Pulmonary Epithelial Neuropilin-1 Deletion Enhances Development of Cigarette Smoke–induced Emphysema

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Rationale: Cigarette smoke (CS) exposure is an important risk factor for chronic obstructive pulmonary disease; however, not all smokers develop disease, suggesting that other factors influence disease development.

Objectives: We sought to determine whether neuropilin-1 (Nrp1), an integral component of receptor complexes mediating alveolar septation and vascular development, was involved in maintenance of normal alveolar structure, and/or altered susceptibility to the effects of CS.

Methods: Transgenic mice were generated to achieve inducible lung-specific deletion of epithelial *Nrp1*. We determined whether conditional *Nrp1* deletion altered airspace size, then compared the effects of chronic CS or filtered air exposure on airspace size, inflammation, and the balance between cell death and proliferation in conditionally *Nrp1*-deficient adult mice and littermate controls. Finally, we evaluated the effects of *Nrp1* silencing on cell death after acute exposure of A549 cells to cigarette smoke extract or short chain ceramides.

Measurements and Main Results: Genetic deletion of epithelial *Nrp1* in either postnatal or adult lungs resulted in a small increase in airspace size. More notably, both airspace enlargement and apoptosis of type I and type II alveolar epithelial cells were significantly enhanced following chronic CS exposure in conditionally *Nrp1*-deficient adult mice. Silencing of *Nrp1* in A549 cells did not alter cell survival after vehicle treatment but significantly augmented apoptosis after exposure to cigarette smoke extract or ceramide.

Conclusions: These data support a role for epithelial *Nrp1* in the maintenance of normal alveolar structure and suggest that dysregulation of *Nrp1* expression may promote epithelial cell death in response to CS exposure, thereby enhancing emphysema development.

Keywords: chronic obstructive pulmonary disease; genetically modified mice; apoptosis

Chronic obstructive pulmonary disease (COPD), defined by the presence of chronic bronchitis and/or emphysema, is a progressive disorder of the airways, characterized by a gradual loss of lung function. At least 24 million adults nationally are estimated to carry this diagnosis, which has been the fourth leading cause of death in the United States since 1991 (1). The single most

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

An imbalance between pathways mediating alveolar cell proliferation and death may lead to airspace destruction and the generation of emphysema. Disruption of growth factor signaling cascades critical for lung development and postnatal homeostasis may promote the development of emphysema in response to cigarette smoke exposure.

What This Study Adds to the Field

This study suggests that loss of pulmonary epithelial *Nrp1* enhances apoptosis of Type I and II epithelial cells and airspace enlargement in response to chronic cigarette smoke exposure.

prevalent and preventable risk factor for the development of COPD is cigarette smoke (CS) exposure (2, 3), which accounts for more than 80 to 90% of the COPD cases in the U.S. (4). Only approximately 20% of smokers develop clinical evidence of COPD (5, 6), suggesting that other factors, including genetic predisposition, may influence disease development.

Neuropilin is a type I membrane protein that is highly conserved across vertebrate species (7–9). Two members of the neuropilin family have been identified, neuropilin-1 (Nrp1) and neuropilin-2 (Nrp2) (7). Both were initially identified as specific neuronal receptors for a class of secreted signaling proteins, the class 3 semaphorins (Sema3), which guide axonal growth cone collapse (7, 10–12). Neuropilins are also widely expressed in nonneuronal tissue, including epithelium and endothelium, and *Nrp1* gene expression has been described in tissue homogenate from lung, heart, placenta, skeletal muscle, kidney, pancreas, liver, and brain (13, 14).

Nrp1 was independently cloned from neurons as a receptor for Sema3 (7, 11) and from endothelium as a novel vascular endothelial growth factor (VEGF) receptor (14, 15). In the nervous system, Nrp1 mediates semaphorin-induced axonal growth cone collapse (7, 11) and is required for semaphorin-mediated neuronal (16) and neural progenitor cell apoptosis (17). In endothelium, Nrp1 enhances VEGF-mediated phosphorylation and signaling via VEGFR2 (18–20) and enhances VEGF-induced endothelial permeability (18), chemotaxis (18, 19), proliferation, and cell survival (19). Emerging data suggests that although semaphorins can modulate endothelial signaling by competitively inhibiting VEGF signaling (21), they may have independent effects on endothelial function (22, 23).

Several groups have described abundant expression of *Nrp1* in pulmonary epithelium (24, 25), yet little is known about the function of Nrp1 in epithelial cells. Nrp1 may modulate the balance between VEGF and Sema3 signaling to alter cell proliferation and death in nonepithelial cell types (17, 26). Both

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Nrp1 ligands, VEGF, and Sema3A, have independently been shown to contribute to alveolar septation (24, 27–30) and vascular patterning (23, 27, 31), and human (32, 33), animal (34–37), and *in vitro* (38) studies support a possible role for VEGF in protection from acute lung injury and preservation of alveolar cell survival (39). Of note, it was shown that expression of Nrp1 protein was reduced in the lungs from smokers with spirometric evidence of COPD compared with smokers who had normal lung function and nonsmoking control subjects (40). Because Nrp1 is part of essential receptor complexes mediating both VEGF and Sema3A signals, we hypothesized that epithelial Nrp1 might play a role in the maintenance of normal alveolar structure.

