Cigarette smoking is a major factor for the development of pulmonary emphysema because it induces abnormal inflammation and a protease-rich local milieu that causes connective tissue breakdown of the lungs. As a result of its capacity to degrade lung tissue and the high risk of patients lacking α1-antitrypsin to develop emphysema, much interest has focused on neutrophil elastase (NE). Two similar neutrophil serine proteases (NSPs), cathepsin G and proteinase 3, coexist in humans and mice, but their potential tissue-destructive role(s) remains unclear. Using a gene-targeting approach, we observed that in contrast to their wild-type littermates, mice deficient in all three NSPs were substantially protected against lung tissue destruction after long-term exposure to cigarette smoke. In exploring the underlying basis for disrupted wild-type lung air spaces, we found that active NSPs collectively caused more severe lung damage than did NE alone. Furthermore, NSP activities unleashed increased activity of the tissue-destructive proteases macrophage elastase (matrix metalloproteinase-12) and gelatinase B (matrix metalloproteinase-9). These in vivo data provide, for the first time, compelling evidence of the collateral involvement of cathepsin G, NE, and proteinase 3 in cigarette smoke—induced tissue damage and emphysema. They also reveal a complex positive feed-forward loop whereby these NSPs induce the destructive potential of other proteases, thereby generating a chronic and pathogenic protease-rich milieu. (Am J Pathol 2014, 184: 2197–2210; http://dx.doi.org/10.1016/j.ajpath.2014.04.015)
related and rely on the same His-Asp-Ser triad for their catalytic activities. The enzymes coexist in man and mouse and are stored together in primary granules in fully processed, readily active forms at relatively high concentrations. NSPs are excreted on cell activation and are known to cleave, processed, readily active forms at relatively high concentrations. The enzymes coexist in man and mouse and are stored together in primary granules in fully processed, readily active forms at relatively high concentrations.

As a result of its capacity to efficiently degrade elastin and the high risk of patients lacking α1-antitrypsin to develop emphysema, much interest has focused on NE. Unlike NE, the roles of CG and PR3 in emphysema have not been clarified. Rather than investigating the pathogenic roles of individual proteases or combinations of two NSPs, we sought to generate mice deficient in all three NSPs, CG-NE-PR3 [NSP-knockout (KO)], and to expose them to long-term CS.

**Materials and Methods**

**Generation of Mice Deficient in CG, NE, and PR3**

Simultaneous deficiency of the Prtn3 and Ela2 gene cluster (129S6/SvEv), deficiency in CG (129S6/SvEv-C57BL/6J), and deficiency in NE (129S6/SvEv-C57BL/6J) were generated by targeted mutagenesis as described elsewhere. Regarding NE-PR3 double deficiency, gene targeting resulted in the deletion of exons 2 to 5 of the Prtn3 gene and exons 1 to 3 of the Ela2 gene, whereas the neighboring genes remained unchanged. NE deficiency was obtained by deleting part of intron 1 and exon 2 of the Ela2 gene, and CG deficiency resulted from the removal of a fragment containing the 3' end of exon 3, intron 3, and the 5' end of exon 4. NE-PR3−/− deficient mice and CG-deficient mice were crossbred to generate heterozygote-deficient progeny (F1). The F1 progeny were intercrossbred to generate mice deficient in NE, PR3, and CG, referred to as NSP-KO. All offspring were genotyped by PCR on tail genomic DNA using specific oligonucleotide primers (Table 1). Mouse strains were subsequently backcrossed (eight generations) on a pure C57BL6/J background. Mice were housed in a pathogen-free facility with food and water ad libitum and a 12-hour light/dark cycle.

NSP-KO mice and their wild-type (WT) littermates were further characterized by mRNA and protein levels using RT-PCR and Western immunoblot analysis. Protease activities were tested using elastin zymography or kinetic assay as described in Enzymatic Activities and Zymography.

Animal handling and procedures were approved by the Animal Studies Committee at our institution (Health and Animal Protection Office, Châlons-en-Champagne, France; Authorization number: 51-31) in accordance with the guidelines of the Federation of European Laboratory Animal Science Associations and following the European Directive 2010/63/EU on the protection of animals used in scientific procedures.

**CS Model**

Twenty-five NSP-KO mice and 27 WT littermates (8 to 10 weeks of age) were subjected to two filtered cigarettes per day [3R4F research cigarettes, University of Kentucky; total particulate matter (means ± SEM): 11.0 (0.33) mg per cigarette] for 5 days per week for 6 months in a specially designed vented nose exposure smoking chamber. Twenty-four sham and non-CS-exposed age-matched littermates were used as controls. Mice tolerated CS exposure, and the average level of serum carboxyhemoglobin after exposure to two cigarettes was approximately 8%. Two NSP-KO mice and one WT mouse died during the smoking period. At the end of the CS exposure period, the mice were sacrificed, and their bronchoalveolar lavage (BAL) fluids and lungs were processed for inflammatory and histologic analyses.

