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A short-term model of COPD identifies a role for mast cell tryptase

Emma L. Beckett, B Biomed Sci^{a,1}, Richard L. Stevens, PhD^{b,c,1}, Andrew G. Jarnicki, PhD^{a,1}, Richard Y. Kim, B Biomed Sci^a, Irwan Hanish, BSc^a, Nicole G. Hansbro, PhD^a, Andrew Deane, BSc^a, Simon Keely, PhD^a, Jay C. Horvat, PhD^a, Ming Yang, MD, PhD^a, Brian G. Oliver, PhD^d, Nico van Rooijen, PhD^e, Mark D. Inman, MD, PhD^f, Roberto Adachi, MD^g, Roy J. Soberman, MD^{b,h}, Sahar Hamadi^c, Peter A. Wark, MD, PhD^{a,i}, Paul S. Foster, PhD^a, and Philip M. Hansbro, PhD^a

^aPriority Research Centre for Asthma and Respiratory Disease and Hunter Medical Research Institute, The University of Newcastle, Newcastle

^bDepartment of Medicine, Harvard Medical School, Boston

^cBrigham and Women's Hospital, Boston

^dSydney Medical School and Woolcock Institute of Medical Research, University of Sydney, Sydney

^eDepartment of Molecular Cell Biology, Vrije Universiteit Medical Center, Amsterdam

^fFirestone Institute for Respiratory Health, St Joseph's Healthcare, Hamilton

^gDepartment of Pulmonary Medicine, The University of Texas MD Anderson Cancer Center, Houston

^hMassachusetts General Hospital, Boston

ⁱDepartment of Respiratory and Sleep Medicine, John Hunter Hospital, Newcastle

Abstract

Background—Cigarette smoke-induced chronic obstructive pulmonary disease (COPD) is a life-threatening inflammatory disorder of the lung. The development of effective therapies for COPD has been hampered by the lack of an animal model that mimics the human disease in a short time-frame.

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Corresponding author: Professor Philip Hansbro, PhD, Priority Research Centre for Asthma and Respiratory Disease and Hunter Medical Research Institute, The University of Newcastle, Newcastle, NSW 2308, Australia. Philip.Hansbro@newcastle.edu.au.

¹Authors contributed equally.

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Objectives—To create an early onset mouse model of cigarette smoke-induced COPD that develops the hallmark features of the human condition in a short time-frame. To use this model to better understand pathogenesis and the roles of macrophages and mast cells (MCs) in COPD.

Methods—Tightly controlled amounts of cigarette smoke were delivered to the airways of mice, and the development of the pathological features of COPD was assessed. The roles of macrophages and MC tryptase in pathogenesis were evaluated using depletion and *in vitro* studies and MC protease-6 deficient mice.

Results—After just 8 weeks of smoke exposure, wild-type mice developed chronic inflammation, mucus hypersecretion, airway remodeling, emphysema, and reduced lung function. These characteristic features of COPD were glucocorticoid-resistant and did not spontaneously resolve. Systemic effects on skeletal muscle and the heart, and increased susceptibility to respiratory infections also were observed. Macrophages and tryptase-expressing MCs were required for the development of COPD. Recombinant MC tryptase induced pro-inflammatory responses from cultured macrophages.

Conclusion—A short-term mouse model of cigarette smoke-induced COPD was developed in which the characteristic features of the disease were induced more rapidly than existing models. The model can be used to better understand COPD pathogenesis, and we show a requirement for macrophages and tryptase-expressing MCs.

Keywords

cigarette smoke; COPD; inflammation; emphysema; airway remodeling; lung function; macrophage; mast cell; protease; mMCP-6; hTryptase- β

INTRODUCTION

Cigarette smoke-induced chronic obstructive pulmonary disease (COPD) is a debilitating disorder of the lung. It is the 4th leading cause of chronic morbidity and death worldwide, and its prevalence is increasing.¹ The disease is characterized by chronic airway inflammation, mucus hypersecretion, airway remodeling, and emphysema that lead to reduced lung function and breathlessness.^{2,3} Systemic effects also are observed in the skeletal muscle, heart, and other organs. Moreover, COPD patients are more susceptible to respiratory infections. Because the mechanisms that lead to COPD and its sequelae are poorly understood at the molecular level, there are no effective treatments.

A major factor that has hampered the study of COPD is the lack of a small animal model that recapitulates the hallmark features of the disease in a reasonable time frame. While lipopolysaccharide and elastase have been used to induce lung damage in rodents that somewhat resemble COPD in humans, such single-factor approaches are not representative of the complex pathology that occurs in those patients who smoke for many years.³ Current models of smoke-induced COPD involve whole body or nose-only exposure of mice to cigarette smoke.³ Acute models of 4 days to 4 weeks duration have been valuable for evaluating the early smoke-induced inflammatory responses in the lung. However, the smoke-exposed mice in these models do not develop emphysema or have diminished lung function.⁴⁻⁸ Chronic models of >6 months duration result in airway remodeling and

emphysema, but only induce mild alterations in lung function,^{4,9–12} and the prolonged time needed to induce these features greatly restricts the use of these models for extensive therapeutic and mechanistic research. This is especially relevant considering the short life-span of the mouse and the substantial animal and labor expenses needed for 6-month experiments. Thus, there is a need for a mouse model of cigarette smoke-induced COPD that has all of the major features of the human condition that are induced in a shorter time frame.

Although mast cells (MCs) have been casually linked to the pathogenesis of COPD, the relevant factors exocytosed from these immune cells have not been identified.¹³ MC numbers are increased in inflammatory infiltrates in COPD patients, which is associated with reduced lung function, airway remodeling and emphysema.^{14,15} The levels of MC-derived human (h)Tryptase- β in sputum correlated with the severity of COPD in one study,¹⁶ and the exposure of IL-3-dependent MCs to cigarette smoke-treated culture medium resulted in increased expression of mouse MC protease-6 (mMCP-6).¹⁷ mMCP-6 is also known to promote inflammation, chemokine expression and macrophage and neutrophil chemotaxis,¹⁸ which are all hallmark features of COPD. Nevertheless, the roles of hTryptase- β and mMCP-6 in COPD pathogenesis have not been investigated in depth.

Here we report the development of a mouse model of COPD in which we deliver tightly controlled amounts of cigarette smoke directly into the airways. The exposed mice exhibit the major characteristic features of COPD observed in humans after only 8 weeks of smoke exposure, thereby facilitating the discovery and/or testing of the efficacy of new therapeutics. The model also enables us to elucidate the cellular, biochemical and molecular mechanisms that underpin the pathogenesis of COPD. In that regard, we now show for the first time detrimental roles for a MC-restricted tetramer-forming tryptase in experimental COPD.

METHODS

Additional details are described in the Journal's Online Repository at www.jacionline.org.

Smoke exposure

Wild-type (WT) BALB/c, WT C57BL/6 (B6), and mMCP-6^{-/-} B6 mice¹⁸ were used in the study. In each experiment, 12 mice were simultaneously exposed to cigarette smoke [twelve 3R4F reference cigarettes (University of Kentucky, Lexington, Ky) twice/day, 5 times/week, for 1–12 weeks] using a custom-designed and purpose-built nose-only, directed flow inhalation and smoke-exposure system (CH Technologies, Nj) housed in a fume and laminar flow hood (Fig S1). Each exposure lasted 75 minutes. All experiments were approved by our institutional animal ethics committee.

Airway and lung inflammation, airway remodeling, and emphysema

Airway inflammation was assessed by differential enumeration of inflammatory cells in bronchoalveolar lavage fluid (BALF).^{19–22} Parenchymal inflammation was assessed by counting the inflammatory cells in 10 randomized fields (x100 magnification) of whole lung sections.²³ RNA was extracted and transcript levels were assessed by standard real-time quantitative (q)PCR assays,²⁴ using the primers described in Table S1.

The numbers of macrophages and MCs in lung homogenates were assessed by flow cytometry and histochemistry.^{25–27} Airway remodeling was determined by measuring the number of mucus-expressing goblet cells around the airways and by assessing airway epithelium thickening.^{22,23,28–30} Emphysema was assessed using the mean linear intercept technique, which is a standard method of assessing alveolar diameter and emphysema in mice.²⁸

Lung function

Forced oscillation and forced maneuver techniques were used to assess lung function parameters.^{25,31}

Glucocorticoid treatment

Dexamethasone (1 mg/kg in 50 μ L sterile water, Sigma, St Louis, MO) was administered intranasally 3 times/week.³² Controls were sham-treated with sterile water.