To test this hypothesis, we generated transgenic mice in which tissue-selective conditional deletion of *Nrp1* in the pulmonary epithelium could be achieved, and determined the effects of lung epithelial *Nrp1* deletion on lung morphometry during either the postnatal period or adulthood. Because this series of experiments demonstrated that loss of epithelial *Nrp1* resulted in a phenotype of mild airspace enlargement, we then evaluated whether pulmonary epithelial *Nrp1* deletion in adult mice enhanced the injurious effects of chronic CS exposure, promoting alveolar epithelial cell death and the development of emphysema in these animals. Finally, *in vitro* experiments were performed to confirm a role for *Nrp1* in alveolar epithelial cell survival. Some of the results of these studies have been previously reported in the form of abstracts (41, 42).

METHODS

Genetically Modified Mice

Animal studies were approved by the Johns Hopkins Animal Care and Use Committee. Inducible lung cell-specific deletion of *Nrp1* was achieved using the Cre-lox system (43). Details regarding the generation of mice for these experiments are provided in the online supplement.

Effects of Conditional Pulmonary Epithelial *Nrp1* Deletion in Adult Mice

Inducible pulmonary epithelial *Nrp1* deletion was achieved by administration of doxycycline-containing chow (0.625 g/kg; Harlan-Teklad, Madison, WI). To determine the effects of neonatal *Nrp1* deletion, chow was administered to CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice and littermates at Postnatal Day 7 (P7), and continued for 12 weeks before killing (*n* = 5–7/group). To assess the effects of pulmonary epithelial *Nrp1* deletion after postnatal alveolarization was complete, adult CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice and littermate controls received doxycycline-containing chow beginning at 9 to 10 weeks of age and continuing for 12 weeks before killing (*n* = 6/group).

Chronic CS Exposure

CS exposure was performed as previously described (44) and outlined in the online supplement. Briefly, at 10 weeks of age CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice (*n* = 16) and littermate controls (*n* = 17) were divided into two groups. Half of the animals of each genotype were exposed simultaneously to CS (5 h/d, 5 d/wk) and doxycycline-containing chow for 12 weeks. The remaining mice served as controls and were maintained on the same chow in a filtered air (FA) environment for the specified time period.

Morphometry

For histologic analysis, animals were anesthetized, the trachea cannulated, and the right hilum ligated, then the right lung was freeze-clamped in liquid nitrogen for biochemical studies, and the left lung was inflated for morphometry and immunohistochemical analysis (see online supplement). Lung inflation was performed with 0.5% low-melting agarose at a constant pressure of 25 cm H₂O, as previously described (45). Four-millimeter transversal sections of the left lung were then fixed *in toto* in 4% paraformaldehyde overnight and subsequently

embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin. The lung sections in each group were randomly coded, and representative images (20 per lung) were acquired with a Nikon E800 microscope (lens magnification 10×; 100–200 alveoli per field) by an investigator who was masked to the identity of the slides. Mean linear intercept (MLI), mean chord length (MCL), and surface to volume ratio (S:V) were determined by computer-assisted morphometry with MetaMorph (Molecular Devices, Downingtown, PA).

Detection of Cell Proliferation and Apoptosis after FA and CS Exposure

Cell turnover was evaluated by immunostaining lung sections with an antibody recognizing proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA), and an antibody that recognizes the cleaved (17/19 kD) fragment of activated caspase 3 (Cell Signaling Technology, Boston, MA). Lung sections were randomly coded, and the relative expression of both PCNA and active caspase 3 was determined by normalizing the number of PCNA- or caspase 3-positive cells to the total cell number, identified by nuclear staining with 4'-6'-diamidino-2-phenylindole (DAPI). At least 10 random fields (60×) were captured from the distal lung parenchyma of each mouse, and a minimum of 500 nuclei were counted for each lung. Staining was quantified by an investigator blinded to the identity of the slides relative to experimental group.

To determine the cell type undergoing apoptotic cell death, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed. After antigen retrieval, the TUNEL reaction was performed by incubating the sections with 96 μ l equilibration buffer, 2 μ l biotinylated nucleotide mix, and 2 μ l rTdT enzyme (Promega, Madison, WI) for 1 hour at 37°C followed by incubation with Alexa Fluor 647 streptavidin conjugate (Invitrogen, Carlsbad, CA) for 1 hour at room temperature. Type I and II epithelial cells were detected by coimmunostaining with hamster monoclonal anti-T1 α (kindly provided by Dr. Mary Williams) and rabbit anti-prosurfactant Protein C (Chemicon, San Diego, CA), respectively. Control sections were incubated with nonimmune IgG in place of the primary antibody. Slides were randomly coded, analyzed using a Zeiss LSM 510 META confocal microscope at 100× magnification, and five random fields from each slide were captured for analysis. Quantification was performed by an investigator masked to the identity of the slides relative to experimental group.