**Lipopolysaccharide and NSP Intranasal Instillation Models**

Mice were anesthetized using 50 mg/kg of ketamine hydrochloride and 5 mg/kg of xylazine hydrochloride, followed by intranasal (i.n.) administration of 10 μg of *Pseudomonas aeruginosa*−derived lipopolysaccharide (LPS) per mouse in 50 μL of phosphate-buffered saline (PBS). Two groups (n = 6 per genotype) of WT and NSP-KO mice received one dose of LPS and were sacrificed 1 day after challenge. In a parallel experiment, two additional groups of mice were i.n. instilled with LPS twice a week and were sacrificed 3 weeks after. In separate experiments, mice (n = 12 per genotype) were anesthetized, followed by i.n. challenge with 50 μL of PBS containing LPS-free NE (10 μg per mouse) or a mix of LPS-free NSPs (NE, CG, and PR3, each at 10 μg). Control mice (n = 5 per genotype) received sterile PBS. Three weeks later, mice were sacrificed and processed.

**BAL and Tissue Processing**

The mice were sacrificed, and the lungs were gently perfused with saline via the right ventricle. The trachea was exposed through a midline incision and was cannulated using a sterile 22-gauge catheter (BD Biosciences, Franklin Lakes, NJ). In all the experiments, the lungs were lavaged in situ (BAL), with 1 mL of PBS, pH 7.4, cycled in three times. Identical recoveries of BAL (700 μL per mouse) were obtained for each mouse. Total cell and differential counts were immediately performed on aliquots of BAL fluids.

Regarding mice that were exposed to CS, BAL fluids were prepared as follows: Three pools of equal volumes of cell-free BALs (two pools of eight samples and one pool of seven samples for the NSP-KO genotype and two pools of nine samples and one pool of eight samples for the WT genotype) were prepared.

Twelve NSP-KO and WT lavaged lungs were processed for histologic analysis. The remaining 11 NSP-KO and 14 WT lavaged lungs were snap frozen in liquid nitrogen for protein and RNA extraction. Three pools of equal lung protein or total RNA amounts were subsequently prepared (two pools of four aliquots and one pool of three aliquots for NSP-KO samples and two pools of five aliquots and one pool of four aliquots for WT samples). All the samples were aliquoted and stored at −80°C until use. When appropriate and to ensure equal protein loading, the total protein...
concentrations of the samples were determined using an RC DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

For histologic analysis, lungs were processed as previously described, with the following modifications. After CS exposure, each mouse was sacrificed by an i.p. injection with an overdose of pentobarbital. The trachea was exposed through a midline incision and was cannulated using a sterile 22-gauge catheter (BD Biosciences). The lungs were perfused through the right ventricle to remove blood. Next, they were inflated in situ by instilling 10% formalin at a constant fluid pressure of 25 cm for 5 minutes, ligated through the trachea, and removed. Inflated lungs were fixed for 48 hours before embedding in paraffin. Serial lung tissue sections were processed for hematoxylin and eosin staining and were examined by light microscopy.

With respect to protease i.n. instillation experiments, six NSP-KO and WT lavaged lungs were processed for histologic analysis. The remaining lavaged lungs were snap frozen in liquid nitrogen and stored at −80°C.

Morphometry

Morphometric assessment of emphysema was performed by determination of the average interalveolar distance estimated by calculating the mean linear intercept (Lm). Briefly, for each mouse, 10 random digitized images of representative hematoxylin and eosin—stained lung tissues were captured in a blinded manner by two investigators (N.G. or L.M.) using a DM750 microscope coupled to a digital camera module ICC50 (Leica Microsystems, Inc., Buffalo Grove, IL) and were analyzed using the image analysis software ImageJ version 1.33 (NIH, Bethesda, MD). Air space enlargement was quantified by measuring Lm.

Cytokine Levels

Cell-free WT and NSP-KO BAL fluids of CS-exposed mice were processed to assess the levels of various cytokines using the RayBio mouse cytokine antibody array 6 (RayBiotech, Tebu-Bio, Le Perray-en-Yvelines, France) according to the manufacturer’s instructions. Cell-free BAL fluids of CS-unexposed mice were analyzed as well. Briefly, equal volumes of cell-free BAL fluids (400 μL) were added to antibody-coated membranes, and detection of immunoreactive cytokines was performed after sequential incubations of the membranes with biotinylated anticytokine antibodies and streptavidin—horseradish peroxidase and visualization by enhanced chemiluminescence. Images were obtained using a ChemiDoc XRS imaging system (Bio-Rad Laboratories). Semiquantitative analysis by densitometry was performed on captured images using Quantity One 1-D analysis software version 4.5.2 (Bio-Rad Laboratories). Spots of interest were normalized to an internal control after subtraction of representative background sample. Cytokine antibody array assays were performed on all BAL fluid pools.

Enzymatic Activities

Lungs of mice that were sacrificed 1 day after i.n. LPS instillation were lavaged with saline, and cells (predominantly
neutrophils) were counted and aliquoted (1 × 10⁶). Lung tissue extracts of control and CS-exposed mice were prepared by homogenizing perfused and frozen lungs in 5 mL of 50 mmol/L Tris, 10 mmol/L CaCl₂, and 750 mmol/L NaCl buffer using a Tissue-Tearor (BioSpec Products Inc., Bartlesville, OK). After three successive freezing/thawing periods, centrifugation (13,000 × g, 5 minutes, 4°C), and lyophilization, aliquots with equal total lung protein concentrations were prepared.²³ Aliquots of equal volumes of cell-free BAL fluids from control and LPS-challenged mice were prepared as well.

Zymography
Aliquots of equal volumes of cell-free BAL fluids or lung tissue extracts were subjected to 10% SDS-PAGE using 3 mg/mL of elastin or 1.5 mg/mL of gelatin as substrates for zymography.²⁴ The gels were then stained with Coomassie blue and destained in 5% acetic acid and 10% methanol. Active proteases appear as transparent lysis bands at their respective molecular sizes.