Respiratory infections

Mice were infected with mouse-adapted strains of *Streptococcus pneumoniae* intratracheally or influenza virus intranasally. Pathogen load was determined by culture or plaque assays of lung homogenates, respectively.^{33–36}

Macrophage and neutrophil depletion

Lung macrophages and neutrophils were depleted by intranasal administration of liposome-encapsulated clodronate or intraperitoneal injection of anti-Ly6G antibody (1A8, Bioxcell, Lebanon, Nh), respectively, 3 times/week.^{25,37}

Transcript expression in tryptase-treated macrophages

B6 mouse bone marrow-derived macrophages were cultured in the absence or presence of recombinant hTryptase- β (0.8 μ g/ml, 25 nM, Promega, Madison, Wi). RNA was isolated, and qPCR assays were used to evaluate the levels of tumor necrosis factor- α (TNF- α), Cxcl1/KC, and interleukin (IL)-1 β transcripts.

Statistical analyses

Data are presented as mean \pm SEM (n=6–8). Comparisons between two groups were made using a two-tailed Mann-Whitney Test. Multiple comparisons were made by one-way ANOVA with Tukey's post-test, or Kruskal-Wallis with Dunn's post-test, where non-parametric analyses were appropriate. Weights were assessed using one-way ANOVA (repeated measures). Analyses used GraphPad Prism Software (San Diego, CA).

RESULTS

Nose-only exposure of WT BALB/c mice to cigarette smoke induces the hallmark features of COPD

We delivered tightly controlled amounts of cigarette smoke into the nares of WT BALB/c mice for 1–12 weeks and assessed the hallmark features of COPD. Weight loss was evident

within the 1st week of exposure (Fig 1, A). Animals lost 10% of their initial weight after 3 weeks, and only re-gained 5% of this initial weight over the remaining exposure period. In contrast, age-matched non-exposed mice steadily gained weight. Four days of exposure to cigarette smoke led to acute inflammation in the airways, characterized by increased numbers of macrophages and neutrophils in the BALF (Fig 1, B). Inflammation persisted and increased with the additional involvement of lymphocytes (particularly CD8⁺ T cells) after 8-weeks. Smoke exposure also induced progressive increases in chronic parenchymal inflammation (Fig 1, C). This was accompanied by increased expression in the lung of the transcripts that encode TNF- α (Fig 1, D), Cxcl1 (Fig 1, E), and IL-1 β (Fig 1, F), but not IL-6, IL-10, IL-13, or interferon- γ (IFN- γ) (data not shown). There were signs of airway remodeling with increased numbers of MSCs in the airways from week 6 and thickening of the airway epithelium from week 8 (Fig 1, G and H). Airway remodeling was accompanied by alveolar enlargement (increases in alveolar diameter, representative of emphysematous tissue destruction) after 8 weeks, which increased in severity by week 12 (Fig 1, I). Although there likely was a steady continuum of pathologic changes taking place in the smoke-exposed lungs of the WT mice, the results revealed the induction and progression of the disease over weeks 4–6 and 8–12, respectively.

Smoke exposure of the airways of mice resulted in reduced lung function similar to that observed in humans with COPD

Since 8 weeks of exposure was required to induce key features of COPD, other parameters were assessed at this time-point. We next investigated the effects of smoke exposure on parameters of lung function. Exposure decreased hysteresis, transpulmonary and airway-specific resistance (RI), tissue damping, and forced expiratory volume in 100 milliseconds/functional vital capacity ratio (FEV₁₀₀/FVC, representative of FEV₁/FVC ratio in humans), but increased dynamic compliance (C_{dyn}), work of breathing, functional residual capacity (FRC), and total lung capacity (TLC) (Fig 2, A–J). These adverse changes in lung function likely resulted from the combination of chronic inflammation, airway remodeling, and emphysematous lesions with associated reductions in alveolar tissue and supporting airway attachments.

COPD features in the experimental model are glucocorticoid-resistant

While glucocorticoids are used to treat patients with COPD to reduce acute inflammation and the frequency of acute exacerbations,^{38,39} they have limited efficacy.^{40–42} Glucocorticoid treatment (either throughout, or for the last 6 weeks of 12 weeks of smoke exposure) did not prevent chronic inflammation or emphysema, nor did they suppress declines in lung function (Fig 3, A–F).

Smoke exposure of the airways has systemic effects

The lung is not the only organ adversely affected in humans with COPD, and systemic inflammation, loss in skeletal muscle mass, and cardiovascular disease often occur in this disease.⁴³ In our model, 8 weeks of exposure also induced systemic changes with significant alterations in the proportion of leukocytes in the blood. The percentage of monocytes decreased but the percentage of neutrophils and lymphocytes increased (Fig 4, A).

Furthermore, skeletal muscle mass (i.e., the quadriceps) was reduced (Fig 4, *B*). Moreover, the hearts of smoke-exposed mice also were enlarged by ~25% and had more surrounding fatty tissue (Fig 4, *C*).

Induction of experimental COPD exacerbates respiratory infections

Chronic microbial colonization of the airways and infection-induced exacerbations are common in COPD patients.⁴⁴ When we infected mice previously exposed to smoke for 8 weeks with *S. pneumoniae* or influenza virus (and smoke exposure discontinued), pathogen burden increased ~10- and ~2.5-fold, respectively (Fig 4, *D* and *E*).

Experimental COPD does not rapidly resolve following smoking cessation

Once patients develop COPD, their clinical conditions generally do not improve significantly after smoking cessation, and often lung function further deteriorates.² In our model, smoke exposure for 8 weeks followed by cessation for 4 weeks did not improve airway inflammation, emphysema, lung function, or circulating leukocyte abnormalities (Fig 5, *A–F*). In regard to airway inflammation (Fig 5, *A*), the numbers of macrophages continued to increase, suggesting the presence of a macrophage-rich pro-inflammatory environment in the lung that underpins the progression of disease.

Experimental COPD is macrophage dependent

Numerous studies have indicated that pulmonary macrophages have prominent pathologic roles in humans with COPD.^{7,9,45,46} Since increased numbers of macrophages were found in the lungs of our smoked-exposed WT mice (Fig 1, *B*), we assessed the importance of these phagocytes in the development of experimental COPD by reducing their numbers during smoke exposure using the clodronate-depletion method. Liposome-encapsulated clodronate were administered into the airways 3 times/week throughout 8 weeks of smoke exposure. Control animals were sham-treated with empty liposomes. The numbers of macrophages were then quantified in the BALF by morphology and in dispersed whole lung by flow cytometry (F4/80⁺). Macrophages were depleted by 61±4% and 73±5% in smoke-exposed mice and by 25±3% and 36±2% in non-exposed controls in BALF and lung tissue, respectively. In contrast, the percentage of monocytes in the peripheral blood was unaffected. Macrophage depletion in the lung resulted in reduced smoke-induced epithelial thickening and emphysema, and protection against alterations in lung function (Fig 6, *A–E*). In contrast, depletion of neutrophils using the anti-Ly6G antibody approach did not suppress the effects of smoke exposure (Fig S2). The accumulated data suggest a central role for pulmonary macrophages in our animal model of cigarette smoke-induced COPD, thereby supporting the clinical data of others that have implicated these cells in the pathogenesis of COPD in humans.⁷

Exposure of WT B6 mice to cigarette smoke also induces COPD

WT BALB/c and B6 mice respond differently in numerous disease models. To assess the general applicability of our model to another commonly used mouse strain, we examined the effects of smoke exposure on the lungs of WT B6 mice. Exposure for 8 weeks resulted in a similar profile and magnitude of weight loss, parenchymal inflammation, airway

remodeling, emphysematous destruction (alveolar enlargement), and altered lung function in B6 mice compared to BALB/c mice (Fig S3, A–N).

