Cigarette Smoke-Induced Lung Endothelial and Alveolar Epithelial Injury

Cigarette smoke exposure aggravates air space enlargement and alveolar cell apoptosis in Smad3 knockout mice

Laszlo Farkas,1,2,3 Daniela Farkas,1,2,3 David Warburton,4 Jack Gauldie,1 Wei Shi,4 Martin R. Stampfli,1,2 Norbert F. Voelkel,3 and Martin Kolb1,2

1Departments of Medicine, Pathology, and Molecular Medicine, McMaster University, Hamilton; 2Firestone Institute for Respiratory Health, St. Joseph’s Healthcare, Hamilton, Ontario, Canada; 3Virginia Commonwealth University, Richmond, Virginia; and 4The Saban Institute, Developmental Biology Program, Los Angeles Children’s Hospital, Los Angeles, California

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Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory lung disease characterized by a spirometric obstructive lung function pattern that is not fully reversible (25, 33). Although COPD imposes a major burden on our society, there is no specific therapy available (41). Pulmonary emphysema shows a progressive destruction of the peripheral lung architecture with loss of alveolar walls and air space enlargement (40a). One current pathogenic concept suggests that emphysema develops from chronic lung inflammation, and associated imbalance of proteases and antiproteases; this concept is particularly supported by the prevalent emphysema in smokers with hereditary α1-antitrypsin deficiency (41). Clearly, cigarette smoke (CS) is the single most important and preventable risk factor in the development of emphysema (14, 49). Nevertheless, only ~20% of cigarette smokers develop clinically detectable airflow limitation and symptoms of COPD, strongly suggesting genetic susceptibility factors (40, 41). Over the past 10 years, increasing evidence has been generated that apoptosis of alveolar septum cells is involved in alveolar destruction and formation of emphysema (20, 21, 31, 51). Several studies have established a link between impaired signaling pathways, postnatal lung structure abnormalities, and increased apoptosis of alveolar wall cells. In emphysema, this is frequently associated with impairment of VEGF gene expression and VEGF-related signaling (8, 20, 21, 23, 31, 34, 44, 50). In addition, autoimmune processes involving autoantibodies and specific lymphocytes have been implicated in alveolar cell apoptosis and emphysema (11, 45–47).

Transforming growth factor-β (TGF-β), a cytokine with a central role for tissue homeostasis and repair, signals via the 1) Smad, 2) MAPK, 3) phosphatidylinositol 3-kinase, and 4) JNK pathways (16, 37). After TGF-β binds to its receptors, phosphorylation of Smad2 or Smad3 results in binding to Smad4, nuclear translocation of this complex, and alteration of TGF-β target gene expression (38). We have previously shown that ubiquitous knockout of the Smad3 gene in mice leads to development of progressive air space enlargement in adult mice, associated with elevated matrix metalloproteinase (MMP) activity and diminished tissue inhibitor of metalloproteinases-1, collagen-1, and connective tissue growth factor (CTGF) expression (2). Pathologically increased lung compliance is found as a functional correlate in the Smad3+/− mice (15).

Our present study investigated whether Smad3−/− mice are more prone to accelerated development of air space enlarge-
ment following CS exposure and to what extent apoptosis of alveolar cells is involved in this process. We found a reduction in VEGF expression and activity and an increase of apoptotic alveolar wall cells in the lungs of adult Smad3−/− mice. We also found an increase in Smad2 protein expression and phosphorylation. After CS exposure, air space enlargement in Smad3−/− mice worsened further, together with increased alveolar septum cell apoptosis, and further augmented Smad2 expression and phosphorylation, with endothelial injury beginning in advance of epithelial injury and early-onset inflammation.

MATERIALS AND METHODS

Smad3 transgenic mice. Smad3−/− mice were originally generated by deletion of exon 8 of the Smad3 gene in mice of background 129SV/EV × C57BL/6 by Yang et al. (55). Breeding and genotyping were published previously (2).

