

Inhibition of Allergen-Induced Airway Remodeling in Smad 3-Deficient Mice¹

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Intracellular signaling pathways that converge on Smad 3 are used by both TGF- β and activin A, key cytokines implicated in the process of fibrogenesis. To determine the role of Smad 3 in allergen-induced airway remodeling, Smad 3-deficient and wild-type (WT) mice were sensitized to OVA and challenged by repetitive administration of OVA for 1 mo. Increased levels of activin A and increased numbers of peribronchial TGF- β 1⁺ cells were detected in WT and Smad 3-deficient mice following repetitive OVA challenge. Smad 3-deficient mice challenged with OVA had significantly less peribronchial fibrosis (total lung collagen content and trichrome staining), reduced thickness of the peribronchial smooth muscle layer, and reduced epithelial mucus production compared with WT mice. As TGF- β and Smad 3 signaling are hypothesized to mediate differentiation of fibroblasts to myofibroblasts in vivo, we determined the number of peribronchial myofibroblasts (Col-1⁺ and α -smooth muscle actin⁺) as assessed by double-label immunofluorescence microscopy. Although the number of peribronchial myofibroblasts increased significantly in WT mice following OVA challenge, there was a significant reduction in the number of peribronchial myofibroblasts in OVA-challenged Smad 3-deficient mice. There was no difference in levels of eosinophilic airway inflammation or airway responsiveness in Smad 3-deficient compared with WT mice. These results suggest that Smad 3 signaling is required for allergen-induced airway remodeling, as well as allergen-induced accumulation of myofibroblasts in the airway. However, Smad 3 signaling does not contribute significantly to airway responsiveness. *The Journal of Immunology*, 2007, 178: 7310–7316.

Airway remodeling is a characteristic feature of the airways of a subset of individuals with chronic asthma (1). The profibrotic cytokine, TGF- β , and its close homolog, activin A, both of which activate cellular responses through the Smad signaling pathway, are hypothesized to play an essential role in promoting the structural changes of tissue remodeling (2). Increased TGF- β mRNA levels have been observed in bronchial biopsy sections of asthmatics compared with normal subjects, and levels of TGF- β correlate with the depth of subepithelial fibrosis (3). Likewise, increased levels of TGF- β 1 have been noted in the bronchoalveolar lavage fluid (BALF)³ of asthmatic patients (4), and levels of TGF- β are significantly increased in severe asthmatics with a prominent eosinophil influx in the airways (5).

Recent evidence also suggests a role for activin A in fibrosis. Activin A, a member of the TGF- β superfamily with structural and functional homology to TGF- β , is believed to affect the growth and differentiation of a variety of target cells. It has been impli-

cated in the inflammatory and repair processes of different organs, including cutaneous wound repair (6), liver cirrhosis (7), and pulmonary fibrosis (8), but activin A has not yet been reported to be expressed in the remodeled airway in asthma. Activin A is expressed by human mast cells and promotes the proliferation of fibroblasts and airway smooth muscle cells (9). Serum activin A levels are increased in patients with untreated moderate asthma compared with treated asthmatics or normal controls (10).

The identification of the Smad family of cytoplasmic signal transducer proteins has unraveled novel mechanisms by which TGF- β receptors signal from the cell surface to the nucleus (11). The activated TGF- β receptor complex induces phosphorylation of intracytoplasmic Smad 3 and its closely related homolog Smad 2, which then bind to the common mediator Smad 4. The Smad heterooligomers subsequently translocate from the cytoplasm to the nucleus where they direct transcriptional activities in response to TGF- β stimulation. Although activin A appears to bind its own specific receptor distinct from that of TGF- β , both activin A and TGF- β share the same intracellular Smad signaling pathway (12).

Smads have been implicated but not yet proven to play a role in airway remodeling and fibrosis in chronic asthma. In mouse models of asthma, the Smad pathway is activated following allergen challenge through phosphorylation of Smad 2 (13, 14). Smad signaling is also increased in the airways of asthmatic subjects compared with controls (15). In vitro studies using human lung fibroblasts have furthermore shown that TGF- β signaling through Smad 3 is a key event mediating fibroblast α -smooth muscle actin (SMA) expression (16), a marker of myofibroblast differentiation.