Chromogenic Peptide Substrate Assay
Aliquots of neutrophils with equal numbers were further activated using LPS and formyl-methionyl-leucyl-phenylalanine and were incubated next with 0.2 mmol/L specific substrates for CG, NE, and PR3 at 37°C in a total volume of 1 mL of Tris-NaCl buffer (0.1 mol/L Tris, 1 mol/L NaCl, pH 7.4). Changes in the absorbances of the reactions were recorded every 2 minutes for 10 minutes at λ 410 nm. Absorbance values were corrected for nonspecific activity by subtracting the values of NSP-KO reactions from those of WT reactions. The chromogenic peptide substrates were N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide, and N-Boc-Ala-ONp for CG, NE, and PR3, respectively (Elastin Products Company Inc., Owensville, MO).

Congo Red—Conjugated Elastin Assay
NE, CG, or PR3 or pooled NSP (NE, CG, and PR3, each at 10 μg) was incubated overnight with 3 mg of Congo red elastin (Elastin Products Company Inc.) in 1 mL of Tris buffer (100 mmol/L, pH 8.0).²⁴ After centrifugation (1 minute/14,000 rpm), the elastolytic activity was the absorbance of the supernatant at 495 nm. Absorbances were corrected for nonspecific activity by subtracting the value of controls (Congo red elastin alone) from those of protease-treated Congo red elastin reactions.

Western Blot Analysis
Immunoblotting experiments were performed on aliquots with equal total protein amounts of bone marrow or lung tissue protein pools.²⁰,²³ The membranes were sequentially incubated with designated anti-mouse primary antibodies [anti-NE, anti-PR3, anti-CG (dilution 1:2000), and anti-myeloperoxidase (MPO; dilution 1:2000)]²⁰,²⁴ or anti—matrix metalloproteinase (MMP)-12 (dilution 1:1000)] followed by their respective secondary horseradish peroxidase—conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). When indicated, the membranes were stripped (100 mmol/L β-mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris-HCl, pH 6.7, for 30 minutes at 50°C) and immunoblotted using primary rabbit polyclonal anti-mouse albumin antibody (Rockland Immunochemicals Inc., Gilbertsville, PA) and its corresponding horseradish peroxidase—conjugated secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ).

RT-PCR and Real-Time RT-PCR
RNA Extraction
Total RNA isolation from bone marrow cells or lung tissues was performed using the MasterPure RNA purification kit (Epicentre, Illumina, Madison, WI), as described by the manufacturer’s protocol.

Figure 1 Characterization of NSP-KO mice. A: PCR genotyping of mice with disrupted CG, Ela2 (for NE), and Prtn3 (for PR3) genes (NSP-KO). Unlike the CG gene (CG), the Ela2 and Prtn3 genes are clustered on the same chromosome (NE-PR3). Mice were genotyped for the CG gene and the Ela2-Prtn3 cluster using specific primers. The primers for the Ela2-Prtn3 cluster amplify a fragment encompassing parts of both genes. Shown are representative ethidium bromide—stained agarose gel micrographs of amplified PCR fragments: WT mice with a WT CG gene (lane 1) and a WT Ela2-Prtn3 cluster using specific primers. The primers for the Ela2-Prtn3 cluster amplify a fragment encompassing parts of both genes. Shown are representative ethidium bromide—stained agarose gel micrographs of amplified PCR fragments: WT mice with a WT CG gene (lane 1) and a WT Ela2-Prtn3 cluster (lane 2); heterozygous mice for the CG gene (lane 3) and the Ela2-Prtn3 cluster (lane 4); and Homozygous mice with a mutated CG gene (lane 5) and an Ela2-Prtn3 cluster (lane 6). B: RT-PCR of equal amounts of total RNA from bone marrow of WT and NSP-KO mice. Shown is a representative ethidium bromide—stained agarose gel micrograph of amplified PCR products. Note the loss of NE, PR3, and CG expression in NSP-KO mouse. Expected amplicon sizes (Bp) are indicated on the left. C: Immunoblotting on equal protein amounts of WT and NSP-KO bone marrow lysates using specific antibodies to NE, CG, PR3, and MPO. Shown is a representative Western blot micrograph. MPO was used for internal control. No immunoreactive bands were detected for NE, CG, and PR3 in NSP-KO bone marrow lysates. In A–C, experiments were repeated at least three times with comparable results. βACT, β-actin for internal control; M, molecular mass standards in kilodaltons.

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RT-PCR
Total bone marrow–derived RNA samples (1 μg) were reverse transcribed, and cDNAs were amplified by PCR using specific primers for Ela2 (NE), CG (CG), Prtn3 (PR3), and β-actin (internal control) (Table 1)20 (40 cycles starting with cDNA denaturation for 2 minutes at 94°C; each cycle corresponded to denaturation for 15 seconds at 94°C, primer annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds). RT-PCR products were analyzed by electrophoresis on 1.5% agarose gels and a gel documentation system using Quantity One software version 4.6.1 (Bio-Rad Laboratories).

