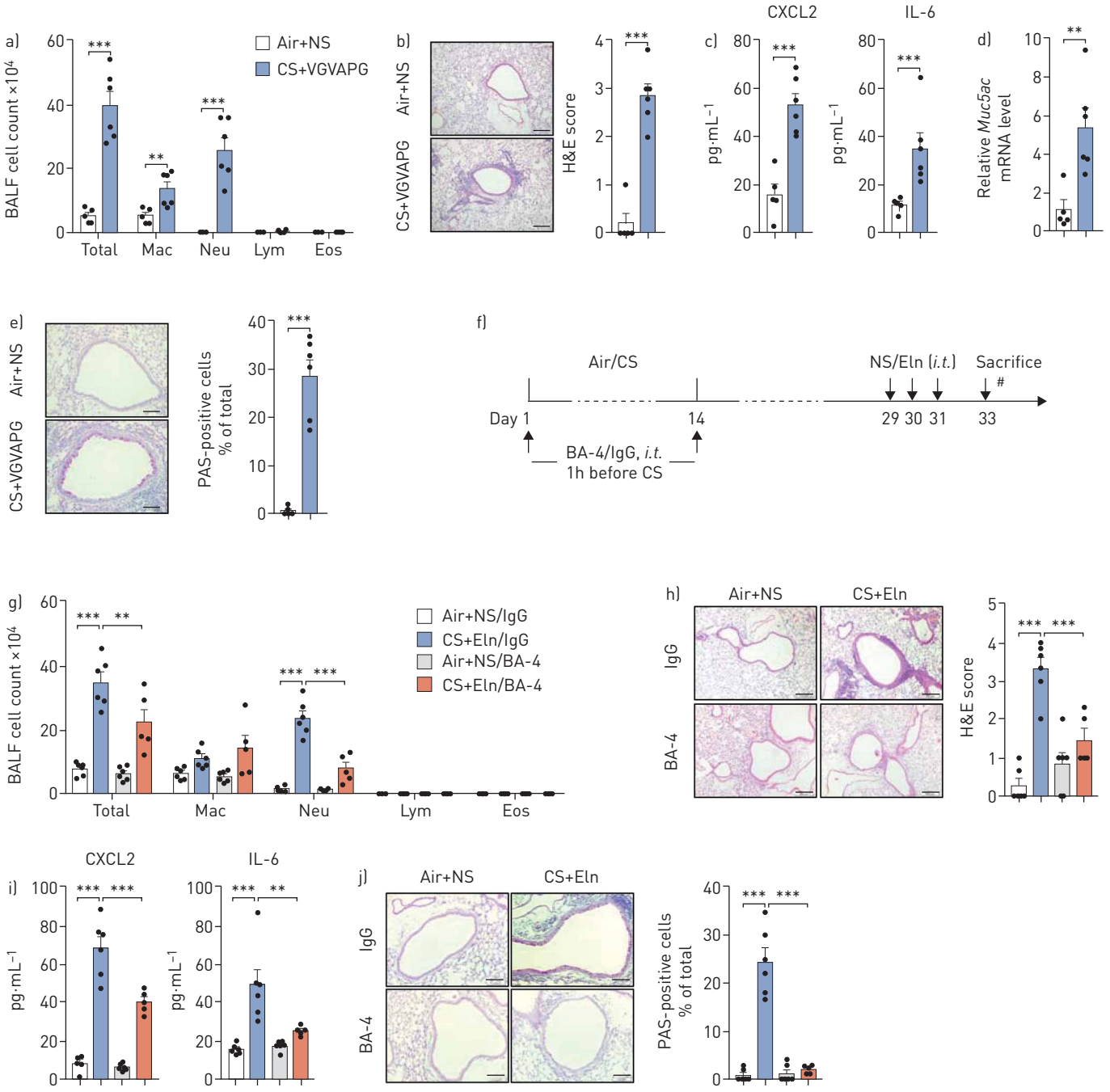

FIGURE 5 Cigarette smoke-induced elastin fragments drive the subsequent airway inflammation and mucus production. a–e) Airway inflammation induced by elastin peptides containing repeated VGVAPG sequences. Experimental protocol was as shown in figure 1a, except using elastin peptides for challenge instead of elastin protein. a) Inflammatory cell counts in the bronchoalveolar lavage fluid (BALF). b) Representative images and semi-quantified scoring of haemotoxylin and eosin (H&E) staining of mouse lung sections. c) The concentrations of CXCL1 and interleukin (IL) 6 in BALF. d) Expression of *Muc5ac* mRNA transcripts in mouse lungs. e) Representative images and the semi-quantified scorings of periodic acid–Schiff (PAS) staining in mouse lung sections. f) Protocol for BA4 antibody treatment. BA4 antibody (10 μg) was administrated intratracheally 1 h before everyday cigarette smoke exposure. g–j) BA4 attenuated the airway inflammation induced by cigarette smoke-sensitisation and elastin challenge. g) Inflammatory cells in BALF. h) Representative images and semi-quantified scoring of H&E staining of mouse lung sections. i) The concentrations of CXCL2 and IL6 in BALF. j) Representative images and the semi-quantified scorings of PAS staining in mouse lung sections. Mac: macrophages; Neu: neutrophils; Lym: lymphocytes; Eos: eosinophils. Data are representative of 5–6 mice and were replicated in at least three independent experiments. Data are presented as mean±SEM. **: p<0.01; ***: p<0.001 by one-way ANOVA.

selected an HLA-A*02:01 allele which presents a relatively high affinity to human elastin peptide GVAPGVGVAPGV to synthesise a tetramer. Peripheral blood mononuclear cells (PBMCs) from COPD patients with this allele exhibited significantly higher positive tetramer staining relative to those from