Protein Expression and Activity

Measurement of VEGF protein in tissue lysates was performed using a commercially available sandwich ELISA (R&D, Minneapolis, MN). Expression of *Nrp1*, VEGFR2 and phosphorylated VEGFR2 (p-VEGFR2) was determined using Western blot analysis, using primary antibodies from Santa Cruz Biotechnology (VEGFR2, p-VEGFR2), or provided by David Ginty (Nrp1 [7]). Expression of p-VEGFR2 was normalized to total VEGFR2 expression, and Nrp1 and VEGFR2 expression were normalized to expression of GAPDH on the same membranes.

Following FA or CS exposure, MMP-2 and MMP-9 activity were assessed using gelatin zymography (*n* = 3/group; see online supplement). MMP-12 activation (*n* = 4–6/group) was determined indirectly by Western blot analysis, using primary antibodies (Santa Cruz Biotechnology) that detect both full-length and cleaved MMP-12.

Lung Lavage

A separate series of adult CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice and their littermates were exposed to 12 weeks of CS or FA (*n* = 4–5/group), then lung lavage was obtained by the gentle instillation then aspiration of 0.3 ml ($\times 3$) phosphate buffered saline (37°C). Total cell count was obtained using a hemocytometer, a cytospin was performed to assess differential cell count, and the remaining fluid was centrifuged and supernatant frozen at –80°C for measurement of VEGF protein levels with a commercially available sandwich ELISA (R&D Systems, Minneapolis, MN).

Inflammatory Mediator Expression in Lung Tissue

Tissues from FA- and CS-exposed CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice and littermate controls (*n* = 6–7/group) were homogenized in

Tris buffer containing protease inhibitor cocktail (Sigma Chemical, St. Louis, MO) and phosphatase inhibitors Na_3VO_4 (100 mM) and NaF (50 mM), then protein concentration was determined (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA) by comparison with bovine serum albumin (BSA) standards. Expression of the following cytokines and chemokines (IL-1 β , IL-10, IL-12 [p40], IL-13, IL-18, IFN- γ , KC, MIP-1 α , MIP-1 β , MIP-2, MCP-1, and RANTES) was determined using the Bioplex assay system (Bio-Rad Laboratories).

Cell Culture Experiments

To confirm the effects of epithelial *Nrp1* deletion *in vivo*, *in vitro* experiments were performed in A549 cells, a type II alveolar cell line. Cells were purchased from ATCC (Manassas, VA), and maintained according to the manufacturer's instructions. *Nrp1* silencing was achieved using siRNA (Dharmacon, Thermo Scientific, Lafayette, CO) (see online supplement for further details).

Assessment of Apoptotic Cell Death *In Vitro*

The effects of *Nrp1* silencing on cell death were determined after treatment with 10% CS extract (CSE) (see the online supplement for further details), or 10 μM ceramide 8:0 (Avanti Polar Lipids, Alabaster, AL) for 24 hours. Results were normalized to cells undergoing mock transfection and vehicle treatment (fetal bovine serum-free medium for CSE, or 0.1% ethanol for ceramide) for the same duration and compared with cells transfected with nontargeting siRNA. Both CSE and ceramide were previously reported to produce endothelial and alveolar septal cell apoptosis and airspace enlargement in rodent models (44, 46) as well as endothelial and epithelial cell death *in vitro* (47–50). Apoptosis was quantified using the Apopercantage kit (Bio-color Ltd., Accurate Chemical and Scientific Corporation, Westbury, NY) following the manufacturer's instructions. Quantification of released intracellular dye was performed using a microplate fluorimeter. In addition, apoptotic nucleosomal release in cell lysates was measured using the Cell Death ELISA^{PLUS} kit (Roche Diagnostics, Indianapolis, IN). Pan-caspase inhibition was achieved using a 30-minute pretreatment with 50 μM carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (ZVAD-fmk; Promega).

Statistical Analysis

Statistical analysis was performed using the StatView program (SAS Institute, Cary, NC). For studies evaluating the effects of conditional *Nrp1* deletion in neonatal or adult mice on airspace size, comparisons between groups were made using one-way analysis of variance (ANOVA). The combined effects of conditional *Nrp1* deletion and CS exposure were evaluated by two-way ANOVA. Two-way ANOVA was also used for comparisons between groups for *in vitro* studies evaluating the effects of *Nrp1* silencing in A549 cells. When significant variance ratios were obtained, least significant differences were calculated to allow comparison of individual group means. *P* values of 0.05 or less were considered significant.

RESULTS

Effects of Pulmonary Epithelial *Nrp1* Deletion in Neonatal and Adult Mice

Conditional deletion of alveolar epithelial *Nrp1* was confirmed by immunohistochemical staining (see Figure E2 in the online supplement). Inducible deletion of pulmonary epithelial *Nrp1* in either the postnatal period (Figure E3) or adulthood (Figure 1) caused a small but statistically significant increase in airspace size. Representative morphology from each condition is shown in the top panels (Figure E3A and Figure 1A), whereas average MLI and MCL for all animals, assessed by computer-assisted morphometry, is shown in the bottom panels (Figure E3B and Figure 1B). As has been previously reported (51, 52), the presence of the CCSP-rtTA transgene in littermates resulted in airspace enlargement relative to control mice that were CCSP-rtTA negative despite similar expression of *Nrp1*. CCSP-rtTA⁺ littermates were therefore used as controls for