To determine the role of Smad 3 in airway remodeling, we used Smad 3-deficient mice in a mouse model of allergen-induced airway remodeling, which is associated with increased expression of TGF- β and activin A. We show that Smad 3-deficient mice have significantly decreased measures of airway remodeling (collagen deposition, smooth muscle layer, and mucus production), which is

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³ Abbreviations used in this paper: BALF, bronchoalveolar lavage fluid; AHR, airway responsiveness; Col-1, collagen-1; ECM, extracellular matrix; KO, knockout; MBP, major basic protein; MCh, methacholine; pSmad, phosphorylated Smad; SMA, smooth muscle actin.

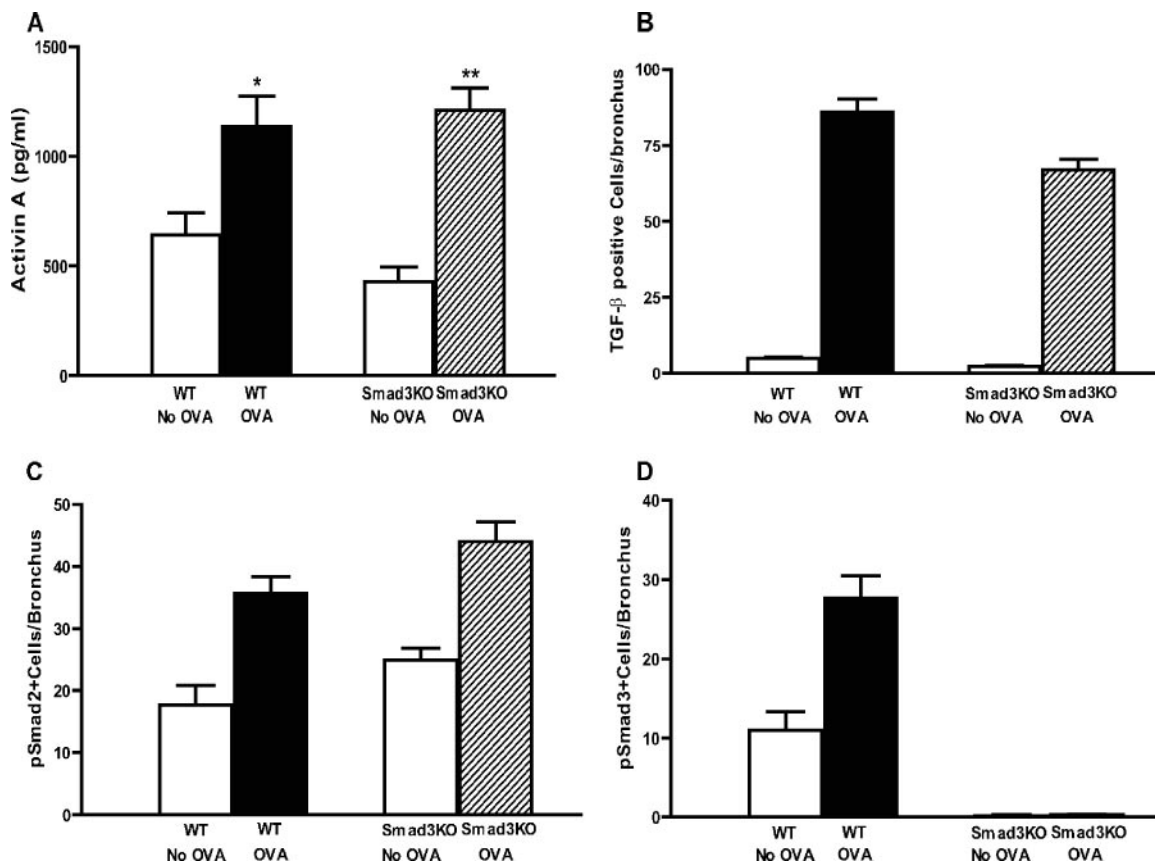


FIGURE 1. Quantitation of activin A, TGF- β 1, pSmad 2, and pSmad 3 in WT and Smad3-deficient mice repetitively challenged with OVA. **A**, Activin A. Repetitive OVA challenge induced a significant increase in the concentration of activin A (measured by ELISA) in the BALF of WT mice (*, WT OVA vs WT no-OVA; $p = 0.01$), as well as of Smad 3-deficient mice (**, Smad3 KO OVA vs Smad3 KO no-OVA; $p = 0.001$). There was no significant difference between the BALF concentrations of activin A in WT vs Smad3 OVA-challenged mice. **B–D**, TGF- β 1, pSmad2, and pSmad3. Lung sections were processed for TGF- β 1 (**B**), pSmad2 (**C**), or pSmad3 (**D**) immunohistochemistry. Repetitive OVA challenge induced a significant increase in the number of peribronchial TGF- β 1⁺ cells/bronchus in WT mice (WT OVA vs WT no-OVA; $p = 0.01$) as well as in Smad3 deficient mice (Smad3 KO OVA vs Smad3 KO no-OVA; $p = 0.01$) (**B**). There was no significant difference in the number of peribronchial TGF- β 1⁺ cells/bronchus in WT vs Smad3 OVA-challenged mice. Repetitive OVA challenge induced a significant increase in the number of peribronchial pSmad 2⁺ cells/bronchus in WT mice (WT OVA vs WT no-OVA; $p = 0.01$) as well as in Smad 3-deficient mice (Smad3 KO OVA vs Smad3 KO no-OVA; $p = 0.01$) (**C**). pSmad 3 is significantly induced in WT mice challenged with OVA (**D**). However, pSmad 3 is not detectable in either non-OVA-challenged Smad 3-deficient mice (**D**) or OVA-challenged pSmad 3-deficient mice (**D**).

associated with a significant decrease in the number of peribronchial myofibroblasts. However, despite significant reductions in airway remodeling, allergen-challenged Smad 3-deficient mice do not have significant reductions in airway responsiveness (AHR) compared with WT mice.

Materials and Methods

Mouse model of OVA-induced airway remodeling