Real-Time RT-PCR
Total lung tissue RNA was reverse transcribed into cDNA using the same procedure as used in RT-PCR. Next, real-time PCR amplification was performed using specific primers for MMP-9 and MMP-12 and normalized to glyceraldehyde-3-phosphate dehydrogenase amplicon (Table 1). PCR amplification conditions were as follows: initial DNA denaturation for 5 minutes at 94°C, primer annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, for a total 40 cycles. Data analysis was performed using the SDS software version 2.4.1 (Applied Biosystems, Foster City, CA).

Transmission Electron Microscopy
WT and NSP-KO mice were i.p. instilled with glycogen (15%) and were sacrificed 4 hours later. Aliquots of the peritoneal lavage were centrifuged, the supernatants removed, and the cells fixed in 2% glutaraldehyde and processed for electron microscopy.20

Bacterial Uptake
WT and NSP-KO mice were i.p. injected first with glycogen and 4 hours later with P. aeruginosa H103. After 30 minutes, cells were collected in gentamicin-containing Hanks’ balanced salt solution, washed, and cytopsins were prepared and Wright stained.29

Detection of Reactive Oxygen Species
The production of reactive oxygen species was examined using 3′-(p-aminophenyl) fluorescein (Molecular Probes Inc., Invitrogen, Eugene, OR) according to the manufacturer’s instructions. Glycogen-elicited peritoneal WT and NSP-KO neutrophils30 were incubated with 10 μmol/L 3′-(p-aminophenyl) fluorescein for 1 hour at 37°C. After washing with PBS (twice), the cells were incubated with 100 nmol/L phorbol-12-myristate-13-acetate for an additional 30 minutes. Fluorescence was monitored at 520 nm (with excitation at 485 nm) using a Gemini EM fluorescence microplate reader (Molecular Devices Inc., Sunnyvale, CA).

Exposure of Epithelial Cells to NSPs
Mouse alveolar cell line MLE15 [a gift from Jeffrey Wittset (University of Cincinnati, Cincinnati, OH)] was grown to

Figure 2  Absence of active CG, NE, and PR3 in cell-free BAL fluids or neutrophils of NSP-KO mice. A: Representative Coomassie blue—stained protein gel micrograph of elastin zymography on equal volumes of cell-free BAL fluids obtained 1 day after i.n. challenge of mice (n = 1 per lane) with LPS. As expected, active NSPs were detected as clear areas of lysis in WT but not NSP-KO BAL fluid. B: Representative Coomassie blue—stained protein gel micrograph showing a similar migration profile of individual (CG, NE, PR3) or pooled purified proteases (NSP). In A and B, experiments were repeated at least twice with comparable results. Representative graphs of kinetic measurements for NE (C), CG (D), and PR3 (E) activities with specific chromogenic peptide substrates using LPS and formylmethionyl-leucyl-phenylalanine–activated BAL neutrophils (10⁶ cells). Note the increased absorbance values in function of time reflecting peptide substrate cleavage in WT (solid lines) but not NSP-KO (dashed lines) neutrophils. Experiments were performed in triplicate with comparable results. Ctrl, purified NE (250 ng) as control; M, molecular mass standards in kilodaltons; OD, optical density.
confluence in culture plates (12 wells) at 37°C and 5% CO₂ in red phenol–free RPMI 1640 culture media supplemented with penicillin/streptomycin and 10% fetal calf serum. Before any treatment, cells were washed three times with sterile PBS and were cultured for 1 hour in the absence of fetal calf serum. Next, cell monolayers were cultured alone or in the presence of a designated concentration of purified NE (10 μg) or a mix of proteases (NE, CG, and PR3, each at 10 μg) for 3 hours. In parallel, NE or pooled proteases were heat denatured (5 minutes at 95°C) before addition to cells. At the end of the treatment time, culture supernatants were collected and centrifuged to remove cell debris, and aliquots were processed for the lactate dehydrogenase (LDH) activity assay. Adherent cells were trypsinized and counted.

**LDH Activity Assay**

The activity of LDH in culture media was determined by the LDH kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions. LDH catalyzes the oxidation of lactate to pyruvate. This reaction is coupled with a reduction of NAD to NADH, which is followed spectrophotometrically at 340 nm. Briefly, 100 μL of culture medium was added to 900 μL of LD-L reagent, and the LDH activity (Δ absorbance per minute) was determined.

**Statistical Analysis**

Data were analyzed using GraphPad Prism version 4.0 (GraphPad Software Inc., La Jolla, CA) and are expressed as means ± SEM. Analysis of variance was used in multigroup comparisons, with follow-up with the Tukey-Kramer test. Wilcoxon exact tests were used for the comparison of two groups. Two-sided P values are reported throughout, and a conservative significance threshold of P < 0.001 was used to account for multiple testing.

**Results**

**Mice Develop Normally in the Absence of the NSPs CG, NE, and PR3**

Mice deficient in the CG, Ela2, and Prtn3 genes (NSP-KO) were generated (Figure 1A). NSP-KO mice underwent normal embryonic and postnatal development and were fertile. Their bone marrow cells completely lacked mRNA transcripts and proteins of CG, NE, and PR3 (Figure 1, B and C). Unlike WT mice, NSP-KO mice had virtually no detectable enzymatic activity.
activities of these proteases (Figure 2). Also, analysis of transmission electron micrographs of NSP-KO bone marrow–derived neutrophils revealed normal numbers of electron-dense (azurophil) granules and normal neutrophil morphologic features (Figure 3A). The cells exhibited no defect in phagocytosis, and their respiratory burst functions as well (Figure 3, B and C).