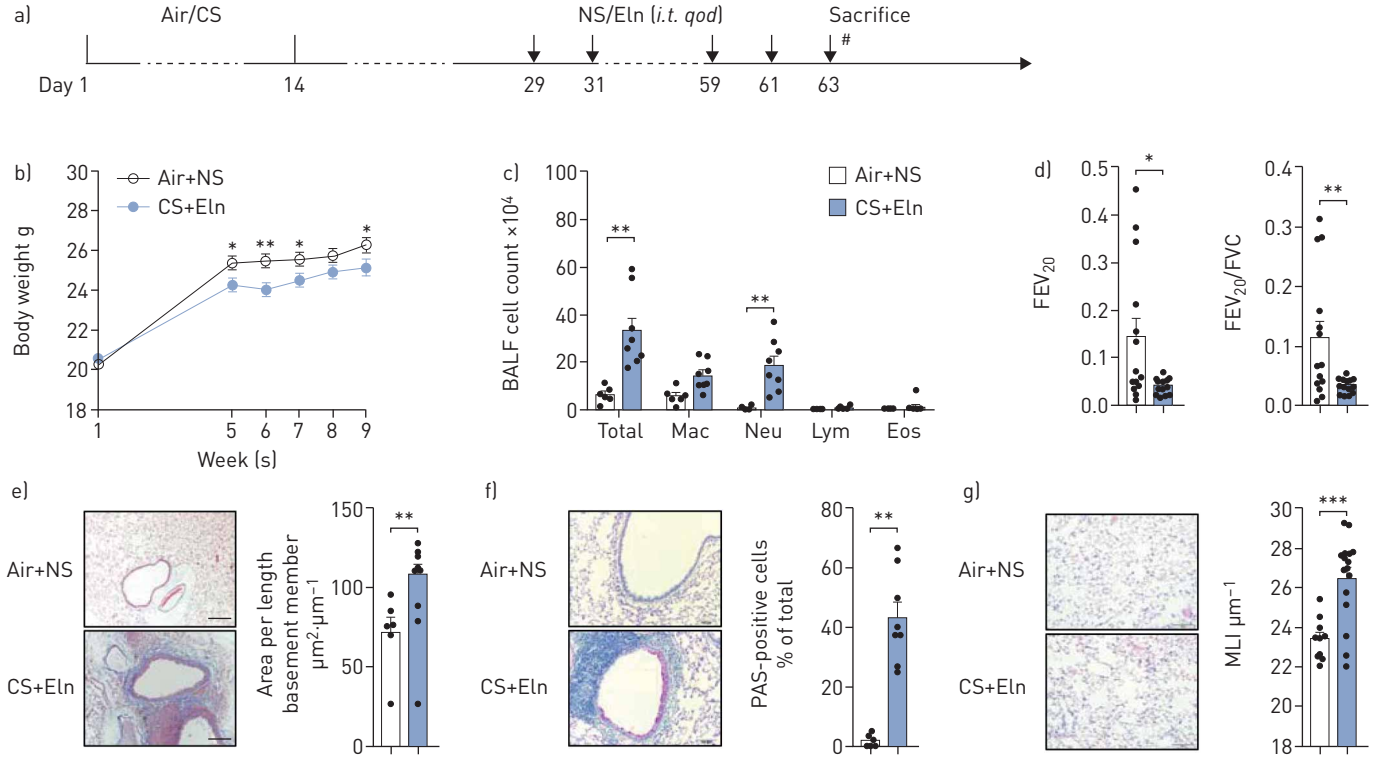

FIGURE 6 Cigarette smoke sensitisation and sub-chronic elastin challenge induce an emphysema-like phenotype in mice. a) Experimental outline. Mice were exposed to cigarette smoke or room air for 2 weeks and were hosted at room air for another 2 weeks. Mice were then challenged with elastin (Eln, 100 μg) or normal saline (NS) intratracheally (*i.t.*) every other day for 30 days, and were sacrificed 48 h after the last elastin challenge. b) Mouse weight at indicated time points. c) Inflammatory cell counts in the BALF. d) Lung function of mice sub-chronically challenged with elastin (n=14). Representative images of Masson's trichrome staining (e) and periodic acid–Schiff (PAS) staining (f) of mouse lung sections and the semi-quantified scoring. g) Representative images of airspace and measurement of the mean linear intercept (MLI) of mouse lungs (n=10–16). Mac: macrophages; Neu: neutrophils; Lym: lymphocytes; Eos: eosinophils; FEV_{20}: forced expiratory volume in 20 s; FVC: forced vital capacity. Data are representative of 6–8 mice unless otherwise indicated, and were replicated in at least three independent experiments. Data are presented as mean±SEM. *: p<0.05; **: p<0.01; ***: p<0.001 by student's t-test.

healthy controls with the same allele (figure 7d). Taken together, these clinical data are in line with our observations in our animal studies, suggesting that elastin drives IL17A-predominant auto-immune processes in COPD.

Discussion