The tetramer-forming tryptase mMCP-6 contributes to macrophage accumulation and inflammation in the airways, and is required for experimental COPD

Macrophage accumulation in the lungs of smoke-exposed mice could be caused by unknown factors released from activated MCs. Although MCs also have been implicated in the pathogenesis of COPD, the link between these two cell types and smoke-induced COPD has not been demonstrated, and the relevant MC-derived factors have not been identified.¹³ Following 8 weeks of smoke exposure, the numbers of macrophages and MCs in the lung of WT B6 mice (i.e., the largest lobe in the multi-lobed right lung by flow cytometry or in the single-lobed left lung by histochemistry) increased ~3 fold in both instances (Fig 7, A–C and Fig S4).

To provide further evidence for the importance of macrophages and MCs in pathogenesis we also assessed their levels in the smoking cessation and clodronate depletion studies. In the cessation studies, the numbers of both of these cell types were elevated concomitant with the maintenance of disease (Fig 7, A–C). In the depletion studies, the suppression of macrophages but not MCs in the lung correlated with the prevention of COPD (Fig 7, A–C). These studies provide further evidence for the pivotal role of macrophages in the pathogenesis of experimental COPD.

mMCP-6 and hTryptase- β have prominent roles in innate immunity and inflammation.^{18,47} Thus, we hypothesized that mMCP-6 might play a critical role in experimental COPD, as occurs in experimental arthritis and colitis. We therefore subjected WT and mMCP-6 null ($-/-$) B6 mice¹⁸ to smoke exposure. We could not detect mMCP-6 mRNA by qPCR in the lungs of non-treated and smoke-treated WT mice, and the levels of mMCP-6 protein were below the limits of detection by SDS-PAGE immunoblot analysis. The numbers of MCs in the lungs of smoke-exposed mMCP-6 $-/-$ mice were not affected and were similar to those in the lungs of smoke-exposed WT mice. However, smoke-exposed mMCP-6-null mice had significantly fewer macrophages (Fig 8, A and B). The reduction in macrophage accumulation in the airways of the tryptase-deficient mice correlated with reductions in neutrophil numbers in the BALF, inflammation of the parenchyma, pro-inflammatory cytokine (e.g., TNF- α and IL-1 β) and chemokine (e.g., Cxcl1) mRNA levels, and emphysematous lesions (Fig 8, C–H). While airway remodeling also was abrogated, there were no obvious differences in the lung function parameters measured between smoke-treated WT and mMCP-6 $-/-$ mice (Fig 8, I and J). While our accumulated data revealed adverse roles for mMCP-6 in the COPD model, other factors that remain to be identified must contribute to the deterioration in lung function in the smoke-exposed mice.

hTryptase- β induces macrophages to increase their expression of pro-inflammatory cytokines and chemokines

To confirm the link between MC tryptases and the pro-inflammatory activity of activated macrophages, we next treated cultured mouse bone marrow-derived macrophages with recombinant hTryptase- β . The tryptase induced these macrophages to markedly increase the

levels of the transcripts that encode TNF- α , Cxcl1, and IL-1 β (Fig 8, *K–M*). These changes did not occur if the tryptase was boiled for 5 minutes before treatment. Although the mechanism by which MC tetramer-forming tryptases induce macrophages to increase the expression of these cytokines and chemokines remains to be determined at the molecular level, the collective data suggest that mMCP-6 and hTryptase- β are associated with macrophage accumulation and macrophage-dependent inflammation, remodeling, and emphysema in COPD.

DISCUSSION

COPD is a major respiratory health problem worldwide² that is usually caused by the chronic inhalation of cigarette smoke.^{48,49} Although a heterogeneous disease, COPD is characterized by chronic airway inflammation (bronchitis) and/or emphysema, as well as reduced lung function.⁴⁹ The chronic inflammation that occurs in the smoke-exposed lung is thought to drive the progressive mucus hypersecretion, airway remodeling, and destruction of alveolar tissue that synergize to reduce pulmonary function.^{48,49} While inhaled glucocorticoids and bronchodilators are used therapeutically to treat the symptoms and exacerbations of COPD,³⁹ there is no effective treatment that prevents the induction of the disease or halts its progression.⁴¹

The lack of a cigarette smoke-induced animal model of COPD of short duration that recapitulates the major features of the human condition has hindered our understanding of the disease. To address this deficiency, we developed a short-term mouse model of cigarette smoke-induced COPD that has most of the key pathological and clinical features of the human disease. Importantly, our *in vivo* model gradually progresses to overt disease over 6–8 weeks (Fig 1) and does not rapidly resolve (Fig 5). The hallmark features of the disease are induced within 8 weeks (Figs 1, 2, and S3), providing opportunities to identify therapeutic targets for both the induction and progression phases of the disease. As occurs in humans who smoke,^{14,47,50} the direct delivery of smoke to the airways of WT BALB/c and B6 mice resulted in acute and chronic inflammation that was dominated by neutrophils, macrophages, and eventually CD8⁺ T cells (Figs 1 and S3). The disease also had a MC component (Figs 7 and 8).

Inflammation was associated with airway remodeling, emphysematous changes, and reduced lung function (Figs 1, 2, S2, and S3). The airway remodeling, alveolar enlargement and emphysema were likely due to inflammation-induced damage of the parenchymal walls. Together, the airway remodeling and emphysema in our model led to reduced lung function, as occurs in humans with COPD (Figs 2 and S3).

The only feature not consistent with human COPD was a decrease in airway resistance (Fig 2, *B* and *C*). This finding suggests an absence of obstruction despite the presence of goblet cell metaplasia and airway remodeling. Because mice have a greater proportion of parenchyma to airway tissue compared to humans, the reduced resistance might be the result of emphysema and associated reductions in tissue attachments. Alveolar distension and hyperinflation led to widening of the airways during ventilation and reduced resistance in other mouse models of emphysema.⁵¹

Glucocorticoids have limited efficacy in treating COPD symptoms in humans. They partially suppress chronic inflammation, but do not reverse the tissue lesions or modify factors that drive chronic disease, and have no effect on the decline in lung function.^{40–42} As occurs in COPD patients, dexamethasone did not suppress inflammation, emphysema, or alterations in lung function in our model (Fig 3). COPD has a systemic component with changes in circulating leukocyte populations and loss of skeletal muscle mass.⁴³ Both of these features were observed in our model (Fig 4, A and B). COPD is also linked to cardiovascular disease, and the hearts of smoke-exposed WT mice had pathologic changes (Fig 4, C).

COPD patients have a 3–6 fold increased risk of *S. pneumoniae*-induced pneumonia.⁵² They also are more susceptible to influenza virus and suffer more severe symptoms when infected.⁵³ Respiratory infections induce further inflammation and acute exacerbations of COPD symptoms which, in turn, increase the rate of disease progression.⁵⁴ The development of experimental COPD was associated with enhanced respiratory infection by *S. pneumoniae* and influenza virus (Fig 4, D and E). The numbers of CD8⁺ T cells that infiltrated the lungs of smoke-exposed mice were elevated but these lymphocytes had reduced expression of the activation marker CD98 (data not shown), which may contribute to impaired pathogen clearance. Although inflammation was increased, the latter findings suggest that immune function was suppressed, thereby predisposing the diseased mice to more severe pulmonary infections.

Once COPD develops in humans, the clinical condition and features of disease often deteriorate further even after the cessation of smoking.² After 8 weeks of smoking and 4 weeks of cessation in our model, macrophage accumulation in the airways of WT mice actually increased compared to that observed immediately after 8 weeks of smoking (Fig 5, A). It is possible that the increase in the number of macrophages in the lungs underpins the progression of COPD even after smoking cessation. This macrophage accumulation may be additionally exacerbated by infection that could further increase the rate of disease progression. There were no signs of resolution of any features of disease, at least 4 weeks after smoking cessation (Fig 5).

We then performed macrophage-depletion studies in WT mice to demonstrate that these phagocytes were essential for smoke-induced emphysema and reduced lung function (Fig 6). The clodronate method is the only method that can be used to reliably deplete the number of macrophages in the lungs of mice. Although clodronate might deplete some dendritic cells, there was no reduction in neutrophils by the method. Furthermore, the direct depletion of neutrophils did not suppress changes in lung structure or function (Fig S3).