Animal experiments. All procedures were approved by the Animal Research Ethics Board of McMaster University and conducted in accordance with the guidelines of the Canadian Council of Animal Care and in accordance with the American Physiological Society’s Guiding Principles in the Care and Use of Animals. Littermate Smad3+/+ mice were used as controls. Mice were housed under specific pathogen-free conditions on a 12-h light-dark cycle. Food and water were provided ad libitum.

CS exposure of female Smad3−/− and Smad3+/+ mice was performed with a whole body CS exposure system (SIU-48, Promech Lab, Vintrie, Sweden) as published previously (3). Eight-week-old mice were acclimatized over 3 days prior to CS exposure. CS exposure was done by using 12 2R4F reference cigarettes with filters removed (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY) over 50 min, twice a day for 4 days or 5 days/wk for 4 or 8 wk. Animals exposed to filtered room air were used as sham controls. The animals were euthanized by abdominal bleeding. Lungs were removed en bloc and 250 μl sterile PBS was applied twice via tracheal tube to obtain bronchoalveolar lavage (BAL) fluid (BALF). Right and left lung were used for molecular biology and histology, respectively.

BALF was centrifuged at 1,500 rpm for 10 min. Cells were counted in a hemocytometer, and cytospins were prepared and stained with a modified Wright-Giemsa staining (Sigma-Aldrich, Oakville, ON, Canada) according to manufacturer’s instructions. A total of 400 cells were counted for differential cell counts.

Lung tissue processing and morphometry. The left lung was inflated with 10% formalin at a pressure of 20 cmH2O and fixed for 24 h. Lungs were paraffin embedded, cut at a thickness of 5 μm, and stained with hematoxylin and eosin.

For immunohistochemistry or immunofluorescence stainings, 3-μm transversal sections of the left lung were deparaffinized and rehydrated. Immunohistochemistry and immunofluorescence were performed according to previously published standard protocols (9). This was followed by blocking of the endogenous peroxidase for 30 min in a freshly prepared methanol H2O2 solution. For antigen retrieval, slides were placed in a preheated steamer in 0.01 M citrate buffer (pH 6.0) for either 20 min (PCNA (no. 2586, Cell Signaling Technology, Danvers, MA), phospho-VEGF receptor-2 (pVEGFR-2, sc-101819, Santa Cruz Biotechnology, Santa Cruz, CA), and VEGF (AF-493-NAA, R&D Systems, Minneapolis, MN), or 45 min [cleaved caspase-3 (no. 9664, Cell Signaling)]. Then sections were blocked for 15 min with 1% normal swine serum (NSS) in Tris-buffered saline (TBS), followed by the primary antibody overnight at 4°C in 1% NSS-TBS. After incubation with a secondary biotin-conjugated antibody (Chemicon/Millipore Billerica, MA) for 1 h at room temperature in 1% NSS-TBS, sections were treated with streptavidin-horseradish peroxidase solution (Vector Laboratories, Burlingame, CA) at 1:600 for 45 min in 1% NSS-TBS at room temperature.

The sections were developed in diaminobenzidine chromogen substrate (Sigma-Aldrich) for 10 min and counterstained in Mayer’s hematoxylin, finally dehydrated and mounted using Vectamount permanent mounting medium (Vector Laboratories). Negative controls with nonspecific Ig were run in parallel. Pictures were taken with an Axioscope microscope, Axioimac MRc and Axiovision 3.1 software (Carl Zeiss, Toronto, ON, Canada).

TUNEL. The In Situ Cell Death Detection kit, TMR red (Roche Applied Science) was used for terminal deoxynucleotidyl transferase dUTP-mediated nick end labeling (TUNEL)/CD34, TUNEL/T1α, and TUNEL/surfactant protein-C (SP-C) double-immunofluorescence stainings according to manufacturer’s instructions. A heat-induced retrieval method in citrate buffer (20 min) was used prior to TUNEL technology with a red fluorescence dye, which was followed by indirect labeling with primary antibody [anti-CD34 (553731, BD Pharmingen, Franklin Lakes, NJ), anti-T1α (AF-3243, R&D Systems), or anti-SP-C (sc-7705, Santa Cruz)] in PBS overnight at 4°C and anti-rat FITC (for CD34, Jackson ImmunoResearch, West Grove, PA) or anti-goat FITC (for T1α or SP-C, Jackson ImmunoResearch) antibody in PBS for 4 h at room temperature. Slides were mounted with Vectashield with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Positive (DNAse-treated), and negative controls (terminal transferase omitted) were run in parallel, as well as a negative control with nonspecific IgG. Pictures were taken with an Axiosvert microscope, Axioimac MRm, and Axiovision 3.1 software (Carl Zeiss).