The nature of the molecular mechanisms that drive cigarette smoke-induced airway inflammation remains a central question in COPD research. Building on previous reports that elastin may drive autoimmune processes in COPD [6, 7], we demonstrate that, in mice exposed to cigarette smoke, elastin serves as a self-antigen and drives adaptive immune responses that facilitates sensitisation to subsequent elastin-induced COPD-like pathologies.

Recent evidence has suggested that elastin-mediated autoimmunity may be associated with COPD pathogenesis, as anti-elastin antibodies were present in patients with COPD [7], and elastin peptides could act as cognate antigens to stimulate Th1 and Th17 differentiation in subjects with emphysema [8]. Deslee *et al.* [6] observed an increase in elastin in very severe COPD patients. In line with these observations, we found that antibodies against elastin were significantly elevated in COPD patients and that T cells from COPD patients bear T cell receptors that recognise elastin peptides. Moreover, we demonstrate that elastin sensitisation and challenge induced the Th1-predominant immune response and subsequent bronchitis-like phenotypes, demonstrating that elastin, as a self-antigen, is able to elicit autoimmune responses in mice. Finally, cigarette smoke-exposed mice were sensitised to elastin, providing experimental evidence that elastin-mediated autoimmunity may contribute to the pathogenesis of COPD. Gu *et al.* [18] have recently demonstrated that $CD4^+$ T cells from cigarette smoke-exposed mice were reactive to elastin fragments *in vitro*, and that mice immunised with a combination of human and rat elastin fragments showed increased infiltration of innate and adaptive immune cells to the lungs and developed emphysema, completely in agreement with our conclusions.