Figure 1. Lung morphometry after conditional pulmonary epithelial *Nrp1* deletion in adult mice. (A) Representative sections (10 $\times$) of lungs from littermate controls (top panel), CCSP-rtTA/tetO-Cre/*Nrp1*^{lox/+} (bottom left) and CCSP-rtTA/tetO-Cre/*Nrp1*^{lox/lox} (bottom right) mice treated with doxycycline chow from 6 weeks of age for 12 weeks (Bar = 100 μm). (B) Average ($\pm$ SE) mean linear intercept (MLI) and mean chord length (MCL) increased significantly in CCSP-rtTA/tetO-Cre/*Nrp1*^{lox/lox} mice after 12 weeks of conditional *Nrp1* deletion ($n = 6$ mice/group). * $P \leq 0.01$ vs. littermates; $\phi P \leq 0.01$ vs. CCSP-rtTA/tetO-Cre/*Nrp1*^{lox/+}; black bars = MLI; gray bars = MCL.

subsequent experiments. However, *Nrp1* deletion in CCSP-rtTA/tetO-Cre/*Nrp1*^{lox/lox} mice caused additional airspace enlargement, which was demonstrated by significant increases in MLI and MCL following doxycycline treatment, when compared with both littermate controls and CCSP-rtTA/tetO-Cre/*Nrp1*^{lox/+} mice. No significant inflammatory infiltrates were noted by histologic evaluation after conditional *Nrp1* deletion in either postnatal or adult animals. These data suggest that *Nrp1* plays a role in alveolar structural maintenance both in the postnatal and adult mouse.

Effects of CS Exposure and Pulmonary Epithelial *Nrp1* Deletion on Alveolar Structure

There is evidence that disruption of structural maintenance programs in the lung may predispose animals to CS-induced alveolar destruction (53, 54). Because we noted mild airspace enlargement after conditional deletion of epithelial *Nrp1* in

both neonatal and adult animals, we sought to determine whether conditional epithelial *Nrp1* deletion exacerbated the injurious effects of CS in the lung. Adult (8–9 wk of age) CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice and littermate controls were used for these experiments. Mice were exposed to CS or FA for 12 weeks. Doxycycline-containing chow was initiated 3 days before CS or FA exposure and was maintained for the duration of the exposure. As shown in Figure 2, this duration and dose of CS did not cause morphologic alterations in littermates, which is consistent with previous reports that indicate that up to 6 months of CS exposure may be required for the development of an emphysematous phenotype (44, 55). In contrast, loss of epithelial *Nrp1* significantly enhanced the development of airspace enlargement (MLI, 47.4 ± 1.7 vs.

52.4 ± 1.3 ; S:V ratio, 0.043 ± 0.002 vs. 0.039 ± 0.001 ; $P < 0.05$) in response to CS exposure for 3 months. These data convincingly demonstrate that the effects of chronic CS exposure on alveolar structure were potentiated after conditional epithelial *Nrp1* deletion.

Effects of CS Exposure and Pulmonary Epithelial *Nrp1* Deletion on Indices of Cell Proliferation and Death

Augmented apoptosis (39, 44, 46) and attenuated proliferation (56, 57) of alveolar cells were shown to be critical for the maintenance of normal alveolar structure in other animal models of emphysema. Evidence suggests that high levels of *Nrp1* may attenuate apoptosis (58). We therefore tested whether conditional *Nrp1* deletion altered the balance between CS-induced cell death and proliferation in our model, thereby leading to altered alveolar structure. Cell proliferation was assessed by immunostaining lungs for PCNA, and apoptosis was determined by immunostaining for active caspase 3 and *in situ* DNA fragmentation (TUNEL). As shown in Figure 3, a trend for fewer PCNA positive nuclei was noted in CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice exposed to both FA and CS, although these differences did not achieve statistical significance. In contrast, as shown in Figure 4, pulmonary epithelial *Nrp1* deletion caused increased CS-induced apoptosis, which was demonstrated by expression of active caspase 3. To determine the specific cell types undergoing apoptotic cell death, TUNEL staining was performed with colocalization for type I and II alveolar epithelial cell antigens and sections were analyzed using confocal microscopy. CS exposure markedly enhanced apoptosis of both type I and II alveolar epithelial cells in lungs from CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice, as shown in Figure 5.

Figure 2. Lung morphometry after cigarette smoke (CS) exposure and conditional pulmonary epithelial *Nrp1* deletion. (A) Representative histology (10 $\times$) from CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice (bottom panels) and littermates (top panels) after conditional pulmonary epithelial *Nrp1* deletion and simultaneous CS or filtered air (FA) exposure for 12 weeks. Prominent intraalveolar macrophages in CS-exposed *Nrp1*-deficient mice are highlighted (insert, lower right panel) (Bar = 100 μ m). (B) Average ($\pm$ SE), mean linear intercept (MLI) increased and surface-to-volume ratio (S:V) decreased after simultaneous pulmonary epithelial *Nrp1* deletion and CS exposure ($n = 8$ –9 mice per group). White bars = CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox}; black bars = littermates. * $P \leq 0.05$ vs. littermates; $^{\phi}P \leq 0.05$ vs. CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox}, FA.