The mechanisms that drive COPD pathogenesis are incompletely understood. Chronic influx of inflammatory cells into the lungs in COPD leads to the generation and release of pro-inflammatory cytokines, chemokines, and leukotrienes⁵⁵ that are thought to contribute to tissue destruction and the development of COPD. However, abnormalities in the protease:protease inhibitor balance in the lung also is important.⁵⁶ In that regard, MCs and their tryptase•serglycin proteoglycan complexes promote inflammation in numerous diseases,^{47,57,58} and MCs have been implicated in COPD pathogenesis.¹³ MCs are common

in inflammatory infiltrates in COPD, and increases in their numbers correlate with reduced lung function and airway remodeling.¹⁴ The numbers of MCs detected in smoke-exposed mice were not high relative to other cell types (Fig 7). Nevertheless, it is well known that MCs release potent pro-inflammatory mediators, and that even small numbers of these cells can have devastating effects *in vivo* as occurs in systemic anaphylaxis.

hTryptase- β ^{59,60} comprises up to 50% of the protein content of a human MC. Its ortholog mMCP-6⁶¹ has beneficial roles in the control of infections,^{18,62} but adverse roles in MC-dependent inflammation of the airways,⁵³ joints,^{47,57} and colon in mice.^{58,63} However, the roles of MCs and their granule mediators in COPD pathogenesis have not been elucidated. In our model, there were no detectable increases in mMCP-6 mRNA or protein levels. This was not surprising since MC tryptases are pre-formed and stored in the cell's granules. Thus, changes in their mRNA levels are not as important as their release or the activation state of the MC. Only low levels of MCs were observed and mMCP-6 protein levels were below the limits of detection by immunoblot in whole lung tissues. There are no other more sensitive tests, such as ELISA, available. Nevertheless, Mortaz and coworkers, have demonstrated that cigarette smoke conditioned media induced the expression of mMCP-6 protein in primary cultured mast cells.¹⁷

Despite these data, the generation of mMCP-6^{-/-} B6 mice¹⁸ allowed us the opportunity for the first time to definitively evaluate the importance of this MC-restricted tryptase in experimental COPD. The MCs in WT BALB/c mice express mMCP-6⁶¹ and the other tryptase family member mMCP-7.⁶⁴ In contrast, the MCs in WT B6 mice lack mMCP-7 due to a splice-site mutation in its gene.⁶⁵ The finding that all the features of COPD were similar in WT BALB/c (Fig 1) and B6 (Fig S3) mice indicates that mMCP-7 is not essential in our experimental model.

Smoke exposure resulted in enhanced pro-inflammatory cytokine and chemokine expression (Fig 1, D-F and 8, E and F) and increased the numbers of macrophages and MCs in the lungs of WT B6 mice (Fig 7). We then employed our mMCP-6^{-/-} B6 mice to demonstrate that the presence of this tryptase is required for smoke-induced pro-inflammatory cytokine and chemokine expression and accumulation of macrophages in the lung, and for airway epithelial thickening and emphysematous damage (Fig 8). There were no statistically significant differences between neutrophil numbers or chemokine mRNA levels in the lung (Fig 8, C and F) or the percentage of leukocytes that were neutrophils in the blood (data not shown) at baseline. Thus, the decreased cellular infiltration in the lungs of the smoke-treated mMCP-6-null mice was not due to a baseline defect. We then showed that recombinant hTryptase- β induced mouse macrophages to increase their expression of the transcripts that encode the cytokines and chemokines that are increased in the lungs of mice with experimental COPD. These findings suggest the tryptase contributes to COPD, at least in part, by directly activating the macrophages in the lungs of the diseased animals. Our discovery that macrophages and tryptase⁺ MCs participate in the pathogenesis of COPD in our model raise the possibility that these cells and their granule tetramer-forming tryptases have comparable adverse roles in human COPD. Thus, the next generation of hTryptase- β inhibitors might have efficacy in the treatment of patients with COPD.

In summary, we developed a short-term cigarette smoke-induced mouse model that has the hallmark features of COPD. This animal model enables the study of the pathogenesis of COPD in a significantly shorter time-frame than existing cigarette smoke-induced models, thereby providing opportunities for pharmacologic intervention. Our model also enables the evaluation of the consequences of COPD on the ability of the lung to combat infections. COPD is the result of a complex interplay of immune dysregulation, and our model allows the evaluation of other important parameters (e.g. systemic consequences) in COPD in the mouse. This study raises the possibility that macrophages and tryptase⁺ MCs might have similar adverse roles in patients with COPD.

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Abbreviations used

BALF	bronchoalveolar lavage fluid
B6	C57BL/6
Cdyn	dynamic compliance
CFU	colony forming unit
COPD	chronic obstructive pulmonary disease
FEV₁₀₀	forced expiratory volume in 100 milliseconds
FRC	functional residual capacity
FVC	functional vital capacity
IFN-γ	interferon- γ
MC	mast cell
mMCP	mouse MC protease
MSC	mucus-secreting goblet cell
qPCR	quantitative polymerase chain reaction
PFU	plaque forming unit
PV	pressure/volume
RI	resistance
TLC	total lung capacity
TNF-α	tumor necrosis factor- α
WT	wild-type

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Clinical implications

We describe a short-term mouse model with the hallmark features of cigarette smoke-induced COPD. We then show that the model can be used to further our understanding of the pathogenesis of COPD. We demonstrate for the first time that a MC-restricted tetramer-forming tryptase has a prominent adverse role in experimental COPD.