Mice Deficient in CG, NE, and PR3 Are Protected from CS-Induced Tissue Destruction

Previously, we reported that NE contributes to CS-induced emphysema in mice. Using the same mouse-adapted smoking apparatus and experimental design, groups of adult NSP-KO mice and their WT littermates were subjected to long-term CS exposure (two cigarettes per day for 5 days per week up to 6 months). During the smoking period, both types of mice appeared grossly normal and showed no signs of distress. Of relevance, the overall lung histologic findings of age-matched sham and non—CS-exposed WT and NSP-KO mice appeared normal (Figure 4, A and B). However, WT mice exposed to CS for 6 months had enlarged alveolar spaces with obvious disrupted alveolar walls compared with their littermate controls (Figure 4, A and C). In contrast, NSP-KO mice exhibited strikingly less altered alveolar dimensions compared with controls (Figure 4, B and D). These observations were further confirmed by calculation of Lm, an estimate of the average wall-to-wall distances of alveoli. Indeed, the Lm of WT lungs increased substantially compared with the Lm of NSP-KO lungs (Figure 4E). Thus, deficiency in CG, NE, and PR3 protected the host from the lung tissue destruction associated with CS.

CG, NE, and PR3 Are Not Required for Inflammatory Cell Recruitment in Response to CS

To explore the underlying basis for the observed tissue damage in WT lungs, we examined first the inflammatory cell response of mice to long-term CS exposure. The types of recruited cells, namely, macrophages, neutrophils, and lymphocytes, in mouse lungs were similar to those reported in human smoker lungs. Also, analysis of BAL fluids showed increased total cell counts, with macrophages being the predominant cells. There was no detectable cell recruitment defect in both genotypes, and MPO levels in whole lungs showed increased but close values in both types of CS-exposed lungs compared with control mice (Figure 5, A and B, and data not shown). To further ensure that CG, NE, and PR3 are not required for immune cell migration irrespective of the type of injuring agent and/or chronicity of inflammation, mice were subjected to acute or chronic lung inflammation models. LPS, a potent virulence factor of Gram-negative bacterial walls, has been used in a variety of animal models to incite an acute or chronic inflammation (data not shown). By 24 hours after LPS i.n. instillation, BAL fluids from both types of mice showed a comparable fulminant leukocyte influx predominated by neutrophils (Figure 5C and Supplemental Figure S1A). Repetitive instillation of LPS twice per week for 3 weeks led to an important increase in leukocyte numbers, and differential cell counts indicated that macrophages represented most of the cells (Figure 5D and Supplemental Figure S1B). These counts were similar in both genotypes of mice as well. Of relevance, comparison of NSP-KO mice with their WT littermates found that unstressed mutant mice have normal circulating leukocyte numbers and differential cell counts compared with WT mice. Also, changes in the hemograms of both types of mice were indistinguishable in the setting of acute or chronic inflammation (data not shown).
shown). Together, these findings suggest that CG, NE, and PR3 are dispensable for inflammatory cell migration at least in these inflammatory lung models.

Because cytokines/chemokines contribute to the recruitment and/or activation of inflammatory cells, we also assessed the levels of various mediators in equal volumes of WT and NSP-KO cell-free BAL fluids using the cytokine antibody array approach. These data revealed changes in the levels of a variety of mediators in the absence of NSPs (Supplemental Figure S2). These cytokine profiles were similar among all BAL pools. Of note, cytokine levels in cell-free BALs of CS-exposed mice were nonsignificant regardless of mouse genotype, and their values were subtracted from those of CS-exposed cell-free BAL fluids. Overall, these changes in cytokine levels had no bearings on cell recruitment to the lungs in response to CS.

Active CG, NE, and PR3 Collectively Cause More Elastin Degradation than Does NE Alone

Next, we checked for the presence of NSPs in lung tissues. Elastin zymography and immunoblotting detected active and immunoreactive NSPs in WT but not in NSP-KO lungs (Figure 6A and data not shown). To confirm the extracellular release of free active NSPs in the setting of lung inflammation and determine their relative semiquantitative levels, WT, NE-KO, and NSP-KO mice were challenged i.n. with LPS, and equal volumes of cell-free BAL fluids were analyzed by zymography. More pronounced NSP activities were detected in WT samples compared with NE-KO samples (Figure 6B). As expected, no NSP activities were detected with NSP-KO samples. Pooled purified CG, NE, and PR3 degraded far better elastin than CG, PR3, or NE alone, further demonstrating that the proteolytic potency of CG, NE, and PR3 as a whole is more striking than individual proteases (Figure 6C). Altogether, these data strongly suggest that active CG, NE, and PR3 are excreted in response to CS and that in contrast to NE alone the three NSPs can collectively cause more lung tissue protein degradation, at least elastin.