Analysis of histology. For mean linear intercept (MLI) measurements, 10 pictures were randomly taken from four transversal sections of the left lung for each mouse at a ×400 magnification with an Axioscope microscope, Axioimac MRc, and Axiovision 3.1 software (all Carl Zeiss), omitting large airways or vessels. MLI was calculated by dividing the number of intercepts on a diagonal line through the total length of the line. MLI measurements from 10 images were averaged for each animal. To ensure objective analysis, the picture sets were coded and analyzed by two blinded investigators using ImageJ image analysis software.

For quantification of alveolar cell proliferation and apoptosis, 10 pictures were randomly taken from four transversal sections of the left lung for each mouse at a ×400 magnification with an Axioscope microscope, Axioimac MRc, and Axiovision 3.1 software (Carl Zeiss), omitting large airways or vessels. Picture sets were coded prior to quantification. Images were loaded into ImageJ software, and a counting frame (600 × 600 pixels) was laid over each image. Cells touching the lower or left margin of the frame were excluded, whereas cells touching the upper or right margin were included. The number of positive cells inside the frame was counted by a blinded investigator. To calculate the tissue area, each image was binarized with a fixed threshold into black (tissue) and white (air space), and the tissue area was quantified from the black area of the binarized image inside the counting frame. The number of proliferating or apoptotic cells was normalized by the tissue area.

For quantification of cleaved caspase-3+ endothelial cells (ECs) in pulmonary arteries, images of 10 pulmonary arteries from four transversal sections of the left lung from each mouse were randomly acquired at a ×400 magnification with an Axioscope microscope, Axioimac MRc, and Axiovision 3.1 software (Carl Zeiss).

For quantification of double immunofluorescence staining, 5–10 image sets (red, green, and blue fluorescence filters) were randomly taken from four transversal sections of the left lung for each animal and staining. Images were coded and analyzed by an investigator blinded to treatment and genotype.

Laser capture microdissection and real-time PCR. Single lobes of the right lung were inflated with 50% optimal cutting temperature (OCT) compound-50% PBS (Tissue-Tek OCT Compound, Sakura Finetek, Torrance, CA), and frozen away fully covered with OCT. All following procedures were done strictly under RNase-free conditions:

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Sections were cut at a thickness of 8 μm in a cryostat at −20°C and stained by use of the HistoGene LCM Frozen Section Staining Kit (Arcturus, Molecular Devices, Sunnyvale, CA). An Arcturus Pixcell II LCM-microscope and CapSure LCM Caps (Arcturus) were used to dissect the lung tissue into three different compartments: airways, pulmonary arteries, and alveolar walls. After RNA isolation with PicoPure RNA Isolation Kit (Arcturus), RNA integrity was determined by a microgel bioanalyzer (Agilent Bioanalyzer 2100; Agilent, Mississauga, ON, Canada) using RNA Nano Chips (Agilent). A one-round linear amplification step with MessageAmp aRNA Amplification Kit (Ambion, Austin, TX) was performed with 800–900 ng of original RNA. The amplified RNA was used to generate cDNA through a reverse-transcrptase reaction by utilizing random hexamer primers according to standard protocols (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was done in the ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA) with gene expression assays from Applied Biosystems as described previously (9). The following assays were used: Mm00437304_m1 (Vegfa), Mm00515790_g1 (CTGF), Mm00801666_g1 (Colla1), and Mm00437762_m1 (B2m). Results were normalized to beta-2 microglobulin. Relative gene expression vs. controls was calculated by the ΔΔCt method (Applied Biosystems).