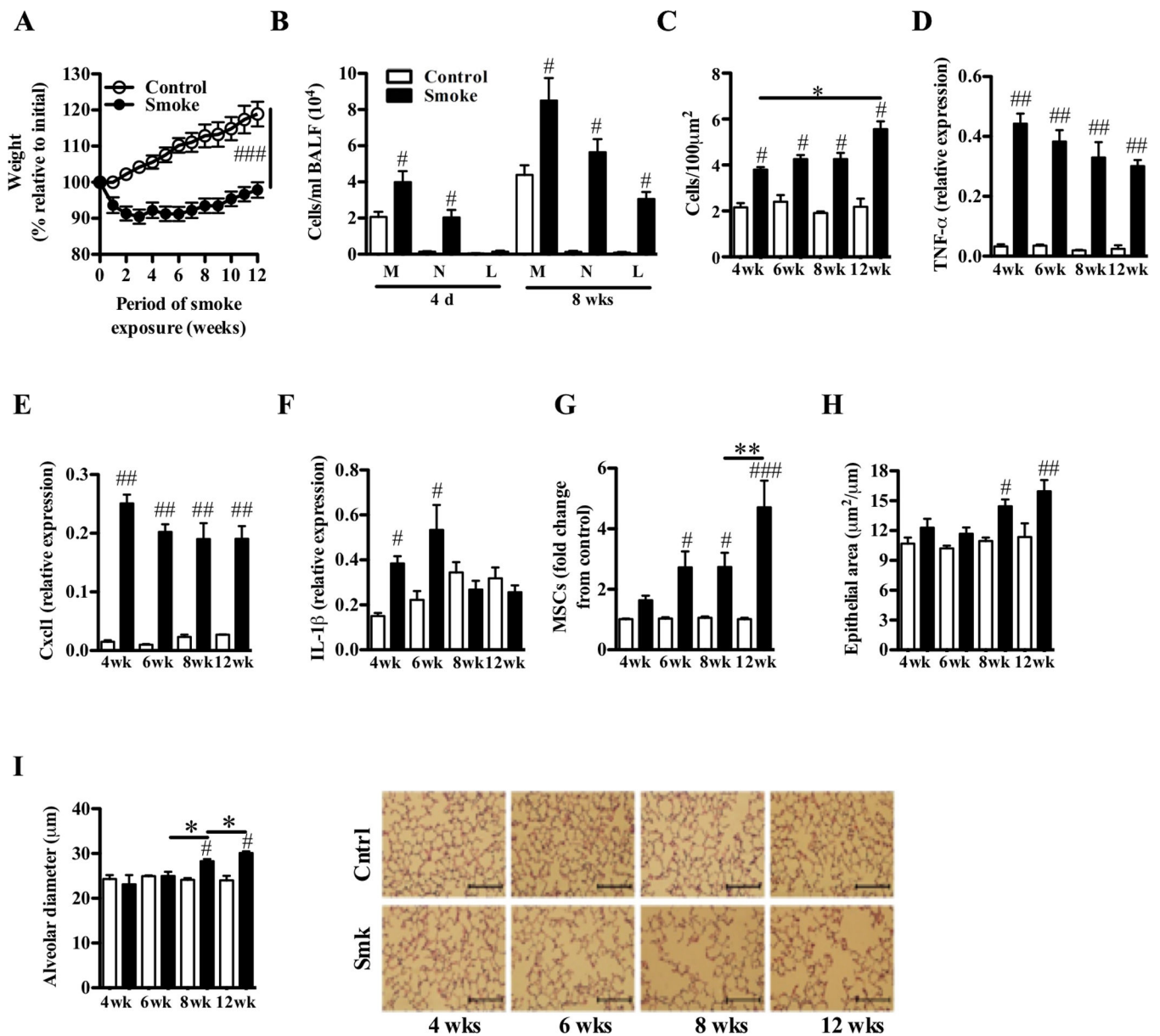
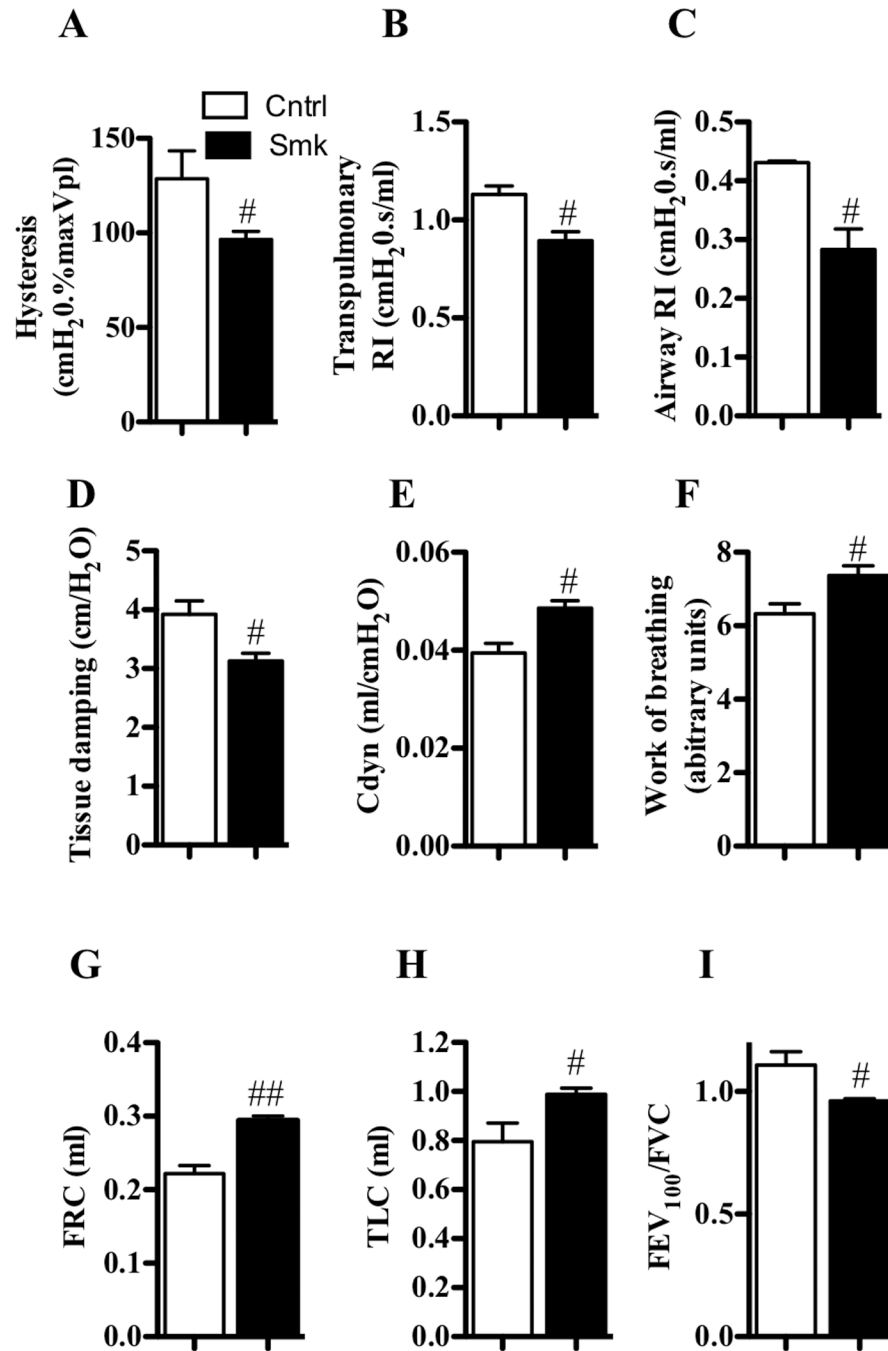


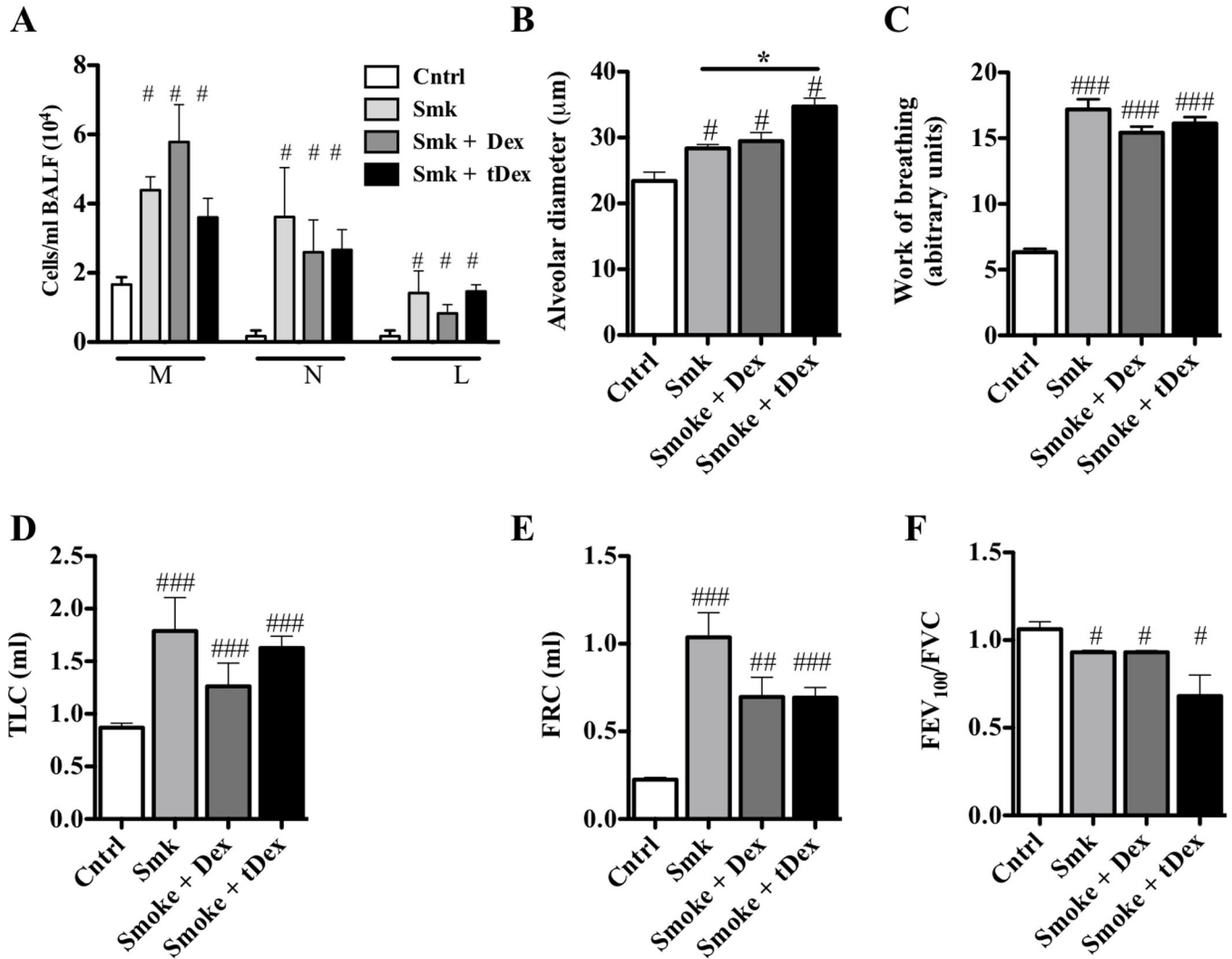
FIG 1. Nose-only exposure of the lungs of BALB/c mice to cigarette smoke induces the hallmark features of human COPD. **A–I**, WT BALB/c mice were exposed to cigarette smoke or normal air for 1–12 weeks. Relative to control mice, smoke-exposed mice had **(A)** reduced weight-gain relative to initial weight; **(B)** acute (after 4 days) and chronic (after 8 weeks) increases in the numbers of macrophages (M), neutrophils (N) and lymphocytes (L) in BALF; **(C)** increased cellular infiltrates in the parenchyma; increased levels of the transcripts that encode **(D)** TNF- α , **(E)** Cxcl1, and **(F)** IL-1 β in lung homogenates; **(G)** increased number of mucus-secreting goblet cells (MSCs) in the airways; **(H)** airway epithelium thickening; and **(I)** alveolar enlargement (scale bar on micrographs = 100 μm). Data are means \pm SEM of 6–8 mice/group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to