Enhanced MMP-9 and MMP-12 Activities in the Presence of Active CG, NE, and PR3

Other elastolytic proteases have also been reported to be involved in the pathogenesis of CS-induced emphysema. Among these proteases, the MMPs macrophage elastase (MMP-12) and gelatinase B (MMP-9) are widely investigated. These data indicate that CS increased secretion/activity levels of these MMPs compared with controls (Figure 7, A and C). There was a considerably higher increase in MMP-9 and MMP-12 levels in WT lungs than in NSP-KO lungs. Previously, we and others have reported that NE has the capacity to induce mRNA expression of various effectors, including proteases, in a mouse model of acute lung inflammation. We then tested transcript levels for MMP-9 and MMP-12 by real-time RT-PCR in the CS-induced chronic lung inflammation model. These findings revealed more pronounced expression of MMP-9 and MMP-12 mRNA transcripts in WT lungs than in NSP-KO lungs concomitant with enhanced secretion/activity levels of their corresponding proteases, respectively (Figure 7, B and D).
CG, NE, and PR3 Better Mediate Severe Lung Injury than Does NE Alone

To convincingly demonstrate whether the cumulative effect of CG, NE, and PR3 better mediates severe lung injury than does NE alone and to circumvent the confounding effect of endogenous NSPs, NSP-KO mice were i.n. challenged with pooled purified CG, NE, and PR3 (NSP) or NE and were sacrificed 3 weeks later. Histologic analyses showed that NE and the NSP pool provoked lung inflammation and injury. BAL fluids of mice that received the NSP pool had a marked increase in recruited leukocytes compared with mice i.n. treated with NE alone (Figure 8, A and B). Light microscopy of tissue sections revealed air space enlargement in NE-challenged lungs compared with saline-instilled lungs (Figure 8, C and D). However, a striking widespread enlargement was observed in NSP pool-challenged lungs compared with NE-challenged lungs (Figure 8, D and E). The Lm value of NSP pool—challenged lungs was greater than that of NE-challenged lungs (Figure 8, D and E).

Figure 6 CS-exposed cell-free BAL fluids contain active NSPs that degrade elastin. A: Elastin zymography showed active NSPs in CS-exposed WT but not NSP-KO lungs. A representative Coomassie blue—stained zymography micrograph. Each lane represents a pool of equal amounts of proteins from lung tissue homogenates. The results were reproducible in all other pools of each genotype. B: Elastin zymography showed more pronounced elastolytic activity in WT cell-free BAL fluid than in NE-KO cell-free BAL fluid. Samples were obtained 24 hours after LPS i.n. challenge of mice. No lytic band was observed in NSP-KO BAL fluid. NSP, purified NE (250 ng), was used as control. Experiments were repeated at least twice with similar findings. C: Pooled proteases degraded elastin far better than individual proteases. There was more marked degradation of Congo red—conjugated elastin by pooled CG, NE, and PR3 (+NSP) than by CG (+CG), PR3 (+PR3), or NE (+NE) alone. The analysis of variance omnibus test (five groups: Congo red—conjugated elastin alone (Ctrl), CG, NE, PR3, and NSP) was significant (P < 0.0001), as were all pairwise comparisons (all P < 0.0001, Tukey-Kramer test). Data are given as means ± SEM of two independent experiments performed in triplicate. ***P < 0.001. M, molecular mass standards in kilodaltons; OD, optical density.

Figure 7 Increased lung levels of MMP-9 and MMP-12 in the presence of active NSPs after long-term exposure to CS. Enhanced MMP-9 gelatinolytic activity (A) and mRNA expression (B) in WT lungs compared with NSP-KO lungs in response to CS. There was a strong immunoreactive band (C) and increased mRNA expression (D) for MMP-12 in WT tissues compared with NSP-KO tissues in response to CS. In A and C, each lane represents a pool of equal amounts of proteins from lung tissue homogenates. Also, zymography and Western blot results were reproducible in all other pools of each genotype. In B and D, changes in gene expression were analyzed by real-time RT-PCR normalized to glyceraldehyde-3-phosphate dehydrogenase. Results are expressed as fold increase of MMP mRNA expression in CS-exposed lungs compared with unexposed lungs. The Wilcoxon exact test revealed significant statistical differences between WT and NSP-KO lungs for MMP-9 (P = 0.00534) and MMP-12 (P = 0.005). Data shown in each lane correspond to one pool of equal RNA amounts from CS-exposed WT or NSP-KO lung tissue homogenates. Similar data were obtained in the other two pools of each genotype. Data represent the means ± SEM of four values per genotype. **P < 0.01. Ctrl, control; M, molecular mass standards in kilodaltons.
similar trend in terms of cell influx and tissue injury was also observed in WT and NE-KO mice after i.n. instillation of NSPs (Figure 9).

CG, NE, and PR3 More Markedly Disrupt the Cultured Epithelial Monolayer than Does NE Alone

Next, the observed air space enlargement after i.n. instillation of proteases in mice prompted us to examine the effect of active NSPs on the integrity of alveolar epithelial cells in a cell culture system. As shown in Supplemental Figure S3A, the untreated cell monolayer exhibited tight cell-cell junctions. The addition of proteases resulted in the appearance of patchy gaps in the monolayer. However, there was more marked monolayer disruption with pooled NE, CG, and PR3 than with NE alone. These changes coincided with greater increases in LDH activity in conditioned cell media and decreases in adherent cell numbers in cultures treated with pooled NSPs than in NE alone (Supplemental Figure S3B and C).

Discussion

Herein, we show that deficiency in CG, NE, and PR3 resulted in the protection of mice against CS-induced emphysema. This protection seems to be more pronounced than that seen in mice deficient in NE only. First, comparison of NSP-KO mouse data with those previously reported about NE-KO mice found that the absence of CG and PR3, in addition to NE, prevented further lung air space enlargement in response to CS (Supplemental Table S1). We then went on to provide compelling evidence that the i.n. instillation of the three proteases clearly precipitates lung tissue destruction commensurate with that produced by NE but with greater severity. Of importance, the concentrations of purified CG, NE, and PR3 that were used are relevant because they can be easily reached if not exceeded in inflamed lungs. It must be emphasized that, to our knowledge, this is the first in vivo study to demonstrate the collateral involvement of CG, NE, and PR3 in CS-induced pulmonary injury and emphysema development.

