Elastin fragments drive disease progression in a murine model of emphysema

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Mice lacking macrophage elastase (matrix metalloproteinase-12, or MMP-12) were previously shown to be protected from the development of cigarette smoke–induced emphysema and from the accumulation of lung macrophages normally induced by chronic exposure to cigarette smoke. To determine the basis for macrophage accumulation in experimental emphysema, we now show that bronchoalveolar lavage fluid from WT smoke-exposed animals contained chemotactic activity for monocytes in vitro that was absent in lavage fluid from macrophage elastase–deficient mice. Fractionation of the bronchoalveolar lavage fluid demonstrated the presence of elastin fragments only in the fractions containing chemotactic activity. An mAb against elastin fragments eliminated both the in vitro chemotactic activity and cigarette smoke–induced monocyte recruitment to the lung in vivo. Porcine pancreatic elastase was used to recruit monocytes to the lung and to generate emphysema. Elastin fragment antagonism in this model abrogated both macrophage accumulation and airspace enlargement.

Introduction
Chronic obstructive pulmonary disease (COPD) currently affects over 18 million Americans and is the fourth leading cause of death in the US. The burden of this disease promises to increase throughout the globe as smoking rates continue to climb in most developing countries (1). Emphysema, a major component of COPD, is characterized by an inflammatory cell infiltrate composed of alveolar macrophages, neutrophils, and CD4+ and CD8+ lymphocytes, and by the presence of proteinases in excess of their inhibitors within the alveolar space, which leads to destruction and permanent enlargement of the peripheral airspaces of the lung (2–7). Macrophages are the predominant inflammatory cell type found in the lung both under normal conditions and in the setting of emphysema (8); however, the mechanisms responsible for the recruitment of peripheral blood monocytes to the lung in the setting of emphysema remain poorly understood.

We have previously shown that mice deficient in macrophage elastase (matrix metalloproteinase-12, or MMP-12) are protected from cigarette smoke–induced emphysema (9). As expected, MMP-12+/+ mice displayed an increase in alveolar macrophage number in response to cigarette smoke when compared with non–smoke-exposed controls, but surprisingly, MMP-12−/− mice did not. This is not simply due to a requirement of MMP-12 for monocytes to properly emigrate from the vasculature to the lung because monocytes do not synthesize MMP-12; only differentiated and activated tissue macrophages produce this proteinase (10). Consistent with this fact, monthly instillations of monocyte chemoattractant protein-1 (MCP-1), concomitant with cigarette smoke exposure, resulted in a significant increase in alveolar macrophages in the MMP-12−/− mice (although they were still protected from the development of emphysema). Thus, MMP-12−/− monocytes are capable of emigrating to the lung if the appropriate stimulus is present.

In addition to C-C chemokine–producing cells, the lung possesses other potential monocyte chemotactic factors that reside within the ECM. Fragments of collagen (11), fibronectin (12), laminin (13), and elastin (14–16) have all been shown to be chemotactic for monocytes in vitro, but data to support a role for these agents in vivo are lacking. In the current study, we show that MMP-12 is required to generate the monocyte chemotactic activity present in a model of cigarette smoke–induced emphysema in the mouse and that elastin fragments (EFs) are required for macrophage accumulation and airspace pathology in this setting.

Results
Bronchoalveolar lavage fluid from MMP-12+/+ but not MMP-12−/− smoke-exposed mice contains monocyte chemotactic activity. Bronchoalveolar lavage fluid (BALF) from WT smoke-exposed mice elicited a 5-fold increase in monocyte chemotaxis in vitro compared with BALF from control mice. In contrast, there was no chemotactic activity in BALF from MMP-12−/− smoke-exposed mice above that seen in non–smoke-exposed controls (Figure 1A). Of note, MMP-12 itself was not chemotactic for monocytes in our assay (not shown). Initially, we assayed for C-C chemokines, substances well known for their monocyte chemotactic properties. Real-time PCR was performed on lung RNA from WT mice exposed to smoke for 1, 3, and 6 months, using primers for IL-1β, MCP-1, MCP-2, MIP-1α, MIP-1β, and RANTES (data not shown). There was no significant increase in RNA expression for any of these chemokines.