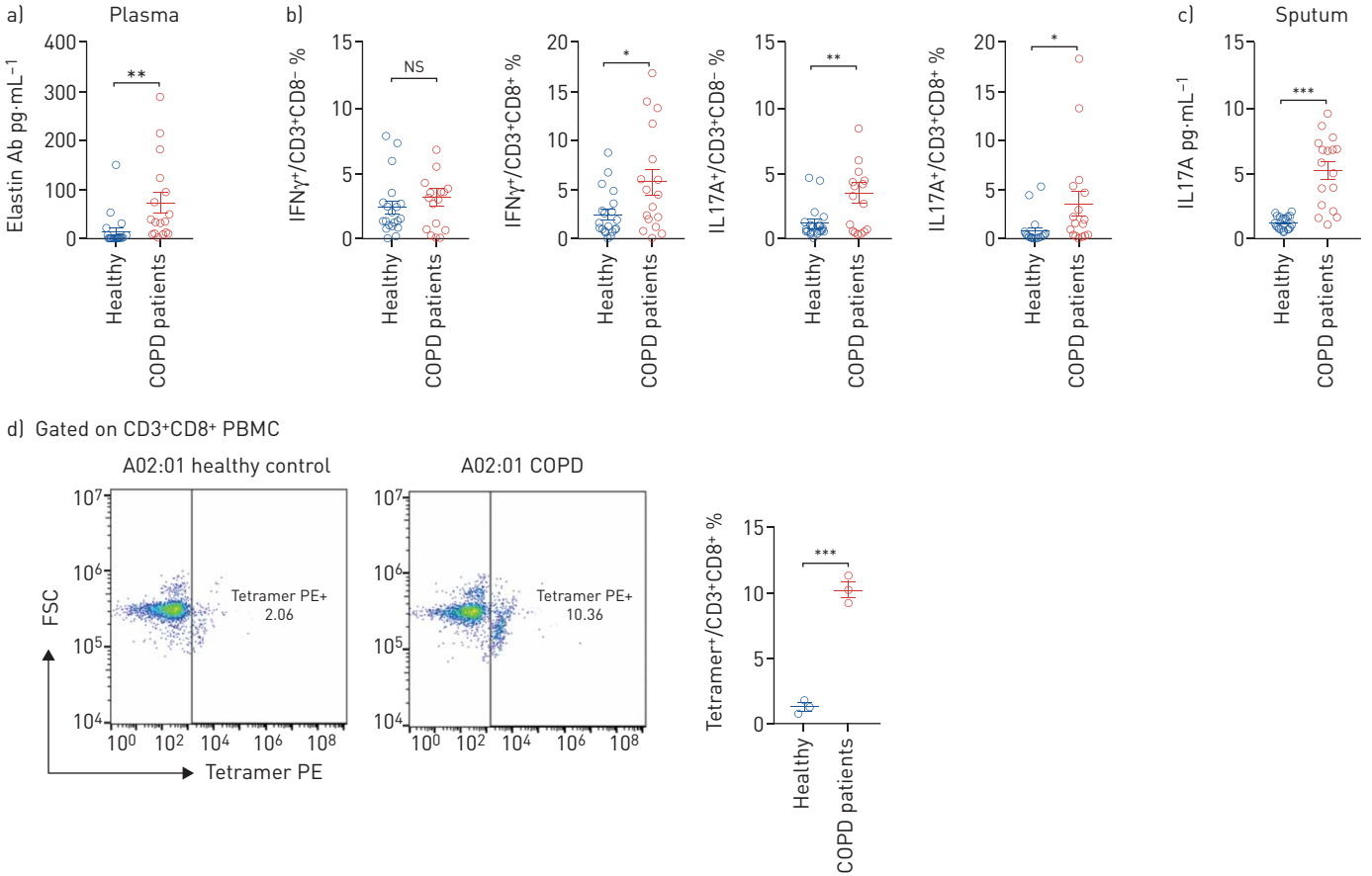

FIGURE 7 Elastin-mediated autoimmunity is elevated in chronic obstructive pulmonary disease (COPD) patients. a) Expression of elastin antibody in plasma of COPD patients or healthy controls. The clinical information of human subjects was shown in Supplementary Table 1. b) Levels of Th1, Tc1, Th17, and Tc17 in peripheral blood from healthy controls (n=20) or patients with COPD (n=17). c) Levels of interleukin (IL)17A in induced sputum from healthy controls and COPD patients. d) Increased levels of elastin-specific T cells in COPD. Peripheral blood mononuclear cells (PBMCs) were collected from human subjects with HLA-A*02:01 genotype (n=3 for either group) and were cultured in the presence of IL2 for 7 days. Cells were then stained with specific tetramer bearing human elastin peptide GVAPGVGVAPGV and were analysed by flow cytometry. Representative images of tetramer staining and quantified results are shown. Data are presented as mean±SEM. *: p<0.05; **: p<0.01; ***: p<0.001; n.s.: not significant, by student's t-test.

Animal models currently used in COPD research have clear limitations, restricting their application to study the pathogenesis of COPD [19–21]. For example, models of cigarette smoke-induced airspace enlargement are time-consuming, requiring in general 6 months of continuous cigarette smoke exposure to induce emphysema formation. Airway inflammation in these models is mild (the proportion of neutrophils in BALF is usually less than 10%) and the mucus expression is rarely evident. Although protease instillation rapidly reproduces many characteristics of human emphysema, these models do not mimic a continuous inflammatory process and there is little evidence that T cell-driven processes are involved. This limits the application of elastase models for studying mechanisms of cigarette smoke-induced airway inflammation and lung pathologies. The animal model presented here overcomes these limitations. We observed moderate levels of neutrophilic airway inflammation (30–60% of neutrophils in BALF, comparable with proportions of eosinophils in asthma models), a Th1/Th17 inflammatory signature, marked mucus hyperproduction, increased airway remodelling, declined lung function and enlarged airspaces. It required 1 month to establish airway inflammation, mimicking human bronchitis, and 2 months to establish parenchymal damage. In addition, our model provides "proof of concept" that autoimmune processes are induced by cigarette smoke exposure and may provide a novel framework for building animal models for other autoimmune diseases. This is of clinical relevance, as cigarette smoke exposure is a known risk factor for a number of autoimmune diseases, such as rheumatoid arthritis.