Because of their ability to degrade various structural proteins, to process mediators to become chemotactic factors, or to activate other proteases in vitro, it has been suggested that NSPs may pave the path for immune cell migration to inflamed tissues. In vivo studies using models of acutely or chronically inflamed lungs demonstrated, however, that the recruitment of inflammatory cells is similar in the presence or absence of NSPs. Also, unlike the previously published study that reported NE contribution to the recruitment of macrophages in response to CS, NE deficiency in NSP-KO mice has no bearing on

Figure 8  Pooled CG, NE, and PR3 collectively causes more lung inflammation and tissue damage in NSP-KO mice than does NE alone. A: Total cell counts and cytospins of BAL fluids 3 weeks after i.n. instillation of NSP-KO mice with purified NE or pooled CG, NE, and PR3. Note the significant increase in BAL cell counts in mouse lungs challenged with pooled proteases (NSP) compared with NE alone. Control mice were i.n. instilled with PBS. The analysis of variance omnibus test for cell counts was highly significant (P < 0.0001). All pairwise follow-up comparisons using the Tukey-Kramer test were significant as well (all P < 0.0001). B: Representative cytospin micrographs from NSP-KO BAL fluids. Lungs that received pooled proteases (NSP) had increased cellularity compared with those that received NE alone. Resident macrophages in BAL fluid of control mice that received PBS. A similar cell pattern was observed in all other BALs. C–E: Protease instillation leads to damaged NSP-KO lung tissues. Shown are representative micrographs of mouse lung tissues after i.n. administration of saline (PBS), purified NE (NE), or pooled CG, NE, and PR3 (NSP). Note the striking damage with the pooled proteases (E) compared with NE alone (D). Similar profiles were observed in the lungs of all other treated mice. Also shows the Lm values of NSP-KO lungs. The analysis of variance omnibus test was highly significant (P < 0.0001), as were all pairwise follow-up comparisons using the Tukey-Kramer test (all P < 0.00001). Data are given as means ± SEM per group of i.n. instilled NSP-KO mice with PBS (n = 5), NE (n = 12), and pooled NSPs (n = 12) (A). Lm values represent means ± SEM per group of i.n. instilled NSP-KO mice with PBS (n = 5), NE (n = 6), and pooled NSPs (n = 6) (C–E). **P < 0.001. Scale bar = 150 μm (C–E). Original magnification, ×200 (B).
inflammatory cell egress. Consistent with the present data, various reports indicate that the absence of NE and/or CG does not impair cell migration in inflammatory situations.\textsuperscript{21,31,48} The present work also highlights for the first time that PR3 may not be required either. Whether CG, NE, and PR3 cross talk to fine-tune inflammatory cell migration merits further investigation, especially now that mice deficient in one, two, or all the NSPs are now available.

With respect to cytokine/chemokine profiles, the changes in their protein levels (increase or decrease) could be explained, in part, by the ability of NSPs to modulate the expression of their corresponding mRNA transcripts. In support of these observations, we recently reported that NE has the capacity to alter the expression of various inflammatory mediators by a mechanism involving, at least in part, Toll-like receptor 4.\textsuperscript{27} The relative contribution of CG and PR3 to this process and associated mechanisms remain to be determined. It must be emphasized that mouse gene targeting allowed us to demonstrate that NSPs contribute to changes in the levels of various mediators in this CS model where other host and/or CS-derived molecules are certainly implicated as well. Probably, the net effect of increased and decreased protein levels of the various cytokines/chemokines contributes to normal inflammatory cell migration. Alternatively, cells egress to sites of inflammation in response to diverse host- and/or insulting agent–derived chemotactic agents without the need for any proteolytic activity.\textsuperscript{49,50}