Having excluded the common chemokines, we began to search for a novel factor that is generated in MMP-12+/+ but not MMP-12−/− mice in the setting of cigarette smoke exposure. BALF from MMP-12+/+ and MMP-12−/− smoke-exposed mice was subjected to gel filtration chromatography. Chemotactic activity was identified only in the BALF from MMP-12+/+ mice, in fractions corresponding to a molecular mass of approximately 45 kDa. Significant chemotactic activity was not present in any fraction of MMP-12−/− BALF (Figure 1B).
EFs are the chemotactic factor present in BALF. Fractions of the BALF demonstrating chemotactic activity for monocytes in vitro were subjected to Western blot analysis using a panel of antibodies against putative chemokine components of the ECM, including thrombospondin, laminin, collagen, fibronectin, and elastin. Only antibodies directed against elastin were detected in chemotactic fractions (Figure 1C).

Additional in vitro assays of monocyte chemotaxis were performed using lung homogenates from MMP-12+/+ and MMP-12–/– smoke-exposed animals and non–smoke-exposed controls. Consistent with the results from the experiments using BALF as the chemoattractant, only the lung homogenates from MMP-12+/+ mice were chemotactic for monocytes. The lung homogenate demonstrated significantly more potent (5-fold higher) chemotactic activity than the corresponding BALF. To show that EFs are the chemotactic factor present in MMP-12+/+ smoke-exposed BALF and lung homogenate, we repeated the monocyte chemotaxis assays with the addition of BA4 mAb (50 nM) to the lower compartments. BA4, an mAb that was derived from an immunization with the α-elastin fraction of insoluble elastin, completely eliminated the activity present in BALF and lung homogenate (Figure 1D). However, BA4 had no effect on formyl-Met-Leu-Phe–induced (fMLP-induced) chemotaxis (not shown).

EF inhibition decreases cigarette smoke–induced lung macrophage accumulation in vivo. To illustrate that EFs influence cigarette smoke–induced lung macrophage accumulation in vivo, we used a 1-week cigarette smoke exposure protocol. Acute cigarette smoke exposure experiments are certainly too brief to produce airspace disease or emphysema, but they have been demonstrated to reliably produce an inflammatory cell infiltrate (17, 18). Using this protocol, we found that the monocyte recruitment elicited by the chemotactic factors generated by cigarette smoke exposure in vivo can be inhibited 66% by BA4 (P < 0.0005) (Figure 2).

EFs are chemotactic for monocytes in vivo. To further prove that EFs are capable of recruiting peripheral blood monocytes to the lung in vivo, we administered purified EFs (no residual elastase in the digest) intratracheally (i.t.) to WT mice. Intratracheal instillation of EFs resulted in a dose-dependent increase in lung macrophage accumulation.
Porcine pancreatic elastase is capable of recruiting monocytes to the lung in vivo. Despite widespread use over the past 30 years, the exact nature of the inflammatory component present in the *porcine pancreatic elastase* (PPE) model of emphysema has yet to be fully characterized. To prove that PPE generates an inflammatory cell infiltrate, we instilled PPE i.t. and quantified the number of macrophages and neutrophils present in the BALF 1, 3, 7, 14, and 21 days later (Figure 3). A single i.t. dose of PPE (1 U = 7.4 μg) produced a significant increase in lung macrophage content at 72 hours (P < 0.01) (Table 1). Furthermore, i.t. administration of the approximately 45-kDa EFs produced a significant increase in lung macrophage content at 72 hours (Table 1). EFs did not recruit neutrophils to the alveolar space above the level seen in controls.

**Table 1**

<table>
<thead>
<tr>
<th>Intratracheal instillation of PBS, EFs, and PPE</th>
<th>Macrophages (×10⁶)</th>
<th>BAL macrophages (×10⁶)</th>
<th>BAL PMNs</th>
<th>PBS</th>
<th>PPE (1 U)</th>
<th>EF peptides (10 μg)</th>
<th>EF peptides (100 μg)</th>
<th>VGVAPG (5 μg)</th>
<th>EF –45-kDa fragment (4 μg)</th>
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<tbody>
<tr>
<td>PBS</td>
<td>1.74 ± 0.06</td>
<td>10.24 ± 1.02</td>
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<tr>
<td>PPE (1 U)</td>
<td>3.99 ± 0.25</td>
<td>18.48 ± 1.79</td>
<td>1.75 ± 0.26</td>
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<tr>
<td>EF peptides (10 μg)</td>
<td>2.88 ± 0.13</td>
<td>19.85 ± 0.75</td>
<td>0</td>
<td></td>
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<tr>
<td>EF peptides (100 μg)</td>
<td>3.77 ± 0.22</td>
<td>19.63 ± 2.95</td>
<td>0.07 ± 0.06</td>
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<tr>
<td>VGVAPG (5 μg)</td>
<td>3.01 ± 0.13</td>
<td>–</td>
<td>–</td>
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<tr>
<td>EF –45-kDa fragment (4 μg)</td>
<td>4.15 ± 0.29</td>
<td>–</td>
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Inflammatory cell counts were tabulated in BAL and on mac-3–stained sections at 72 hours. *P* < 0.01 vs. control. PMN, polymorphonuclear leukocyte.