Protein isolation and Western blotting. One lobe of the snap-frozen right lung was homogenized in RIPA buffer (0.1 g tissue/ml RIPA) for protein isolation. After 30 min incubation in RIPA buffer, lung homogenate was centrifuged at 13,000 g for 10 min. Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Protein lysate was incubated for 10 min at 70°C in SDS loading buffer (Santa Cruz Biotechnology, Santa Cruz, CA), and 30 μg protein lysate was loaded into each well for SDS-PAGE. After electrophoresis, proteins were blotted onto a polyvinylidene difluoride membrane (Bio-Rad), blocked for 1 h in 5% dry milk-PBS-0.1% Tween 20 and incubated with the respective primary antibodies overnight at 4°C in blocking buffer. Secondary horseradish peroxidase-conjugated antibodies were applied for 1 h at room temperature in blocking buffer. Blots were developed with ECL reagent (PerkinElmer, Waltham, MA) on Genemate X-ray films (BioExpress, Kaysville, UT).

Statistical analysis. Data are shown as means ± SE. The following statistical tests were used: Student’s t-test (for 2 groups) and one-way ANOVA followed by Newman-Keuls multiple-comparison test (for more than 2 groups). P values less than 0.05 were considered significant. Statistical analysis and graphs were done with GraphPad Prism 5.0 (GraphPad, San Diego, CA).

RESULTS

Alveolar cell proliferation and apoptosis in naïve Smad3−/− mice. Air space enlargement developed in naïve Smad3−/− mice as previously published (2). There was no difference in the numbers of proliferating (PCNA+) cells in alveolar walls between CS-unexposed Smad3−/− and Smad3+/+ mice at different ages (Fig. 1, A–C). However, the number of apoptotic (cleaved caspase-3+) cells normalized to tissue area was increased in naïve Smad3−/− vs. Smad3+/+ mice, in particular at the ages of 8 and 20 wk, and to a lesser extent (only showing a trend), at 12 wk postnatally (Fig. 1, D–F).

Compartment-specific mRNA levels in airways, pulmonary arteries and alveolar walls. The mRNA expression of Colla1 (pro-collagen Iα1) and CTGF was significantly reduced in airway walls of naïve Smad3−/− mice at the age of 12 wk (CTGF) or 20 wk (Colla1). In pulmonary arteries, Colla1 and

Fig. 1. Proliferation and apoptosis of alveolar septal cells in naïve Smad3−/− mice. There were no changes found in the number of proliferating (PCNA−) alveolar septum cells in Smad3−/− mice: In A and B, representative images of PCNA immunohistochemistry (IHC) is shown for Smad3+/+ (A) and Smad3−/− (B) mice at the age of 8 wk. The quantification of PCNA− cells vs. tissue area in Smad3+/+ and Smad3−/− mice at the age of 8, 12, and 20 wk is shown in C. Apoptosis of alveolar wall cells was increased in Smad3−/− mice: D and E are representative images of cleaved caspase-3 IHC of Smad3+/+ (D) and Smad3−/− (E) mice at the age of 20 wk. Insets in A and E show enlarged PCNA− (A) and cleaved caspase-3+ cell (E) as highlighted in the figures. F: quantification of cleaved caspase-3+ cells vs. tissue area in Smad3+/+ and Smad3−/− mice at the age of 8, 12, and 20 wk. Each bar represents mean ± SE of 4–6 animals/group (C, F); *P < 0.05 (1-way ANOVA). Original magnification (A, B, D, E): ×400. Scale bar: 50 μm (A, B, D, E). Counterstaining with hematoxylin.
CTGF were both downregulated in untreated 12- and 20-wk-old Smad3−/− mice. In alveolar walls, Col1a1 and CTGF mRNA was decreased in Smad3−/− animals of 20 wk (Fig. 2, A and B). The mRNA expression of Vegfa (VEGF) was diminished in airways, pulmonary arteries, and alveolar walls of Smad3−/− mice (Fig. 2C). The reduction of VEGF was verified by immunohistochemistry in alveolar walls (Fig. 2, E and F). In addition, the number of pVEGFR-2 cells/tissue area was significantly decreased in alveolar walls of Smad3−/− animals at the ages of 8 and 20 wk, and to a lesser extent (nonsignificant trend) 12 wk after birth, indicating a TGFβ/Smad3 dependence of VEGF expression and activity (Fig. 2, D, G, H).