mice that breathed normal air, * $P < 0.05$, ** $P < 0.01$ compared to other groups indicated. Statistical significance of the reduced weight gain is for the whole curve.

**FIG 2.**

Nose-only cigarette smoke exposure leads to changes in lung function that are similar to that in humans with COPD. A–I, WT BALB/c mice were exposed to cigarette smoke or normal air for 8 weeks. Relative to control mice, smoke-exposed mice had decreased (A) hysteresis, (B) transpulmonary and (C) airway-specific resistance (RI) and (D) tissue damping, but increased (E) dynamic compliance (Cdyn), (F) work of breathing, (G) functional residual capacity (FRC), and (H) total lung capacity (TLC), and (I) reduced ratio of forced

expiratory volume in 100 milliseconds/forced vital capacity (FEV₁₀₀/FVC). Data are means \pm SEM of 6–8 mice/group, # $P < 0.05$, compared to mice that breathed normal air.

**FIG 3.**

Experimental COPD is glucocorticoid-resistant. **A–F**, WT BALB/c mice were exposed to cigarette smoke or normal air for 12 weeks and were treated with dexamethasone or sham-treated with sterile distilled water either prophylactically throughout (Dex), or therapeutically (tDex) for the last 6 weeks, of the smoking protocol. Steroid treatment had no effect on (**A**) macrophage (M), neutrophil (N) and lymphocyte (L) numbers in the BALF, (**B**) alveolar enlargement or changes in lung function; (**C**) work of breathing, (**D**) total lung capacity (TLC), (**E**) functional residual capacity (FRC) or (**F**) forced expiratory volume in 100 milliseconds/forced vital capacity (FEV₁₀₀/FVC) ratio. Data are means \pm SEM of 6–8 mice/group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to mice that breathed normal air. There were no differences between other groups.

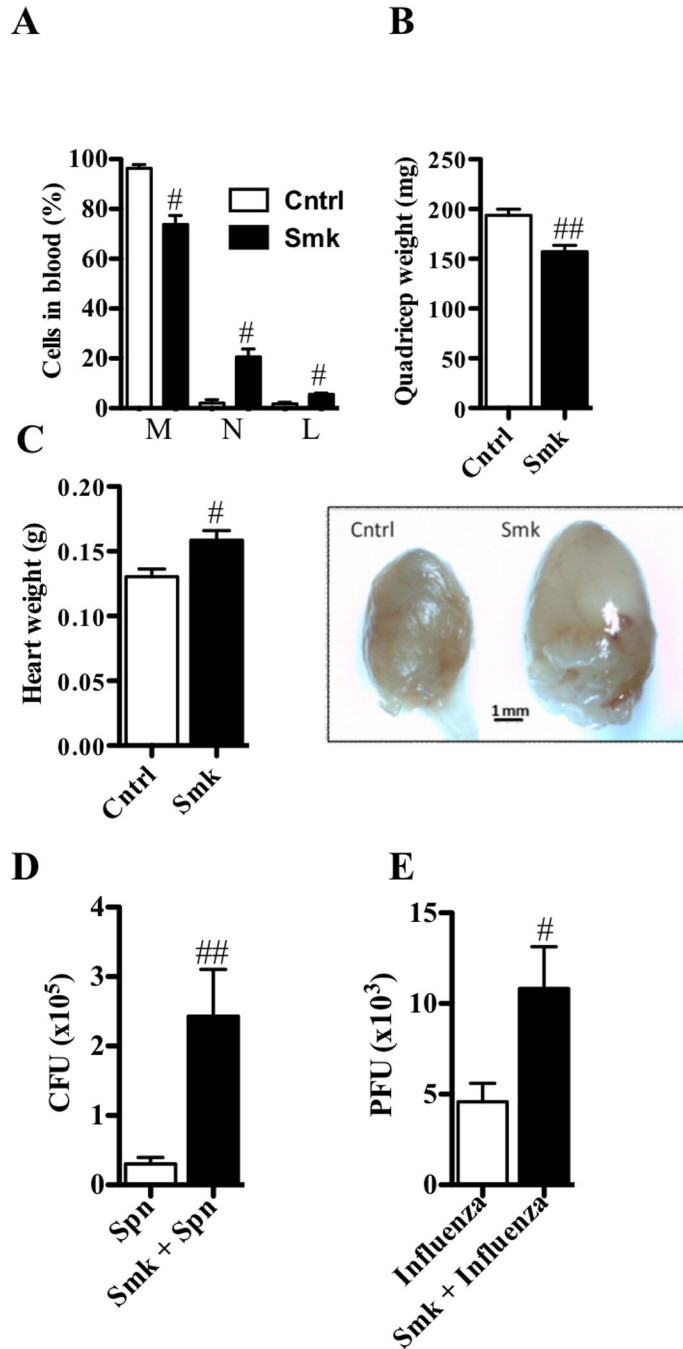
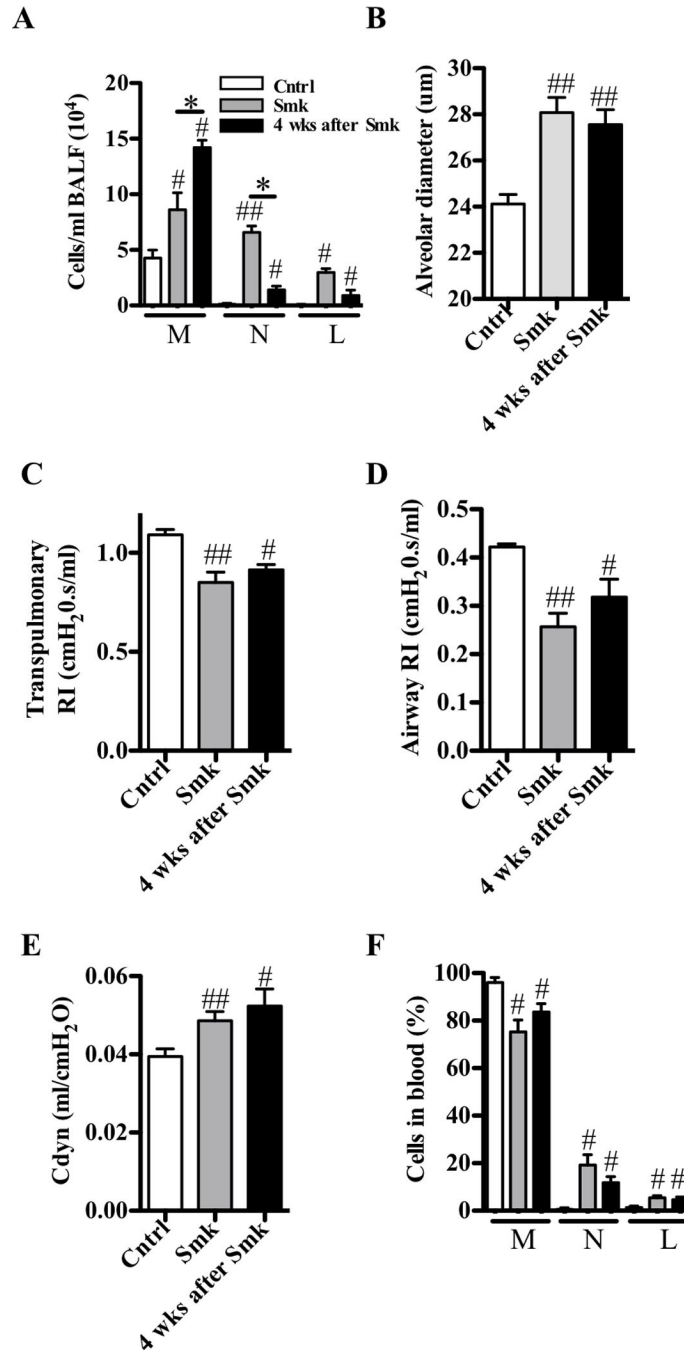