Figure 3. Proliferating cell nuclear antigen (PCNA) immunostaining after cigarette smoke (CS) exposure and pulmonary epithelial *Nrp1* deletion. (A) Representative PCNA (green fluorescence; left panel) and nuclear 4'-6'-diamidino-2-phenylindole (DAPI) (blue fluorescence; middle panel), with merged image (right panel), following CS exposure and conditional *Nrp1* deletion (60 $\times$ magnification). (B) Relative expression (mean $\pm$ SE) of PCNA-positive nuclei, normalized to total cell number (assessed by nuclear staining with DAPI). Data were averaged from 10 random fields per mouse, and a minimum of 500 nuclei were counted for each animal. There tended to be fewer PCNA positive cells in CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice compared with littermate controls, but these differences did not achieve statistical significance. Relative expression of PCNA was not altered by CS exposure in mice of either genotype. White bars = CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox}; black bars = littermates.

Figure 4. Immunostaining for cleaved caspase 3 after cigarette smoke (CS) exposure and pulmonary epithelial *Nrp1* deletion. (A) Representative sections (60 $\times$) of lungs from littermate controls (*top panels*), and CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox} mice (*bottom panels*) after simultaneous CS or filtered air (FA) exposure and doxycycline-induced pulmonary epithelial *Nrp1* deletion for 12 weeks. Green fluorescence demonstrates cytoplasmic staining for cleaved caspase 3 (white arrows). The number of cells expressing cleaved caspase 3 was normalized to total cell number estimated by nuclear staining with 4'-6'-diamidino-2-phenylindole (DAPI) (blue fluorescence). (B) Relative expression (mean $\pm$ SE) of cleaved caspase 3, normalized to DAPI, increased following CS exposure in CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox} mice. Data were averaged from 10 random fields per mouse, and a minimum of 500 nuclei were counted for each animal. White bars = CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox}; black bars = littermates; * $P < 0.05$.

Effects of CS Exposure and Pulmonary Epithelial *Nrp1* Deletion on VEGF Expression and Signaling

Because VEGF, a *Nrp1* ligand, promotes cell survival (38, 59) and acts as a mitogen for both epithelial (60–62) and endothelial (63) cells, we measured the concentration of VEGF in both lung lavage and tissue lysates. As indicated in Figure 6 (*top panel*), levels of VEGF in lung lavage fluid did not differ significantly between conditionally *Nrp1*-deficient mice and littermates maintained in FA. However, although lavage VEGF concentration increased significantly after CS exposure in littermate control mice, this effect was lost in CS-exposed CCSPrtTA/tetOCre/*Nrp1*^{flox/flox} animals ($P \leq 0.05$). Similar trends in VEGF protein concentration were seen in lung tissue lysates,

Figure 5. Increased DNA fragmentation after cigarette smoke (CS) exposure and conditional pulmonary epithelial *Nrp1* deletion. (A) Representative images obtained using confocal microscopy following terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (white fluorescence), and coimmunostaining for type I (T1- α ; red fluorescence) and type II (SP-C; green fluorescence) alveolar epithelial cells. TUNEL-positive type I cells are marked by yellow arrows, and type II cells by white arrows. DNA fragmentation following CS exposure was seen in both type I and II alveolar epithelial cells. TUNEL positive cells were quantified after nuclear staining 4'-6'-diamidino-2-phenylindole (DAPI) (blue fluorescence). Five random fields were captured for each animal. *Top panels* = littermates; *bottom panels* = CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox}. (B) Relative expression (mean $\pm$ SD) of TUNEL positive type I and type II alveolar epithelial cells increased following CS exposure in CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox} mice. Data were normalized to values in littermate controls exposed to filtered air (FA). Black bars = FA-exposed littermates; gray bars = CS-exposed littermates; white bars = FA-exposed CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox}; striped bars = CS-exposed CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox}; * $P < 0.05$.

although lysate differences between mice did not achieve statistical significance (Figure 6, *bottom panel*). We also assayed expression of phosphorylated and total VEGFR2 in whole lung lysates, as *Nrp1* has been shown to alter VEGF signaling primarily via VEGFR2, and pharmacologic inhibition of VEGFR2 has been shown to promote apoptosis and airspace enlargement in rodents. As shown in Figure 7, expression of phosphorylated VEGFR2 was not significantly altered by tissue selective conditional deletion of *Nrp1* or CS, and similarly, total VEGFR2

Figure 6. Effects of cigarette smoke (CS) exposure and conditional pulmonary epithelial *Nrp1* deletion on vascular endothelial growth factor (VEGF) concentration. VEGF concentration (mean $\pm$ SE), measured using ELISA, in lung lavage fluid (*top panel*; $n = 4-5$ /group) and lung tissue homogenates (*bottom panel*; $n = 6-7$ /group). In littermate control mice, VEGF concentration increased significantly after CS exposure compared with filtered air (FA). In contrast, no CS-induced increase in VEGF concentration was seen in *Nrp1*-deficient mice. A similar trend was seen when VEGF concentration was measured in lung tissue homogenate, although these differences did not reach statistical significance. *Top panel*, * $P < 0.05$ vs. littermates; *bottom panel*, $\Phi P < 0.05$ vs. FA. White bars = CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox}; black bars = littermates.

expression did not differ significantly between CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox} mice and littermate controls after FA or CS exposure. As expected, under control (FA) conditions, expression of Nrp1 protein was decreased in the lung lysates from CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox} mice when compared with littermates. Also shown in Figure 7, Nrp1 expression decreased in control mice after chronic CS exposure to levels similar to those seen with conditional *Nrp1* deficiency. These data suggest that conditional deletion of *Nrp1* attenuated CS-induced increases in lung lavage VEGF protein concentration without significantly reducing VEGFR2 expression or phosphorylation.