**Figure 9** Cumulative effect of CG, NE, and PR3 mediates severe lung injury more than NE alone, regardless of mouse genotype. Total cell counts of BAL fluids and lung histologic findings 3 weeks after i.n. instillation of WT and NE-KO mice with purified NE or pooled CG, NE, and PR3. A: Note the significant increase in BAL fluid cell counts in mouse lungs challenged with pooled proteases (NSP) compared with NE alone. The analysis of variance omnibus test for cell counts was highly significant ($P < 0.0001$). All pairwise follow-up comparisons using the Tukey-Kramer test were significant as well (all $P < 0.0001$). B–G: Representative tissue section micrographs from WT and NE-KO lungs. As for NSP-KO lungs, PBS-challenged WT and NE-KO exhibited normal air space (B and C), whereas protease instillation led to damage of lung tissues that is more striking with pooled proteases (F and G) than with NE alone (D and E). Also shown are the $L_m$ values of WT and NE-KO mouse lungs. The analysis of variance omnibus test was highly significant ($P < 0.0001$), as were all pairwise follow-up comparisons using the Tukey-Kramer test (all $P < 0.0001$). Data are given as means ± SEM per group of i.n. instilled WT or NE-KO mice with PBS ($n = 5$), i.n. instilled WT or NE-KO mice with NE ($n = 12$), and i.n. instilled WT or NE-KO mice with pooled NSPs ($n = 12$) (A). $L_m$ values represent means ± SEM per group of i.n. instilled WT or NE-KO mice with PBS ($n = 5$), NE ($n = 6$), and pooled NSPs ($n = 6$). ***$P < 0.001$. Scale bar = 150 μm (C–E).
Although the present findings demonstrate that NSPs are not required for inflammatory cell recruitment to inflamed lungs, their contribution to CS-induced tissue damage is unquestionable. In fact, detection of active NSPs underlies an important aspect relevant to disease development. Neutrophil-derived NSPs are present in significant amounts that overwhelm the lung’s ability to neutralize them, thereby promoting damage to adjacent tissue structures more readily. In support of this, it was previously shown that CS- or host-derived oxidants and local proteases suppress the activities of NSP physiologic inhibitors, namely, z1-antitrypsin and secretory leukocyte protease inhibitor, which again conceivably enhance NSP-mediated lung tissue destruction.\(^3\)\(^5\)\(^1\)\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\) Moreover, the present data from cell-free system studies indicate that unchecked NSPs as opposed to NE alone have a greater capacity to degrade elastin, an important lung structural protein. Also, cell culture results strongly suggest that the presence of active NSPs may severely disrupt lung epithelial integrity. Such disruption could occur, at least in part, through alteration of cell-cell junction proteins known to contribute to the tight physical barrier property of the epithelial lining.\(^5\)\(^3\)\(^4\) Of interest, the increased LDH activity into the culture medium as a consequence of membrane integrity loss suggests that NSPs directly or indirectly contribute to cell death. Whether these proteases mediate cell apoptosis or necrosis merits further investigation.

In addition to NSPs, numerous studies have reported increased levels of various proteases in CS-induced emphysema and their involvement in tissue destruction.\(^1\)\(^4\) These proteases include MMPs, particularly MMP-9 and MMP-12. Importantly, the present study found that in the presence of NSPs, mRNA expression of these MMPs increased conjointly with their activities in the setting of CS-induced emphysema. Recently, we provided evidence that NE mediates the expression of cathepsin B and MMP-2 by a nuclear factor-kB—dependent mechanism, suggesting that extracellularly discharged NE shares at least the same signal transduction to induce the expression of host lung cell—derived MMP-9 and MMP-12.\(^2\)\(^7\)\(^3\)\(^5\)\(^6\) Also, given that NSPs share close catalytic activities, a similar mechanism may be at work for CG and PR3 as well. Regarding MMP-12, the present data are consistent with the previous study showing NE involvement in increased activity of this MMP in response to CS.\(^3\)\(^2\)\(^3\)\(^5\)\(^6\) In that study, such an increase was attributed to the requirement of NE for macrophage recruitment, macrophages being the predominant source for MMP-12 secretion. The present findings reveal, however, that NSPs, including NE, have no bearing on macrophage recruitment but rather induce mRNA expression of MMP-12 of these cells. The underlying mechanism of NSP-induced expression of MMP-9 and MMP-12 awaits further studies. Notwithstanding, we identified interactions between NSPs and MMP-9 and MMP-12 in that NSPs preside over a hierarchy of protease activation (ie, increased expression of MMP-9 and MMP-12 in WT but not NSP-KO mice in response to CS). In this regard, NSPs not only induce the expression of MMPs but also have been reported to inactivate their physiologic inhibitors, namely, tissue inhibitors of metalloproteinases.\(^2\)\(^3\) Of importance as well, MMP-12 and NE have the capacity to inactivate z1-antitrypsin (the major inhibitor of NE) and secretory leukocyte protease inhibitor.\(^2\)\(^3\)\(^5\)\(^2\)

This study sheds light on a potential novel mechanism in the protease-antiprotease hypothesis that leads to tissue destruction in CS-induced emphysema. It reveals that endogenous NSPs amplify the unchecked protease-rich microenvironment through their unopposed proteolytic activity and capacity to unleash the expression of other tissue-destructive proteases, including MMP-9 and MMP-12. Consequently, extracellular matrix proteins, including elastin, are degraded, leading to lung tissue destruction.

Significantly, NE was traditionally considered the key player of the NSP family in the protease-antiprotease hypothesis of emphysema pathogenesis, and therapeutic strategies aiming at inhibiting specifically this protease are continuously pursued. The present findings suggest that these dogmas may no longer be valid, at least in mice, in that the NE sisters unchecked active CG and PR3 are pathogenic proteases that most likely participate in the initiation and/or chronicity of lung inflammation and tissue injury.\(^3\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\) In fact, the inefficacy of the myriad of NE-specific inhibitors in experimental models of emphysema and/or the prospect for their use in patients further reinforce this assumption. Accordingly, because CG, NE, and PR3 share similar substrate binding clefts, the design of a tailor-made and highly optimized inhibitor(s) to target simultaneously all three NSPs may represent an attractive alternative because it may suffice to attenuate the proteolysis-mediated tissue destruction and chronic inflammation that develop with CS and/or persist long after smoking cessation.\(^3\)\(^5\)\(^6\) Such a pharmacological approach might also be beneficial to other neutrophil-associated acute or chronic diseases, including systemic vasculitis and rheumatoid arthritis, in which the overwhelming burden of CG, NE, and PR3 exceeds the level of their physiologic inhibitors.

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**Supplemental Data**

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**References**


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