**Figure 3**

Effects of PPE and EF antagonism on lung macrophage content in vivo. PPE (1 U = 7.4 μg) or PBS control was administered into the lungs of WT mice. BAL was performed before the lungs were inflated and fixed with 10% buffered formalin at days 1, 3, 7, 14, and 21. (A and B) BAL macrophage (A) and BAL neutrophil (B) counts were tabulated using a hemocytometer and HEMA 3–stained cytopsins. (C) Lung macrophage counts are expressed as macrophages per high-powered field adjusted for tissue density on mac-3–stained sections. n = 4 mice per group. Bars represent SEM. *P < 0.05 vs. control. (D) To determine the effect of EF antagonism on monocyte recruitment, PPE-recipient mice received 10 μg BA4 (n = 9), 10 μg anti–collagen type I (col) antibody (n = 4), 10 μg anti–laminin-5 (lam) antibody (n = 4), or 10 μg IgG1 isotype control antibody (n = 8) over the course of the 3-day experiment; and either BA4 or IgG1 isotype control over the course of 21 days. The macrophage counts are expressed as above. Bars represent SEM. *P < 0.01 vs. control; **P < 0.001 vs. control. (E–G) Representative mac-3–stained sections are shown for PBS (E), PPE/IgG1 (F), and PPE/BA4 (G) at the 3-day time point. Magnification, ×40.

EF antagonism reduces PPE-induced accumulation of alveolar macrophages in vivo. Pancreatic elastase causes a direct proteolytic injury to the lung that results in a small increase in airspace size before it is rapidly cleared. The majority of the airspace enlargement present after 21 days (~80%) is the result of the chronic inflammatory process initiated by PPE (19). Therefore, modification of this inflammatory process could result in a reduction in the amount of PPE-induced emphysema. To show the...
dependence of macrophage accumulation and airspace enlargement on the presence of EFs, we repeatedly administered BA4 or isotype control antibody i.t. to PPE-recipient mice and measured the airspace dimensions (CL) and alveolar tissue and airspace areas after 21 days. An additional control group received PBS only at each instillation. BA4 treatment resulted in complete protection from the development of emphysema, with the CL being the same as that in PPE-treated mice after 24 hours, or the part of the injury that is the result of direct proteolysis (Figure 4).

Discussion
Accumulation of macrophages in the interstitium, septum, and alveolar airspace of the lung is one of the characteristic features of pulmonary emphysema. Over 20 years ago, 2 separate reports by Hunninghake et al. and by members of our group (R.M. Senior and R.P. Mecham) demonstrated that EFs possessed chemotactic properties for monocytes in vitro, but, notably, not for mature alveolar macrophages or neutrophils (14–16). That work fueled speculation that EFs may play a role in the recruitment of monocytes to the lung in emphysema, given that the lung represents a significant source of elastin. Although mature, cross-linked elastic fibers are not chemotactic, cryptic sequences of the protein may be released within the ECM upon proteolysis by elastolytic enzymes, such as MMP-12 or neutrophil elastase (NE). Despite these well-characterized chemotactic properties for monocytes in vitro, a role for EFs in vivo has been elusive, partly due to the absence of chemotactic factors that were generated only in the MMP-12+/+ mice. We were able to detect only fragments of elastin, a known substrate for MMP-12 (20), in chemotactic fractions of bronchoalveolar lavage (BAL). Inhibition of monocyte chemotaxis using BA4 confirmed the dependence of this chemotactic activity on EFs. EF inhibition is relevant in vivo as well, given that BA4 administration concomitant with cigarette smoke exposure significantly reduced the accumulation of lung macrophages.