Morphometry and alveolar cell apoptosis after CS exposure. Acute CS exposure (4 days) did not affect MLI in Smad3−/− animals beyond the level of sham-treated Smad3−/− mice (Fig. 3G). In contrast, 4-wk CS exposure resulted in a significant elevation of MLI in Smad3−/− compared with CS exposed Smad3+/− and sham-treated Smad3−/− mice (Fig. 3, B, E, G). This increase was further enhanced after 8 wk of CS exposure (Fig. 3, C, F, G). CS-exposed Smad3+/− mice had no increase in MLI compared with sham Smad3+/− mice (Fig. 3G).
were higher numbers of apoptotic (cleaved caspase-3\(^+\)) alveolar wall cells in Smad3\(^{-/-}\), which further increased after 4 wk and 8 wk of CS exposure, but not after 4 days (Fig. 3H). There was a trend toward elevated alveolar wall cell apoptosis in Smad3\(^{+/+}\) after 8 wk of CS exposure (Fig. 3H).

**BAL cell counts following CS exposure.** Total cell number was moderately increased after 4 days of CS exposure in Smad3\(^{-/-}\), but neither in CS-treated Smad3\(^{+/+}\) nor in sham-treated Smad3\(^{-/-}\) or Smad3\(^{+/+}\) mice (Fig. 4A). The predominant cell types in BAL were monocytes and macrophages, although there was an elevation of neutrophil numbers in CS-exposed mice (Fig. 4, B and C). In addition, CS-exposed Smad3\(^{-/-}\) animals had elevated lymphocyte counts (Fig. 4D).

After 4-wk CS exposure, total BAL cells were dramatically increased in CS-treated Smad3\(^{-/-}\) animals, whereas total BAL cells were only slightly elevated in CS-exposed wild-type control (Fig. 4A). The predominant cell types were again monocytes and macrophages, but CS-exposed Smad3\(^{+/+}\) had a 7.5-fold increase of neutrophils compared with sham (Fig. 4, B and C). The mean BAL neutrophil count of CS conditioned Smad3\(^{-/-}\) was 37.9-fold of sham-Smad3\(^{-/-}\) and 2.6-fold of CS-treated Smad3\(^{+/+}\) (Fig. 4C). Lymphocyte numbers were

![Image of hematoxylin and eosin-stained images from sham Smad3 and CS-exposed Smad3 mice at 4 days, 4 wk, and 8 wk.](http://ajplung.physiology.org/)
generally higher in Smad3\(^{-/-}\) animals, the average lymphocyte number after 4 wk of CS exposure of Smad3\(^{-/-}\) was 6.6-fold of Smad3\(^{+/+}\) mice, whereas it was 2.8-fold of sham-treated Smad3\(^{+/+}\) (Fig. 4D).

After 8 wk, total lung BAL cell numbers were increasing in CS-exposed Smad3\(^{+/+}\), but decreasing in CS-treated Smad3\(^{-/-}\) animals (Fig. 4A). The predominant cells were monocytes and macrophages, but CS-exposed Smad3\(^{+/+}\) had an average 50.6-fold increase of neutrophils compared with sham-treated Smad3\(^{+/+}\), with total cells elevated to 1.9-fold of sham treatment (Fig. 4, B and C). After 8 wk, the average neutrophil count of CS-exposed Smad3\(^{-/-}\) was 13-fold of sham-Smad3\(^{-/-}\) and 0.35-fold of CS-treated Smad3\(^{+/+}\) (Fig. 4C). Lymphocytes in CS-exposed Smad3\(^{-/-}\) animals were similar to CS-exposed Smad3\(^{+/+}\) and sham Smad3\(^{+/+}\) (Fig. 4D).

**Western blotting.** Western blot analysis of Smad3\(^{+/+}\) and Smad3\(^{-/-}\) mice after 8 wk of sham or CS exposure confirmed that Smad3\(^{-/-}\) mice showed increased apoptosis as measured by caspase-3 cleavage in the whole lung protein lysate (Fig. 5).