Eight- to 10-wk-old Smad 3-deficient mice (17) ($n = 13$ OVA, $n = 9$ no OVA) and WT ($n = 11$ OVA, $n = 6$ no OVA in all experiments) on a background of C57/BL/6 (The Jackson Laboratory) were immunized s.c. on days 0, 7, 14, and 21 with 25 μ g of OVA (grade V; Sigma-Aldrich) adsorbed to 1 mg of alum (Aldrich) in 200 μ l of normal saline as described previously (18–20). Intranasal OVA challenges (20 μ g/50 μ l in PBS) were administered on days 27, 29, and 31 under isoflurane (Vedco) anesthesia. Intranasal OVA challenges were then repeated twice a week for 4 wk. Age- and sex-matched control mice were sensitized but not challenged with OVA during the study. Mice were sacrificed 24 h after the final OVA challenge, and the lungs were analyzed. Lungs from the different experimental groups were processed as a batch for either histological staining or immunostaining under identical conditions. Stained and immunostained slides were all quantified under identical light microscope conditions, including magnification ($\times 20$), gain, camera position, and background illumination. Slides were analyzed by three observers, two of whom were blinded. A blinded observer also reviewed slides scored by the unblinded

observer. The scores of the blinded observer were similar to the scores of the unblinded observer. All animal experimental protocols were approved by the University of California, San Diego Animal Subjects Committee.

Measurement of BALF levels of activin A

The concentration of activin A in BAL fluid was assayed by ELISA (R&D systems) as previously described in this laboratory (18).

Peribronchial trichrome staining

Lungs in the different groups of mice were equivalently inflated with an intratracheal injection of a similar volume of 4% paraformaldehyde solution (Sigma-Aldrich) to preserve the pulmonary architecture. The area of peribronchial trichrome staining in paraffin-embedded lungs was outlined and quantified under a light microscope (Leica DMLS; Leica Microsystems) attached to an image analysis system (Image-Pro plus; Media Cybernetics) as described previously (18–20). Results are expressed as the area of trichrome staining per micrometer length of basement membrane of bronchioles 150–200 μ m in internal diameter.

Lung collagen assay

The amount of lung collagen was measured as previously described in this laboratory (18–20) with a collagen assay kit that uses a dye reagent that selectively binds to the [Gly-X-Y]_n tripeptide sequence of mammalian collagens (Biocolor; Newtonabbey). In all experiments, a collagen standard was used to calibrate the assay.