Airway mucus hypersecretion is one of the cardinal features for both COPD and asthma. However, unlike increased mucus hyperproduction in asthma models or by particulate matter [22–24], there is little evidence that cigarette smoke exposure increases mucus production in mouse models. In contrast, acrolein, a reactive α, β-unsaturated aldehyde and component of cigarette smoke, readily induces mucus production in murine airways [25, 26]. Our findings identify elastin as an effective stimulator to trigger mucin expression in cigarette smoke-exposed mice and, thus, provide a novel model for mucus research with relevance to COPD. In fact, we have observed that elastin peptides containing the active GXXPG conformational motif [27, 28], but not elastin protein, induced MUC5AC expression in human bronchial epithelial cells (data not shown), suggesting a critical role of these active peptides in driving mucus production in COPD.

Our current study emphasises the pivotal role of MMP12 in initiating cigarette smoke-induced autoimmune responses. In line with previous reports showing that *Mmp12*$^{-/-}$ mice are protected from cigarette smoke-induced airway inflammation and emphysema [15], *Mmp12* deficiency markedly attenuated elastin-induced airway inflammation and mucus hyperproduction in mice with prior exposure to cigarette smoke. Other MMPs in *Mmp12*$^{-/-}$ alveolar macrophages were not altered (data not shown), and thus we could not rule out the functions of other MMPs in our study. In fact, MMP12 deficiency only partially attenuated inflammatory processes, supporting the participation of other MMPs in cigarette smoke-induced autoimmunity. One of the mechanisms that implicates MMP12 in the pathogenesis of COPD is the production of elastin fragments that contain GXXPG or XGXPG motifs, which exert monocyte chemotactic activity [17]. In our current study, we found that tetramers that contained elastin peptides with these motifs were able to directly bind T cells from COPD patients and induce airway inflammation in cigarette smoke-sensitised mice. We further show that antibodies that target the active motif in elastin effectively attenuated airway inflammation when delivered during cigarette smoke exposure. Thus, the MMP12-generated elastin peptides induced by cigarette smoke exposure not only are chemotactic factors for monocytes, but also initiate T cell responses, thereby driving subsequent airway inflammation, mucus production, and airspace enlargement.

It is noteworthy that COPD/emphysema generally develops decades after smoking cessation, accompanied by increased inflammation in patients' airways. These observations are consistent with our results in the murine model where mice developed airway inflammation following elastin challenge 6 months after an initial 2 weeks of cigarette smoke exposure. Therefore, cigarette smoke exposure may sensitise to elastin forming an immune memory that perpetuates inflammatory processes long after an individual quits smoking.

Accumulating evidence suggests that IL17A-driven immune processes may contribute to the pathogenesis of COPD, as increased levels of IL17A and elevated numbers of IL17A^{+} cells have been detected in bronchial mucosa, peripheral blood, and sputum of COPD patients [29–31]. Similarly, we observed augmented concentration of IL17A in induced sputum and increased levels of Th17 and Tc17 in the blood of COPD patients. Moreover, animal studies have demonstrated that IL17A is increased in cigarette smoke-induced airway injury [32–34] and *Il17a*$^{-/-}$ mice exposed to cigarette smoke are protected from airspace enlargement [35, 36]. In line with these, IL17A was significantly increased in the elastin-driven airway inflammation in mice that were previously exposed to cigarette smoke. Genetic deficiency or neutralisation of IL17A decreased elastin-driven inflammation, indicating a pivotal role of Th17/IL17A in the pathogenesis of COPD.

In conclusion, our findings demonstrate an elastin-mediated autoimmunity in cigarette smoke-exposed mice, which is orchestrated by upstream MMP12 and is mediated by the subsequent IL17A signalling. Based on this, we establish a novel mouse model mimicking most human COPD characteristics, and suggest that MMP12, active elastin peptides, or IL17A could serve as novel therapeutic targets for COPD.

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