**EC apoptosis.** The number of apoptotic (cleaved caspase-3\(^{+}\)) pulmonary artery ECs following acute CS exposure (4 days) increased in Smad3\(^{-/-}\), but not in Smad3\(^{+/+}\) mice (Fig. 6, A, B, G). There was no difference between sham exposed Smad3\(^{+/+}\) and Smad3\(^{-/-}\), as well as CS conditioned Smad3\(^{+/+}\) mice. After 4 and 8 wk of CS exposure, there was a trend toward elevated apoptosis of pulmonary artery ECs in CS-exposed Smad3\(^{-/-}\), but no difference between CS exposed Smad3\(^{+/+}\) and sham Smad3\(^{+/+}\) or Smad3\(^{-/-}\) animals was seen (Fig. 6G). Similarly, the number of apoptotic microvascular ECs (TUNEL\(^{+}\) CD34\(^{+}\) cells) was significantly increased in Smad3\(^{-/-}\) after 4 days of CS exposure (Fig. 6, C–F, H). Smad3\(^{+/+}\) mice showed a trend toward elevation of microvascular apoptosis after 8 wk of CS exposure (Fig. 6).

**Alveolar epithelial apoptosis.** The number of apoptotic alveolar epithelial type 1 cells (AEC1, TUNEL\(^{+}\) T1a\(^{+}\)) was significantly elevated in Smad3\(^{-/-}\) after 4 and 8 wk, but not
after 4 days, of CS exposure (Fig. 7, A–D, I). There was no statistical difference between CS-exposed Smad3+/+ and sham Smad3+/+ or Smad3−/− mice, but CS-exposed Smad3+/+ and sham Smad3−/− animals showed a trend toward higher apoptotic AEC1 counts (Fig. 7I). The number of apoptotic alveolar epithelial type 2 cells (AEC2, TUNEL+ SP-C+) was significantly increased in Smad3−/− after 8 wk, with a trend seen already after 4 wk (Fig. 7, E–H, J). These results indicate elevated alveolar epithelial cell apoptosis after 4 and 8 wk of CS exposure, in particular in Smad3−/− mice.

DISCUSSION

Emphysema is the consequence of a progressive destruction of the distal lung architecture with a loss of alveolar septa and enlargement of the air spaces (40a). The “classical” pathogenesis concept is based on the presence and activity of chronic lung inflammation that eventually leads to an imbalance between proteases and antiproteases (41). CS is by far the most prominent risk factor for the development of emphysema (49), and a genetic predisposition has been proposed, given the fact that only ~20% of cigarette smokers develop clinically relevant COPD (40, 41). Increasing evidence suggests that apoptosis of alveolar wall cells plays a critical role in the pathobiology of emphysema (20, 21, 31, 51). A number of studies have linked several signaling pathways with importance for lung development and adult lung homeostasis to emphysema through postnatal lung structure abnormalities and increased apoptosis of alveolar wall cells (8, 21, 23, 34, 44). We have previously shown that knockout of the TGF-β signaling molecule Smad3 in mice results in postnatal development of air space enlargement. This is associated with an imbalance between protease expression (being enhanced) and antiproteases (reduced), abnormalities in growth factor expression and activity, and also of extracellular matrix (ECM) components (2). Importantly, the structural alterations in the lungs of Smad3−/− mice have a significant impact on lung physiology, illustrating the relevance of this animal model (15). In the present study we tested the hypothesis that CS exposure accelerates the development of emphysema in Smad3−/− mice, and we investigated extent to which this process involves alveolar wall cell apoptosis. We indeed found remarkable alveolar cell apoptosis in the lungs of naive adult Smad3−/− mice at baseline, which was augmented following a period of up to 8 wk of CS exposure; the apoptosis was accompanied by increased air space enlargement and a further elevation of the increased Smad2 levels and phosphorylation in CS-exposed Smad3−/− mice. In wild-type mice 8-wk CS exposure was insufficient to induce structural changes.