FIG 4. Experimental COPD has systemic involvement and exacerbates respiratory infections. **A–E**, WT BALB/c mice were exposed to cigarette smoke or normal air for 8 weeks. Relative to control mice, smoke-exposed mice had **(A)** alterations in the proportions of monocytes (M, decreased), neutrophils (N, increased), and lymphocytes (L, increased) in blood; **(B)** reduced quadriceps weight; **(C)** increased heart weight, size, and fatty deposits; and decreased **(D)** clearance of *S. pneumoniae* (after 48 hours of infection) and **(E)** influenza virus (after 7 days

of infection). Data are means \pm SEM of 6–8 mice/group, # $P<0.05$, ## $P<0.01$, compared to WT mice that breathed normal air.

**FIG 5.**

Experimental COPD does not rapidly resolve following cessation of smoke exposure. **A–F**, WT BALB/c mice were exposed to cigarette smoke or normal air for 8 weeks. Smoke exposed mice were evaluated immediately after the cessation of smoking or 4 weeks later. Relative to control mice, both groups of smoke-exposed mice had (**A**) increased airway inflammation; (**B**) alveolar enlargement; and changes in lung function; decreased (**C**) transpulmonary and (**D**) airway-specific resistance (RI); (**E**) increased dynamic compliance (Cdyn); as well as (**F**) altered leukocyte populations in blood. Mice that had ceased smoking

4 weeks earlier had more macrophages, but fewer neutrophils, in their BALF. Data are means \pm SEM of 6–8 mice/group, # $P<0.05$, ## $P<0.01$, compared to mice that breathed normal air, * $P<0.05$, compared to other groups indicated.

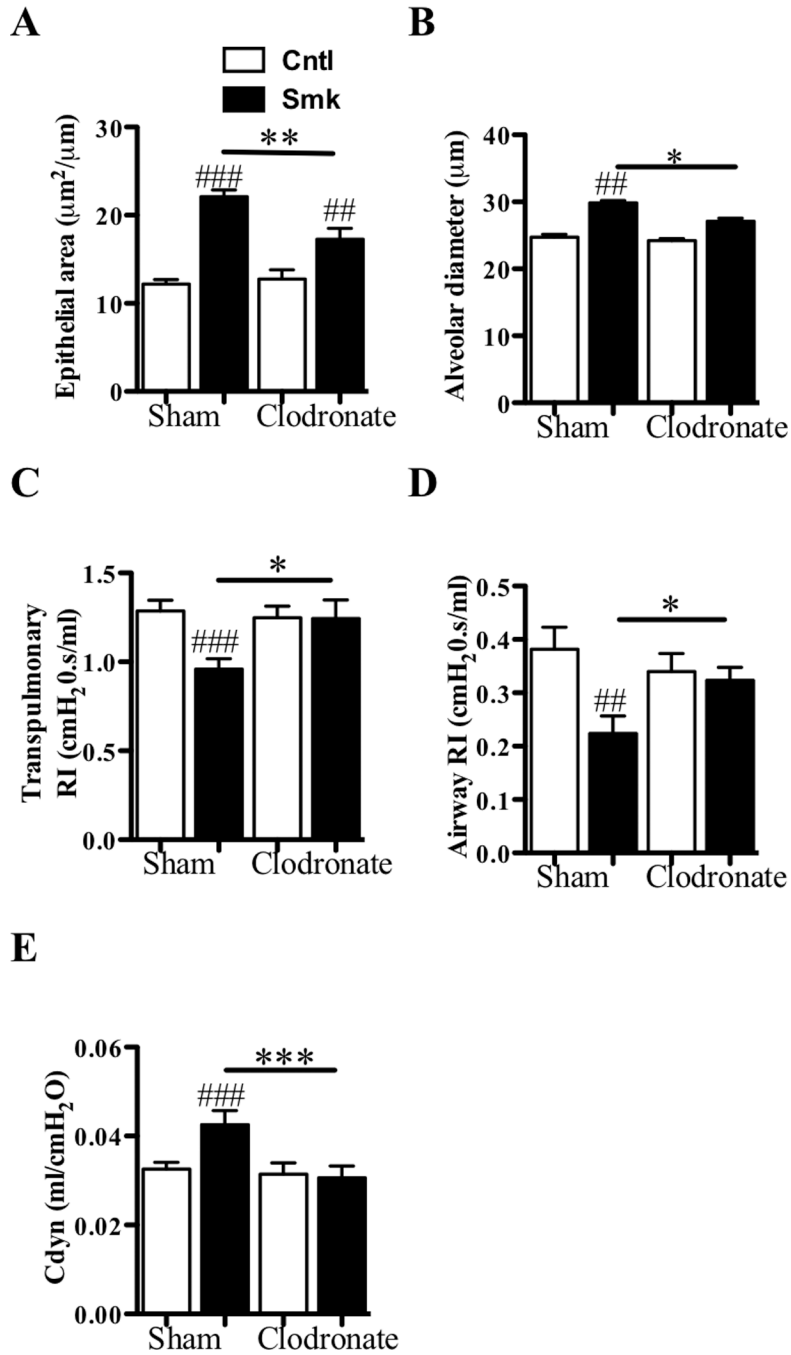
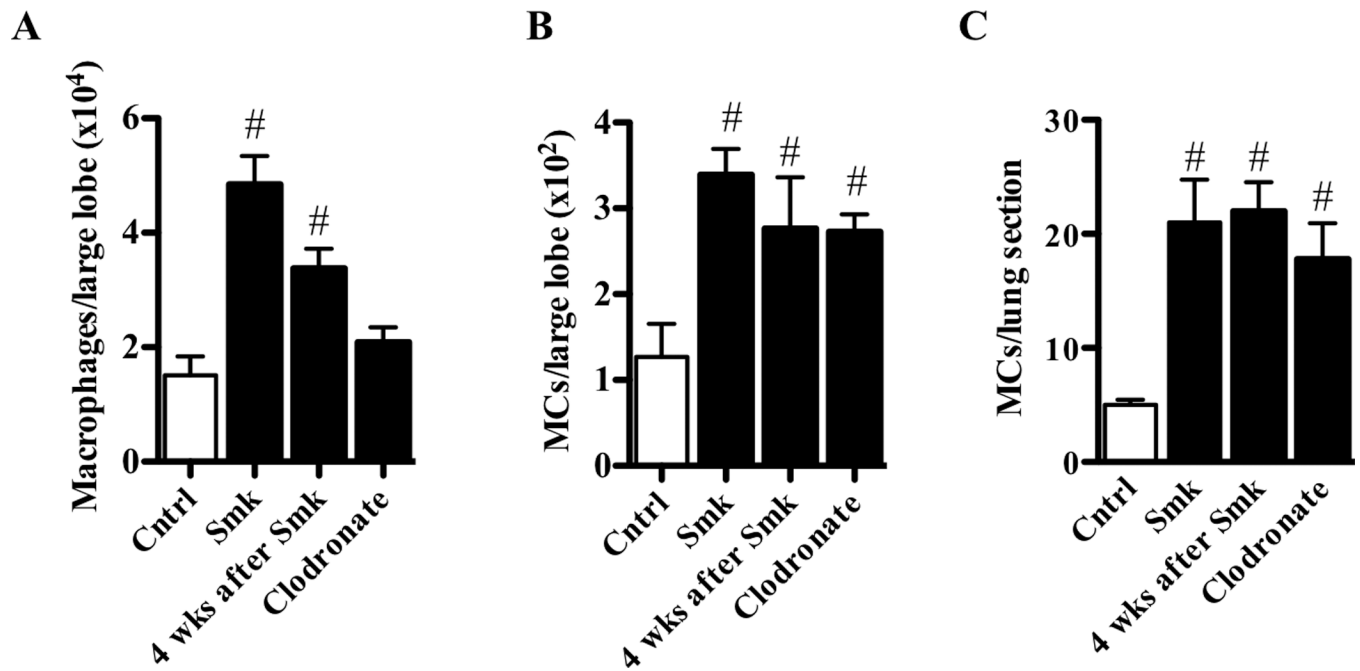