Smooth muscle layer thickness and myofibroblast quantification

The thickness of the airway smooth muscle layer was measured with an image analysis system as described previously (18–20). In brief, the thickness of the smooth muscle layer (the transverse diameter) was measured from the innermost aspect to the outermost aspect of the smooth muscle layer. The smooth muscle layer thickness of 5–10 bronchioles of similar size (150–200 μm) was counted on each slide. Lung sections were also immunostained with both an anti- α -SMA primary Ab (Sigma-Aldrich) and an anti-Col-1 primary Ab (Chemicon International) to distinguish myofibroblasts (α -SMA positive; Col-1 positive) (21, 22) that express collagen (Col-1) from smooth muscle cells (α -SMA positive; Col-1 negative). Species- and isotype-matched Abs were used as controls in place of the primary Abs. The primary Abs were detected with two different HRP enzyme-labeled secondary Abs with signal amplification using tyramide signal amplification (Molecular Probes), according to the manufacturer's instructions. The anti- α -SMA primary Ab was detected with a HRP-labeled secondary Ab (Alexa 546, red color), whereas the anti-Col-1 primary Ab was detected with a different HRP-labeled secondary Ab (Alexa 488, green color). Cells coexpressing α -SMA and Col-1 have a yellow color.

Airway mucus expression

To quantitate the level of mucus expression in the airway, the number of periodic acid Schiff (PAS)-positive and PAS-negative epithelial cells in individual bronchioles were counted as previously described in this laboratory (18–20). Five to 10 bronchioles were counted in each slide. Results are expressed as the percentage of PAS-positive cells per bronchiole, which is calculated from the number of PAS-positive epithelial cells per bronchus divided by the total number of epithelial cells of each bronchiole.

Peribronchial eosinophils

Lung sections were processed for major basic protein (MBP) immunohistochemistry as described above, using an anti-mouse MBP Ab (provided by Dr. J. Lee, Mayo Clinic, Scottsdale, AZ). The number of individual cells staining positive for MBP in the peribronchial space was counted using a light microscope. Results are expressed as the number of peribronchial cells staining positive for MBP per bronchiole with a 150- to 200- μm -internal diameter. Five to 10 bronchioles were counted in each slide.

Quantitation of TGF- β 1⁺ cells, phosphorylated Smad (pSmad) 2⁺ cells, and pSmad 3⁺ cells in the airways of WT and Smad 3-deficient mice

Lung sections were processed for immunohistochemistry to detect either TGF- β 1, pSmad 2, or pSmad 3 as described above, using either an anti-mouse TGF- β 1 Ab (Santa Cruz Biotechnology), an anti-pSmad 2 Ab (Chemicon International), or an anti-pSmad 3 Ab (Calbiochem). The pSmad 2 and pSmad 3 Abs detect nuclear phosphorylation of either Smad 2 or Smad 3, respectively, using methods as previously described in this laboratory (23). Species- and isotype-matched Abs were used as controls. Results are expressed as the number of peribronchial cells staining positive for either TGF- β 1, pSmad 2, or pSmad 3 per bronchiole with a 150- to 200- μm -internal diameter. Five to 10 bronchioles were counted in each slide stained with the anti-TGF- β 1 Ab, and 3 bronchioles were counted in each slide stained with either the pSmad 2 or pSmad 3 Abs.

Determination of AHR to methacholine (MCh) in vivo

AHR to MCh was assessed as previously described (24) 24 h after the final OVA challenge in intubated and ventilated mice (flexiVent ventilator; Scireq) anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. The frequency-independent airway resistance (Raw) was determined using Scireq software in mice exposed to nebulized PBS and MCh (48 mg/ml). The following ventilator settings were used: tidal volume (10 ml/kg), frequency (150/min), and positive end-expiratory pressure (3 cmH₂O).

Statistical analysis

Results in the different groups of mice were compared by ANOVA using the nonparametric Kruskal-Wallis test, followed by posttesting using Dunn's multiple comparison of means. The statistical analysis of lung immunohistology experiments is based on the comparison of the 3–10 bronchiole fields examined per mouse lung in each group of mice (Smad 3-deficient mice $n = 13$ OVA, $n = 9$ no OVA; and WT mice $n = 11$ OVA, $n = 6$ no OVA). All results are presented as mean $\pm$ SEM. A statistical software package (GraphPad Prism) was used for the analysis. Values of $p < 0.05$ were considered statistically significant.