Both structure and cellular composition of alveolar walls are significantly altered in Smad3−/− mice. These mice show
impaired alveolarization during lung development, followed by formation of centrilobular emphysema (5). In the present study, we also observed profound alveolar cell apoptosis. This is in keeping with a number of other reports based on the examination of human emphysema tissue or various animal models (4, 17, 20, 21, 23, 32, 57). One potential limitation of our study results is that 12-wk-old Smad3−/− mice showed a less pronounced alveolar cell apoptosis than 8- or 20-wk-old Smad3−/− mice. Our findings of increased Smad2 and pSmad2 levels in Smad3−/− mice points toward enhanced TGF-β signaling via Smad2 as a potential mechanism for epithelial and endothelial apoptosis (26, 54). However, it is plausible that the alveolar cell apoptosis in Smad3−/− mice may also be the result of decreased production of TGF-β/Smad3-dependent growth factors, such as VEGF, as indicated by reduced VEGF levels and decreased VEGFR-2 phosphorylation (21, 22, 29, 30). Similar to the number of apoptotic alveolar cells, we did not find a significant reduction for pVEGFR-2+ cells in 12-wk-old naive mice. But the reduction of VEGF expression and VEGFR-2 phosphorylation may also be a direct effect of epithelial and endothelial apoptosis, since these cells are the main source and targets of VEGF in the alveolar compartment (10, 12, 20, 21, 23, 50, 52).

We further demonstrate reduced mRNA levels of Col1A1 (pro-collagen 1α1) and CTGF in alveolar walls of Smad3−/−, which demonstrates that Smad3−/− mice do not only exhibit an ECM-degrading environment through increased MMP expression, but also produce less ECM proteins, making these lungs more vulnerable to alveolar injury (2, 5). Churg et al. (7) have demonstrated reduced TGF-β mRNA expression in the lung parenchyma of mice after 5 mo of CS exposure, indicating that prolonged CS exposure can result in reduced TGF-β signaling in wild-type mice.

Our present study investigated how Smad3−/− mice respond to additional damage to the lung by exposing them to CS for up to 8 wk. Several studies have demonstrated that long-term CS exposure can induce pulmonary emphysema in rodents and that alveolar cell apoptosis is involved in the development of emphysema in these studies (35, 51, 58). Reduced VEGF expression and activity has been linked to inhibitory activity of CS extract on VEGF expression in lung epithelial cells in vitro (48). Postnatal cell-specific conditional deletion of genes with importance in alveolar homeostasis can accelerate the development of emphysema following CS exposure through alveolar cell apoptosis, as has been recently shown after deletion of neuropilin-1, a putative modulator of VEGF signaling (23).
Although Le and colleagues (23) used a 12-wk exposure to CS, we were able to induce a significant amplification of air space enlargement after only 8 wk in CS-exposed Smad3−/− mice, on the background of an already damaged lung structure. The more pronounced emphysematous changes correlated with more apoptotic cells in the alveolar walls. This might also point toward premature lung senescence in Smad3−/− mice, characterized by inappropriate injury repair, which has been suggested to sensitize to CS-related emphysema (23, 56). Our quantitative assessment of alveolar endothelial and epithelial cell apoptosis revealed the interesting finding that microvascular and pulmonary artery EC apoptosis occurred early in CS-exposed Smad3−/− mice, whereas apoptosis of alveolar epithelial cells was found later after 4 and 8 wk of CS exposure, when air space enlargement was already significantly increased. Although the extent of alveolar epithelial apoptosis is less after 4 wk than after 8 wk, alveolar epithelial apoptosis may, together with increased MMP activity and reduced elastin synthesis, contribute to the more accentuated air space enlargement in CS-exposed Smad3−/− mice (2, 5). Although CD34, the marker that we used to detect alveolar capillary ECs, is also found on hematopoietic and precursor cells, several studies, including our own work, have demonstrated that CD34 can also be used in the lung to detect microvascular ECs (9, 17, 32). Nevertheless, our data indicating early EC apoptosis are in agreement with reports indicating that ECs are more susceptible to damage and that EC injury may be at the beginning of alveolar wall destruction (17, 20, 21, 43, 45, 47). One potential explanation would be the generation of ceramide following CS exposure, with the induction of early EC apoptosis, followed by epithelial apoptosis later on (24, 32). Our data also support human and animal studies indicating that pulmonary artery EC injury is implicated in the vascular pathobiology of COPD and that effects of CS on pulmonary artery endothelium and vascular abnormalities can precede the clinical signs of vascular dysfunction and pathology (1, 42). Pulmonary artery EC apoptosis might be the beginning of extensive remodeling processes in the pulmonary vasculature, likely through the release of mediators and vascular constriction (1, 9, 39). In addition, we also found elevated expression of Smad2 and augmented Smad2 phosphorylation in Smad3−/− mice, which was further enhanced by CS exposure. This is in line with previous work by Churg and colleagues (53), who showed that CS can stimulate TGF-β release and activity. Increased signaling through the Smad2 pathway (which may represent a counterregulatory mechanism) can contribute to epithelial and endothelial apoptosis (26, 54). Therefore, reduced TGF-β signaling through Smad3 seems to predispose pulmonary artery ECs to apoptotic effects of CS, whereas extensively elevated local levels of active TGF-β also result in EC injury (9). This suggests the importance of a tightly regulated balance between the different TGF-β signaling pathways for pulmonary vascular homeostasis (36).