FIG 6. Depletion of pulmonary macrophages suppresses the development of experimental COPD. **A–E**, WT BALB/c mice were exposed to cigarette smoke or normal air for 8 weeks. These two groups of mice also were treated with either liposome encapsulated clodronate or empty liposomes (Sham) 3 times/week for the duration of the experiment commencing on the 1st day of smoking. Relative to smoke-exposed macrophage-sufficient mice, smoke-exposed macrophage-depleted mice had reduced (**A**) airway epithelium thickening; and (**B**) alveolar enlargement; and altered lung function; increased (**C**) transpulmonary and (**D**) increased

airway-specific resistance (RI), and (E) reduced dynamic compliance (C_{dyn}). The smoke-exposed macrophage-depleted mice had no alveolar enlargement or changes in lung function compared to non-smoke exposed control mice. Data are means±SEM of 6–8 mice/group, ## $P<0.01$, ### $P<0.001$ compared to mice that breathed normal air, * $P<0.05$, ** $P<0.01$ *** $P<0.001$ compared to other groups indicated.

**FIG 7.**

Experimental COPD increases the numbers of pulmonary macrophages and MCs. **A–C**, WT B6 mice were exposed to cigarette smoke or normal air for 8 weeks. Some mice were then rested for 4 weeks after smoking. Other mice were treated with clodronate. Relative to control mice, smoke-exposed mice had increased numbers of **(A)** F4/80⁺ macrophages and **(B)** Kit⁺/FcεRI⁺/IgE⁺ (by flow cytometry in the largest lobe of the multi-lobed right lung) or **(C)** toluidine blue⁺ (by histochemistry in the single-lobed left lung) MCs in the lungs. Smoking cessation did not alter macrophage or MC numbers. Clodronate specifically attenuated macrophage numbers. Data are means±SEM of 6–8 mice/group, # *P*<0.05, compared to mice that breathed normal air.

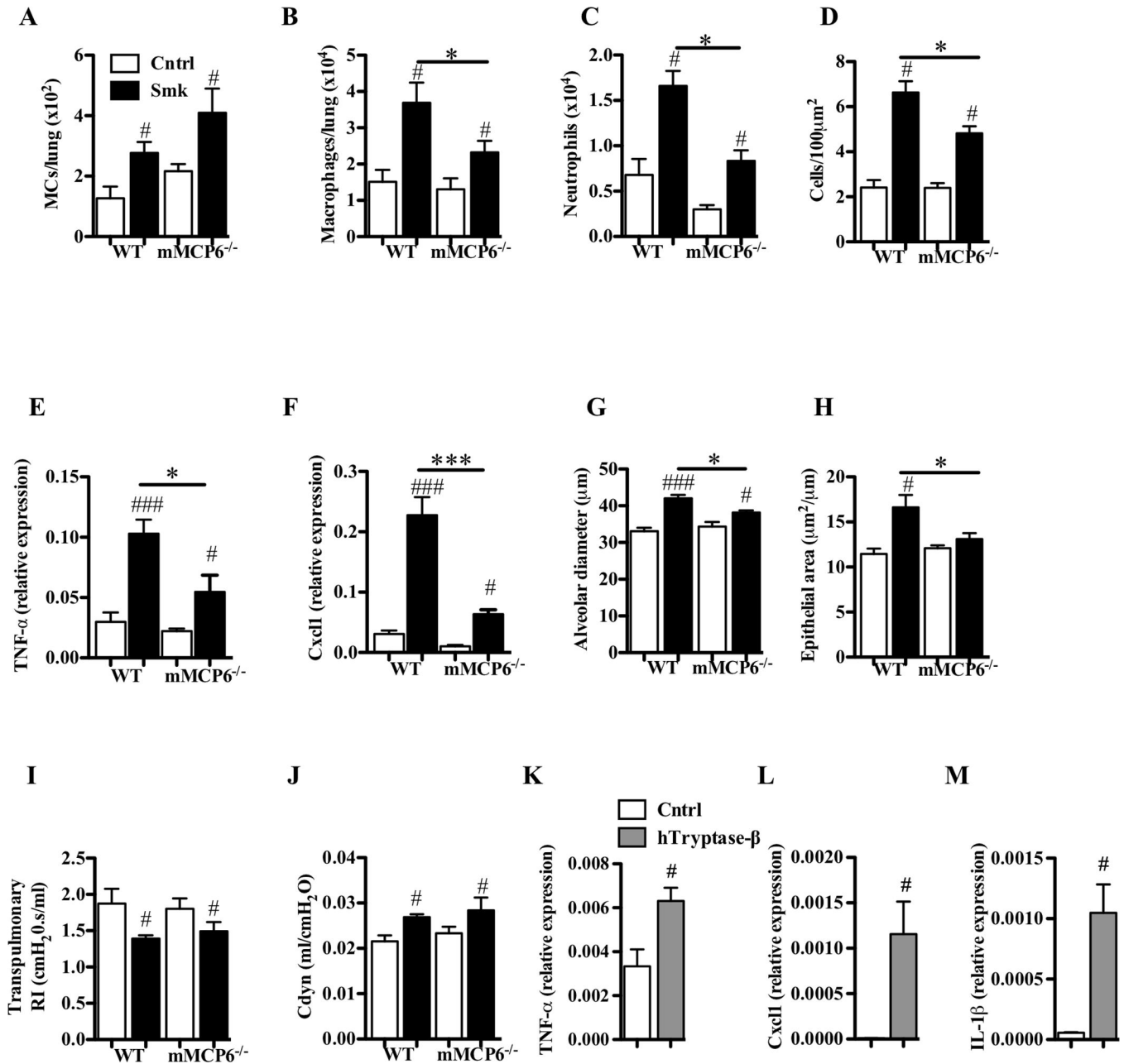


FIG 8.

The tryptase mMCP-6 contributes to pulmonary macrophage accumulation and parenchymal inflammation, and is required for airway remodeling and alveolar enlargement in experimental COPD. A–J, WT and mMCP-6^{-/-} B6 mice were exposed to cigarette smoke or normal air for 8 weeks. Relative to smoke-exposed WT mice, smoke-exposed mMCP-6^{-/-} mice had (A) no change in the number of Kit⁺/FcεRI⁺/IgE⁺ MCs but had reduced (B) numbers of F4/80⁺ macrophages and (C) neutrophils in the lung, (D) less cellular infiltrates in the parenchyma, attenuated (E) TNF-α and (F) Cxcl1 mRNA levels in lung homogenates, (G) diminished alveolar enlargement, (H) no airway remodelling and no differences in lung function [e.g. (I) transpulmonary resistance (RI) or (J) dynamic

compliance (Cdyn)]. **K–M**, B6 mouse bone marrow-derived macrophages were cultured in the absence or presence of recombinant hTryptase- β . Relative to untreated cells, hTryptase- β -treated cells had increased levels of the transcripts that encode (**K**) TNF- α , (**L**) Cxcl1 and (**M**) IL-1 β . Data are means \pm SEM of 6–8 mice/group, or of 3 cell cultures in triplicate (representative of 4 repeat experiments), # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to mice that breathed normal air (**A–J**) or compared to sham-treated macrophages (**K–M**), * $P < 0.05$, compared to other groups indicated.