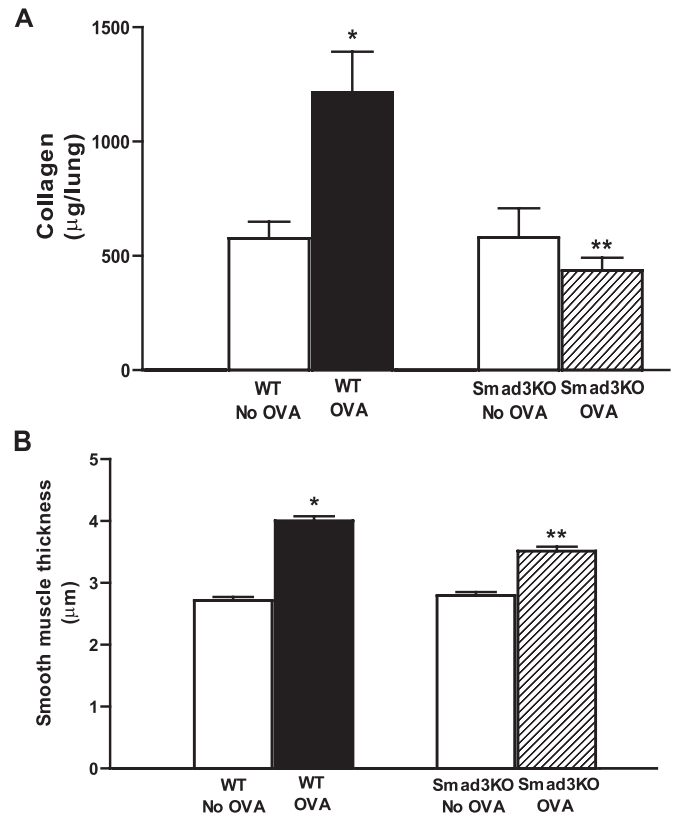


FIGURE 2. Quantitation of total lung collagen and peribronchial smooth muscle layer thickness in WT and Smad 3-deficient mice repetitively challenged with OVA. **A**, Total lung collagen content. Repetitive OVA challenge induced a significant increase in total lung collagen in WT mice (*, WT OVA vs WT no-OVA; $p = 0.02$). Smad 3-deficient mice repetitively challenged with OVA had less total lung collagen compared with WT mice repetitively challenged with OVA (**, Smad3 KO OVA vs WT OVA; $p = 0.002$). **B**, Peribronchial smooth muscle layer thickness. WT mice repetitively challenged with OVA developed significantly increased thickness of the peribronchial smooth muscle layer compared with non-OVA-challenged WT mice (#, WT OVA vs WT no-OVA; $p = 0.001$). In contrast, the thickness of the peribronchial smooth muscle layer in Smad 3-deficient mice challenged repetitively with OVA was significantly reduced compared with WT mice challenged repetitively with OVA (##, Smad3 KO OVA vs WT OVA; $p = 0.01$).

Results

Repetitive OVA challenge induces expression of activin A in remodeled airways

With repetitive OVA challenge, the concentration of activin A increased significantly in the BALF of both WT (643.3 ± 99.2 vs 1135 ± 139.7 pg/ml, WT no-OVA vs WT OVA; $p = 0.01$) (Fig. 1A) and Smad 3-deficient mice (429.1 ± 66.9 vs 1212.0 ± 101.3 pg/ml, Smad 3 knockout (KO) no-OVA vs Smad 3 KO OVA; $p = 0.001$) (Fig. 1A). There was no statistical difference in levels of activin A in the BALF of OVA-challenged WT compared with OVA-challenged Smad 3-deficient mice (1135 ± 139.7 vs 1212.0 ± 101.3 pg/ml, WT OVA vs Smad 3 KO OVA; $p = \text{NS}$) (Fig. 1A).