Augmented inflammation following CS exposure is another hallmark of dysregulated injury repair (23, 56). The BAL differential cell counts revealed an interesting time course of inflammatory cell accumulation in the airway lumen of CS-exposed Smad3−/− mice: The most extensive elevation in total cell counts, macrophages, neutrophils, and lymphocytes was found after 4 wk of CS exposure. Interestingly, cell counts decreased after 8 wk CS in Smad3−/− mice, while they at the same time significantly increased in CS-exposed Smad3+/+. This observation points perhaps toward a dysregulated immune response following continuous CS challenge, which is in line with previous studies showing that TGF-β signaling via Smad3 is required for an appropriate regulation of the immune response, in particular for normal T-lymphocyte and neutrophil activity (13, 28, 55). It is also plausible that release of elastolytic enzymes from neutrophils contribute to the progression of emphysema and perpetuate inflammatory cell recruitment to the lung. Furthermore, alveolar cell apoptosis may be triggered by mediators secreted from neutrophils (41).

Because TGF-β pathways are critically important to maintain tissue homeostasis and the immune responses, mutations in the TGF-β gene or the genes encoding signaling molecules have been investigated in the context of emphysema and COPD. So far, these studies have provided contradictory results, possibly because of the heterogeneous nature of the disease entities captured under the term COPD (18, 19, 59). In Smad3−/− animals, several key components of the emphysema pathobiology are likely weakened leading to the development of air space enlargement, such as increased MMP activity, enhanced inflammation, and alveolar cell apoptosis. In normal lungs, CS exposure disrupts the alveolar maintenance program, which among other factors largely depends on VEGF expression and signaling (27). In the already damaged lungs of Smad3−/− mice, the shift of TGF-β signaling from Smad3 to Smad2 may reduce the activity of VEGF, thereby affecting both epithelial and endothelial homeostasis. Our data highlight the critical importance of a tight regulation of the distinct TGF-β pathways and their activity in the lung.

We have found an important interaction between changes in TGF-β signaling and VEGF expression and signaling in Smad3 knockout-induced air space enlargement. This finding is of considerable translational interest, given the association of reduced VEGF expression and increased alveolar cell apoptosis in human emphysema lungs (20). Our findings that altered TGF-β signaling may substantially contribute to the inhibition of angiogenesis in emphysema, and thereby promote apoptosis and alveolar destruction, may have important translational implications for designing new therapy strategies for human emphysema.

In summary, we demonstrate that impaired TGF-β signaling via the Smad3 pathway is associated with a shift toward the Smad2 pathway, increased alveolar cell apoptosis, and air space enlargement. CS exposure of Smad3−/− mice results in early-onset EC apoptosis and inflammation, enhanced alveolar cell apoptosis, and aggravated air space enlargement. EC injury occurs in advance of epithelial apoptosis. In conclusion, our data support the hypothesis that CS exposure further causes the progression of air space enlargement in an already damaged lung and that augmented alveolar cell apoptosis and inflammation are likely involved in this process.

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