To demonstrate that EF-driven macrophage accumulation can inhibit the development of emphysema, we turned to the PPE model of emphysema. The PPE model, used to reliably generate experimental emphysema for decades, has been criticized in the past for rapidly producing airspace disease via direct proteolytic
numerous other EFs are capable of binding to the EBP. In addition to elastin, is the most well-recognized ligand for this receptor, hexapeptide VGVAPG, a commonly repeated sequence in human PPE-induced lung macrophage accumulation by 65% and 90% at present in the BA4 group being similar to that present 24 hours after the administration of PPE, or the part of the injury that is the direct result of PPE-mediated proteolysis. EF antagonism reduced PPE-induced lung macrophage accumulation by 65% and 90% at 3 and 21 days, respectively. Prolonged treatment with BA4, therefore, achieves a more complete reduction in macrophage number, but there certainly exists an alternative mechanism that would account for the remainder. We have not been unable to detect significant increases in the C-C chemokines, so there is likely an alternate, as-yet unrecognized chemotactic factor.

EFs exert their chemotactic influence on monocytes via interaction with the elastin-binding protein (EBP) (21). Whereas the hexapeptide VGVAPG, a commonly repeated sequence in human elastin, is the most well-recognized ligand for this receptor, numerous other EFs are capable of binding to the EBP. In addition to VGVAPG, all elastin-derived peptides that follow the motif GXXPG or GXGPG (where X is a hydrophobic amino acid) display chemotaxis for monocytes in vitro (22, 23). This is noteworthy because the murine tropoelastin sequence does not contain the VGVAPG sequence; however, it does contain significant quantities of both GXXPG and GXGPG. In fact, these commonly repeated sequences represent 21% of the entire mouse tropoelastin sequence. Also of note, epitope recognition studies have demonstrated that BA4 also binds to all peptides that follow the GXXPG and GXGPG motifs (24), thus explaining why it is an effective inhibitor of the chemotactic EFs generated in the mouse. Although the monocyte chemotactic properties of EFs reside within these hydrophobic pentapeptide and hexapeptide repeats, the EFs generated in vivo tend to be much larger. In the current study, we identified EFs in the BALF from WT smoke-exposed mice in gel filtration fractions of approximately 45 kDa (the broad peak of activity ranged from 35 to 50 kDa). Prior studies have demonstrated a wide range in the size of EFs that possess monocyte chemotactic activity, from 10 to 50 kDa, that seems to depend on the type of elastin digested and the enzyme used to digest it (14–16).

Mature elastin is a complex polymer of tropoelastin monomers that have been irreversibly cross-linked by the enzyme lysyl oxidase. As a result, the mature protein is highly insoluble, and fragments generated from the polymer by proteolysis (Figure 5) are heterogeneous in size and composition (the broad band detected here is likely the result of several distinct fragments between 35 and 50 kDa, not 1 unique EF). Most contain multiple chain fragments joined through 1 or more cross-links, making their characterization by conventional proteomic approaches difficult if not impossible (25). Given the extensive prior work showing that the chemotactic properties of EFs reside within the commonly repeated penta- and hexapeptides, and that BA4 effectively inhibits them both in vitro and in vivo, it is likely that the larger peptides we identified contain the chemotactic sequences cross-linked with other regions of the protein. Furthermore, additional analysis of substrate specificities for MMP-12, within elastin, may provide insight into the unique structural features of EFs generated in vivo.

Based on our previous findings and our current study of MMP-12−/− mice, we hypothesize that alveolar macrophages become activated upon chronic exposure to cigarette smoke; that these macrophages release MMP-12 that degrades ECM components including elastin; and that the degradation of elastin results in the generation of EFs chemotactic for monocytes. The early influx of neutrophils and NE caused by cigarette smoke exposure (17, 18) also contributes to the generation of EFs and provides a possible explanation as to why NE−/− mice are partially protected from both the accumulation of macrophages and the development of airspace enlargement upon exposure to cigarette smoke (26). The interplay between macrophages and neutrophils in this process is unlikely to be limited to the generation of EFs. We have previously shown that NE is capable of degrading the tissue inhibitors of metalloproteinases (TIMPs), the major inhibitors of MMP-12. Furthermore, MMP-12 degrades α1-antitrypsin, whose major function is to inhibit NE. Thus it appears that these proteinases are able to indirectly augment each other’s function by degrading one another’s inhibitors. However, it is not surprising that the lack of MMP-12 would prevent the generation of cigarette smoke–induced EFs in our model, given that the recruitment of neutrophils (and NE) by cigarette smoke in mice is modest, when compared with macrophage accumulation. Therefore, given the sparse representation of neutrophils in the cigarette smoke–induced emphysema in our mice that are housed in a sterile barrier facility, the greater relative importance of the macrophage and its degradative enzymes is not surprising. Whereas PPE administration elicits both macrophage and neutrophil inflammation (Figure 3), the neutrophils are cleared rapidly in this setting, apparently leaving the majority of alveolar tissue destruction to macrophages, which maintain their upregulation, presumably via continued production of EFs. It is also possible that EFs induce MMP biosynthesis (27). This EF chemotactic signal ultimately leads to enhanced lung tissue destruction by placing more macrophages, and thus more MMP-12, within close proximity of the airspace. In the absence of repair, peripheral airspace enlargement is the result (28).