Effects of CS Exposure and Pulmonary Epithelial *Nrp1* Deletion on Inflammation and Inflammatory Mediator Production

See the online supplement for further details.

Effects of *Nrp1* Silencing on Epithelial Cell Apoptosis *In Vitro*

To confirm a role for epithelial Nrp1 deletion in the augmentation of cell death, we used siRNA to silence *Nrp1* in A549 cells, a human alveolar type II cell line, and then evaluated the susceptibility of these cells to apoptosis. As shown in Figure 8, the silencing of *Nrp1* resulted in enhanced apoptosis after exposure of A549 cells to 10% CSE (*top panel*) or ceramide (*bottom panel*) for 24 hours. In contrast, apoptosis of vehicle-treated cells was not altered by transfection with *Nrp1* siRNA in either series of experiments. Enhanced cell death in response to both CSE and ceramide was significantly attenuated by pretreatment with the pan-caspase inhibitor ZVAD-fmk (Figure 9). Taken together, these data support a critical role for *Nrp1* in alveolar epithelial cell survival following exposure to stimuli relevant to emphysema pathophysiology.

Figure 7. Effects of conditional pulmonary epithelial *Nrp1* deletion and cigarette smoke (CS) exposure on expression of *Nrp1*, p-vascular endothelial growth factor (VEGF)R2, and VEGFR2 in lung lysates. Representative expression of Nrp1, p-VEGFR2, and VEGFR2 by Western blot analysis of lung lysates (*top panel*), and mean ($\pm$ SE) changes in expression, assessed by densitometry (*lower panel*; $n = 4-6$ /group). Expression of Nrp1 was significantly lower in lung lysate from CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox} mice after filtered air (FA) or chronic CS exposure when compared with FA-exposed littermates. Exposure of littermate control mice to chronic CS also resulted in decreased *Nrp1* expression when compared with FA. There were no significant differences detected in expression of either p-VEGFR2 or total VEGFR2 as a result of conditional pulmonary epithelial *Nrp1* deletion, or chronic CS exposure in either littermate controls or CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox} mice. 1 = FA, littermate; 2 = FA, CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox}; 3 = CS, littermate; 4 = CS, CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox}. White bars = CCSP-rtTA/tetO-Cre/*Nrp1*^{flox/flox}; black bars = littermates; * $P < 0.05$.

DISCUSSION

Cigarette smoke is the single most prevalent risk factor for the development of COPD, yet not all smokers develop this disorder (5, 6). Several hypotheses have evolved in an attempt to understand how CS causes alveolar destruction and emphysema, and particularly why certain hosts exhibit increased susceptibility to the injurious effects of CS exposure. For some time, investigators have focused on inflammation and protease/antiprotease imbalance as the mechanism underlying emphysema develop-

Figure 8. Effects of *Nrp1* silencing on (top panel) cigarette smoke extract (CSE)- and (bottom panel) ceramide-induced apoptosis of A549 cells. Apoptosis of A549 cells following 24 hours of exposure to CSE (10%; $n = 6$ /condition; top panel) or ceramide 8:0 (10 μ M; $n = 5$ /condition, bottom panel) was augmented after silencing of *Nrp1*. Shown for comparison are results in A549 cells transfected with nontargeting (NT) siRNA. In contrast, silencing of *Nrp1* had no effect on apoptosis (white bars, both panels) of vehicle-treated cells for either series of experiments. Cell death was assessed using the Apopercantage assay, and results were normalized to vehicle treated, mock-transfected cells. Results shown are mean $\pm$ SE. * $P < 0.05$ vs. vehicle; $\phi P < 0.05$ vs. NT siRNA. Top panel, white bars = vehicle; gray bars = 10% CSE. Bottom panel, white bars = vehicle; black bars = ceramide 10 μ M.

ment, and ample clinical and experimental evidence supports this concept (64, 65). Although CS causes enhanced lung inflammation with the potential for an imbalance of protease/antiprotease activity, it remains unclear why emphysema develops only after decades of smoking. Studies using knockout mice have shown that extracellular matrix proteases and inflammatory cells are required for CS-induced emphysema, which in the mouse develops after 6 months of ongoing exposure (44, 65, 66).

Because mounting evidence suggests that the apoptotic machinery is also necessary to generate an emphysema phenotype (64, 67–69), an alternative and potentially complementary hypothesis to explain host susceptibility to the injurious effects of chronic smoke exposure has evolved. This hypothesis suggests that CS-induced alveolar destruction could be exacerbated by abnormalities in pathways critical for lung development and postnatal lung homeostasis, creating an imbalance between cellular programs regulating lung injury and repair (69, 70). Several lines of investigation have supported a role for the disruption of alveolar structural maintenance in the pathogenesis of emphysema. Published literature describes spontaneous airspace enlargement in mice that are conditionally deficient for VEGF (71) or components of other growth factor signaling cascades that are active during normal lung development (72–76). Additional studies demonstrate that dysregulation of programmed cell death in the adult lung may lead to airspace enlargement in the absence of marked inflammation (39, 46, 77,

Figure 9. Effects of ZVAD-fmk on cigarette smoke extract (CSE)- or ceramide-induced apoptosis of A549 cells. Exposure to either 10% CSE ($n = 4$ /condition; top panel, gray bars = 10% CSE; black bars = 10% CSE + ZVAD; white bars = vehicle + ZVAD), or 10 μ M ceramide 8:0 ($n = 8$ /condition; middle panel, gray bars = 10% ceramide 8:0; black bars = ceramide 8:0 + ZVAD; white bars = vehicle + ZVAD) for 24 hours resulted in apoptosis, which was augmented by silencing of *Nrp1* and attenuated by pretreatment of cells for 30 minutes with the pan-caspase inhibitor ZVAD-fmk (50 μ M). Cell death was assessed using the apopercantage assay (top and middle panels), and results were normalized to vehicle-treated, mock-transfected cells. * $P < 0.05$ vs. vehicle + ZVAD, CSE + ZVAD; $\phi P < 0.05$ vs. NT siRNA (top panel). * $P < 0.05$ vs. vehicle + ZVAD, ceramide + ZVAD; $\phi P < 0.05$ vs. NT siRNA (middle panel). To confirm that *Nrp1* silencing enhanced CSE-induced apoptosis, we also evaluated cell death after exposure to 10% CSE for 24 hours by measurement of nucleosome release in cell lysates using the Cell Death ELISA^{PLUS} kit ($n = 8$ /condition; bottom panel, gray bars = 10% CSE; black bars = 10% CSE + ZVAD; white bars = vehicle + ZVAD). Results for this assay were normalized to vehicle-treated, mock-transfected cells. Results shown are mean $\pm$ SE. * $P < 0.05$ vs. vehicle + ZVAD, CSE + ZVAD; $\phi P < 0.05$ vs. NT siRNA (bottom panel).

78). It was also shown that alteration of genes associated with cellular senescence, a state characterized by impaired recovery from injury, may sensitize mice to CS-induced inflammation and emphysema (54). These data suggest that inflammation alone cannot mediate the destructive elements of cigarette smoke in

the lung or that alveolar destruction is triggered by distinct chronic functional and molecular signatures in the inflammatory processes. The present work proposes that disruption of alveolar maintenance signals linked to epithelial Nrp1 signaling exacerbates alveolar injury provoked by CS, leading to airspace enlargement.

Although several groups have described expression of *Nrp1* in alveolar epithelium (24, 25), little is known regarding its function in these cells. VEGF has autocrine effects on growth and proliferation of pulmonary epithelial cells *in vitro* and may protect against oxidant-induced apoptosis of cultured primary lung epithelial cells and epithelial-derived cell lines (38, 60). Moreover, VEGF stimulates branching morphogenesis of renal tubular epithelium in culture (61). Whether Nrp1 is required for these effects of VEGF on epithelial function is unknown. Furthermore, Sema3A, the alternate ligand for Nrp1, was shown to inhibit alveolar septation in cultured fetal lung explants in a *Nrp1*-dependent manner (24), raising the possibility that disruption of the Sema3A–Nrp1 signaling axis may be an important determinant of airspace size. Because Nrp1 is highly expressed in pulmonary epithelium and is an integral component of the cellular receptor complexes involved in both alveolar septation and vascular development, we sought to determine if epithelial *Nrp1* was required for the maintenance of normal alveolar structure and/or altered susceptibility to CS-induced emphysema. Our data from filtered air-exposed mice demonstrate mild airspace enlargement after deletion of pulmonary epithelial *Nrp1* in both postnatal and adult animals compared with littermate controls. One potential limitation of this model is that our data in *Nrp1*-sufficient CCSP-rtTA⁺ littermates corroborate previous reports of nonspecific effects of the CCSP-rtTA transgene on lung morphometry (51, 52). However, our studies revealed that pulmonary epithelial-specific *Nrp1* deletion has additional effects on morphometric measures of airspace size. We therefore believe that our data support a role for epithelial Nrp1 in the development and maintenance of normal adult alveolar structure.

More striking than the effects of conditional pulmonary epithelial *Nrp1* deletion in the FA-exposed neonatal or adult lung were the effects of chronic CS exposure in conditionally *Nrp1*-deficient mice. A potential role for *Nrp1* in CS-induced emphysema is supported by recent data demonstrating reduced expression of Nrp1 protein in the lungs of smokers with COPD compared with lungs from smokers who had normal lung function as well as nonsmoking controls (40). Consistent with this published data in human lungs, our experiments demonstrate decreased Nrp1 expression in tissue lysate from control mice exposed to CS for 3 months compared with those exposed to air. Importantly, however, we found that airspace enlargement in response to chronic smoke was significantly amplified in mice after pulmonary epithelial *Nrp1* deletion.