Although the findings here are limited to a model of emphysema, the concept that degradation products of elastin can drive a macrophage-predominant, chronic inflammatory disease process is potentially applicable to all diseases that occur in elastin-rich organs, including coronary artery disease, peripheral vascular disease, and aortic aneurysm (29). Additionally, EF antagonism represents a potential therapeutic strategy for COPD, a disease for which, to date, there are no available disease-modifying agents.

**Methods**

**Materials.** All reagents were obtained from Sigma-Aldrich unless otherwise noted. BA4 is a mouse anti-bovine IgG1-subclass mAb that was derived from an immunization with the α1-elastin fraction of insoluble elastin. It has subsequently been shown to recognize the VGVAPG hexapeptide found in human elastin, among others (24).

*Mice.* MMP-12−/− deficient mice and WT littermates were used for the experiments involving cigarette smoke exposure. The MMP-12−/− mice were generated in our laboratory as previously described (10). Briefly, intron 1 and

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exon 2 were disrupted by the insertion of the phosphoglycerokinase promoter and neomycin resistance genes (PGK-neo). The construct was electroporated into RW4 ES cells, and correctly targeted clones were microinjected into C57BL/6j blastocysts. Mice homozygous for the MMP-12 mutation were backcrossed 10 generations into the C57BL/6j background. Age- and sex-matched WT littermates were used as controls. All other experiments were carried out in C57BL/6j mice purchased from Jackson Laboratory. The Harvard Standing Committee for Animal Research at Harvard University School of Public Health approved all animal experiments.

Cigarette smoke exposure. MMP-12−/− and MMP-12+/+ mice (n = 10) were exposed to cigarette smoke from 2 unfiltered cigarettes (University of Kentucky) 6 days a week for 1, 2, 3, or 6 months (depending on the experiment) using a smoking apparatus as previously described (9). The animals tolerated the treatment without evidence of toxicity (carboxyhemoglobin levels −10%). We additionally used an acute cigarette smoke exposure protocol in which the mice were subjected to the smoke from 4 cigarettes a day for 7 days.

BALF analysis. At the conclusion of a given experiment, mice were sacrificed before performance of BAL via a 22-gauge i.v. catheter inserted into the trachea. The lungs were lavaged with 1 ml DMEM 3 times to obtain the BALF. The BALF was then centrifuged at maximum speed for 3 minutes, and the supernatant was retained for further analysis. BALF obtained in this way was subjected to gel filtration using an HPLC machine (Waters Corp.) with a HiLoad Superdex 200 column (Amersham Biosciences). Each BAL sample was loaded in a volume of 1 ml into a bed volume of 320 ml. Fractions were collected in 1-ml aliquots using a flow rate of 1 ml/min in a buffer composed of 50 mM Tris (pH 7.5), 10 mM CaCl2, and 150 mM NaCl. Using this technique, the BALF was separated into 56 fractions based on molecular weight. Each of the fractions was assayed for chemotactic properties as described below. Representative fractions (nos. 11 and 37) of the BALF (100x concentration) were then subjected to Western blot analysis using enhanced chemiluminescence. Polyclonal anti-elastin (rabbit anti-mouse) antibody at a 1:1,000 dilution was used for Western blot analysis. BA4 mAb (described above) at a 1:1,000 dilution was used to confirm these fractions. An additional fraction corresponding to a molecular weight of approximately 45 kDa was administered i.t. (see below) to demonstrate the chemotactic properties of EFs. On day 1, WT mice were anesthetized with an i.p. injection of 2.5% Avertin. After the mice were appropriately sedated, they were intubated via direct visualization of the larynx using a 22-gauge i.v. catheter in which the needle point had been filed down to a blunt end and was used as a stylet. One unit (7.4 μg) PPE; 10 μg or 100 μg purified digested bovine ligament elastin (i.e., EFs); 5 μg VVGAPG (all from Elastin Products Co.); the approximately 45-kDa EF (generated as described above); or PBS or Avertin buffer (n = 4–6 for each group) was administered i.t. in a total volume of 40 μl.