Because Nrp1 modulates the balance between cell proliferation and survival in other cell types (16, 17, 19, 58), we compared indices of proliferation and programmed cell death in the lungs of mice after FA and CS exposure. Overall, there tended to be fewer proliferating cells in the distal lung parenchyma of CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice than in littermate controls, but these differences were not statistically significant, and the amount of cell proliferation was not altered by chronic smoke exposure in mice of either genotype. In contrast, apoptosis was markedly enhanced in conditionally *Nrp1*-deficient mice exposed to CS compared with FA controls and CS-exposed littermates. Our *in vivo* colocalization data suggest that *Nrp1* deletion enhanced the susceptibility of both type I and type II alveolar epithelial cells to CS-induced

programmed cell death. Furthermore, *in vitro* experiments confirmed that *Nrp1* silencing significantly augmented apoptosis after acute exposure of A549 cells to either CSE or ceramide (a proposed mediator of airspace remodeling in emphysema [46]), whereas reduction of *Nrp1* using RNA interference did not enhance cell death in vehicle-treated cells. Taken together, these findings suggest that Nrp1 modulates alveolar epithelial cell survival after exposure to oxidative stressors.

Because disruption of VEGFR2 signaling has been shown to enhance alveolar cell apoptosis and alveolar destruction (39, 46, 77), one could speculate that the effects of pulmonary epithelial *Nrp1* deletion are secondary to altered VEGF signal transduction. Our data suggest that lavage VEGF concentration increased in response to CS in littermate control mice. This finding is consistent with the study of Wright and colleagues (79), demonstrating time-dependent up-regulation of VEGF expression from the lungs of smoke-exposed guinea pigs. In contrast, chronic CS exposure had no effect on VEGF concentration in lavage from conditionally *Nrp1* deficient mice. Higher VEGF concentrations after smoke exposure might be predicted to enhance cellular proliferation, or limit apoptosis, in response to injury. It is therefore reasonable to pose a causal link between lower VEGF concentration and increased apoptosis in the alveolar epithelium. However, the effects of VEGF on cell proliferation and survival (15, 30, 39, 60, 61) and airspace enlargement (39, 46, 77) in previous studies were primarily mediated through VEGFR2, and tissue levels of phosphorylated VEGFR2 were not significantly decreased after chronic smoke exposure and conditional epithelial *Nrp1* deletion. Although VEGFR2 protein expression measured in whole lung lysates may not reflect changes in a specific cell compartment, this suggests that changes in lavage VEGF concentration may not explain differences in alveolar epithelial cell death. Because epithelial cells are the primary source of VEGF in lung lavage, an alternative explanation linking conditional epithelial *Nrp1* deletion and decreased lavage VEGF concentrations in CS-exposed CCSP-rtTA/tetO-Cre/*Nrp1*^{fllox/fllox} mice could be that enhanced epithelial cell death decreases VEGF production. Furthermore, although several investigators have demonstrated that the effects of Nrp1 on cell proliferation and death may depend on how this receptor modulates the balance between VEGF and Sema3 signaling (17, 26), other studies also demonstrate that Nrp1 may alter cell survival independent of its effects on VEGFR activation (22, 80–82).

Lung lavage inflammatory cell type and number were not significantly altered by conditional pulmonary epithelial *Nrp1* deletion, although histology suggested a possible mild macrophage accumulation in alveoli. Differences in smoke-induced MMP activity and inflammatory mediator production that resulted from conditional *Nrp1* deficiency were subtle. It is possible that alterations in alveolar maintenance due to disruption of Nrp1 signaling leads to secondary, mild, smoke-induced inflammation, which could play a contributory role to alveolar destruction in this model. Alternatively, it is known that phagocytosis of apoptotic cells by tissue macrophages is associated with minimal proinflammatory cytokine release. A possible explanation for the presence of macrophage infiltration in CS-exposed *Nrp1*-deficient mice, but not in CS-exposed littermates, would be activation, or possible dysregulation, of normal apoptotic clearance pathways (83, 84).

In summary, our data support a role for pulmonary epithelial Nrp1 in the maintenance of normal alveolar structure. Conditional deletion of *Nrp1* in lung epithelial cells exacerbated the injurious effects of chronic CS exposure, promoting the development of apoptosis of Type I and Type II alveolar epithelial

cells, and airspace enlargement. *In vitro* experiments confirmed that loss of *Nrp1* augmented alveolar epithelial cell death following acute exposure to cigarette smoke extract and short chain ceramides. Overall, these data support the hypothesis that dysregulation of key developmental signal transduction pathways in the adult lung may alter the balance between cell injury and repair in response to toxic exposures such as cigarette smoke, thereby accelerating airspace enlargement and the development of emphysema.

Conflict of Interest Statement: A.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.T.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.B. received \$5,001 to \$1,000 in consultancy fees from Merck; up to \$1,000 in lecture fees from Novartis; and more than \$100,000 in industry-sponsored grants from Quark Pharmaceuticals. R.M.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.M.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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