To demonstrate the dependence of monocyte recruitment to the lung on EFs in the PPE model of emphysema, we administered BA4 to inhibit the chemotactic properties of EFs. On day 1, WT mice were anesthetized with 2.5% Avertin and received a dose of 10 μg BA4 (n = 9). 10 μg IgG1 isotype control antibody (n = 8). 10 μg anti-collagen type I antibody (Col-β, forward primer, 5′-CTCGGAGCCTGTAAGCAGT-3′; reverse primer, 5′-TCTCGGAGCCTGTAAGCAGT-3′; MCP-1, forward primer, 5′-TTCTGGGCTGTCCTTGCAC-3′; reverse primer, 5′-GAGCCAACAGTG-GATGCT-3′; MCP-2, forward primer, 5′-CTCTGCCTTAATCTTCCAG-3′; reverse primer, 5′-CAGGGCACTCTGCTTGAAC-3′; MIP-1α, forward primer, 5′-CTGGCCTGTGCTTCTCCATAC-3′; reverse primer, 5′-CAACGATGATGTGGCCTG-3′; MIP-1β, forward primer, 5′-CTCTCCCTCTT-GCTCGTGCC-3′; reverse primer, 5′-TGCTGGTGAAGAACCCTGGA-3′; RANTES, forward primer, 5′-GCAAAGTGCTCAACTTCTGCA-3′; reverse primer, 5′-CTTGGCCGTTCTCCATGAGT-3′. Results are expressed as the fold change from non-smoke-exposed controls.

Intratracheal administration of EFs, PPE, and BA4. WT mice were anesthetized with an i.p. injection of 2.5% Avertin. After the mice were appropriately sedated, they were intubated via direct visualization of the larynx using a 22-gauge i.v. catheter in which the needle point had been filed down to a blunt end and was used as a stylet. One unit (7.4 μg) PPE; 10 μg or 100 μg purified digested bovine ligament elastin (i.e., EFs); 5 μg VVGAPG (all from Elastin Products Co.); the approximately 45-kDa EF (generated as described above); or PBS or Avertin buffer (n = 4–6 for each group) was administered i.t. in a total volume of 40 μl.

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Lung tissue preparation. At the conclusion of each experiment, the lungs were inflated to allow for further analysis. The animals were sacrificed at the appropriate time point, the RV was perfused with normal saline to remove blood, and the lungs were inflated at 25 cm H2O with 10% buffered formalin for 10 minutes via an i.t. catheter. The lungs were then removed and fixed for 24 hours before embedding in paraffin. Serial mid-sagittal sections were obtained for morphological and histological analysis.

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In addition, lung tissue homogenates were prepared from mice exposed to smoke for 2 months, by homogenization of the lungs in 5 ml of 50 mM Tris, 150 mM NaCl, and 10 mM CaCl2 buffer.

**Morphometry.** Mid-sagittal sections stained with a modified Gill’s stain were used to calculate the CLs as previously described (9). Ten randomly selected x200 fields per slide were photographed using MetaMorph (Molecular Devices). The images were analyzed using Scion Image (Scion Corp.). Airway and vascular structures were eliminated from the analysis. The images were manually thresholded. The CLs were determined in both a horizontal and a vertical plane, allowing for the calculation of alveolar airspace areas.

**Immunohistochemistry.** Alveolar, interstitial, and septal macrophages were quantified with mac-3 immunostaining (rat anti-mouse antibody at 1:1,000 dilution; BD Biosciences — Pharmingen) on mid-sagittal sections using the avidin-biotin HRP technique in which 3,3′-diaminobenzidine was the chromogenic substrate. Results are represented as the average count from 5 different high-powered fields per slide that have been corrected for millimeters of alveolar wall using the morphometric data (as described above).

**Statistics.** Data are expressed as the mean value ± SEM unless otherwise indicated. Statistical significance was determined using the Student’s t-test (2-tailed distribution with a 2-sample equal variance). P values less than 0.05 were considered statistically significant.

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