TGF- β 1, pSmad 2, and pSmad 3 expression in the airways of WT and Smad 3-deficient mice

WT mice repetitively challenged with OVA had significantly increased numbers of TGF- β 1⁺ cells/bronchus (4.9 ± 0.3 vs 86.1 ± 4.3 TGF- β 1⁺ cells/bronchus, WT no-OVA vs WT OVA; $p = 0.01$) (Fig. 1B) and pSmad 2⁺ cells/bronchus (17.6 ± 3.2 vs

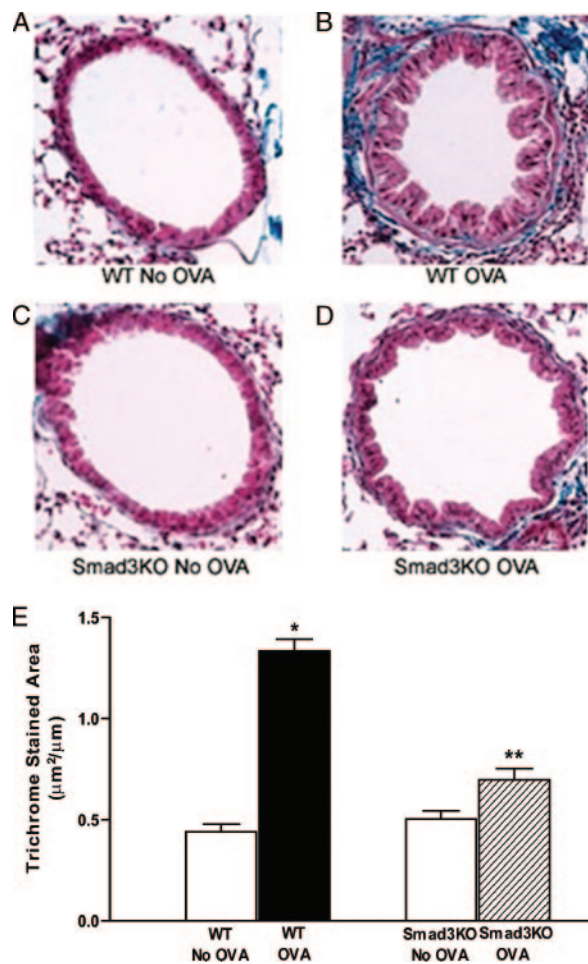


FIGURE 3. Peribronchial trichrome stain in WT and Smad3 deficient mice repetitively challenged with OVA. *A–D*, Representative photomicrographs of trichrome stained lung sections from WT and Smad3 deficient mice. In the absence of OVA challenge, WT (*A*) and Smad 3-deficient mice (*C*) exhibited minimal peribronchial trichrome staining (blue color). In contrast, repetitive OVA challenge in WT mice induced circumferential peribronchial trichrome staining (*B*), which was significantly reduced in Smad 3-deficient mice repetitively challenged with OVA (*D*). *E*, Quantitation of the area of peribronchial trichrome stain. WT mice repetitively challenged with OVA developed an increased area of peribronchial trichrome staining compared with non-OVA-challenged WT mice (*, WT OVA vs WT no-OVA; $p = 0.001$). In contrast, Smad 3-deficient mice repetitively challenged with OVA had significantly reduced areas of peribronchial trichrome staining compared with WT mice repetitively challenged with OVA (**, Smad3 KO OVA vs WT OVA; $p = 0.001$).

35.6 ± 2.7 pSmad 2^+ cells/bronchus, WT no-OVA vs WT OVA; $p = 0.01$) (Fig. 1C). Similarly, Smad 3-deficient mice repetitively challenged with OVA had significantly increased numbers of TGF- $\beta 1^+$ cells/bronchus (2.2 ± 0.3 vs 67.0 ± 3.5 TGF- $\beta 1^+$ cells/bronchus, Smad 3 KO no-OVA vs Smad 3 KO OVA; $p = 0.01$) (Fig. 1B) and pSmad 2^+ cells/bronchus (24.9 ± 2.0 vs 44.0 ± 3.1 pSmad 2^+ cells/bronchus, Smad 3 KO no-OVA vs Smad 3 KO OVA; $p = 0.01$) (Fig. 1C).

Repetitive OVA challenge also significantly increased the numbers of pSmad 3^+ cells in the airways of WT mice (11.0 ± 2.4 vs 27.6 ± 2.9 pSmad 3^+ cells/bronchus, WT no-OVA vs WT OVA; $p = 0.01$) (Fig. 1D). In contrast, pSmad 3 was not detectable in either OVA-challenged or non-OVA-challenged Smad 3-deficient mice (Fig. 1D).

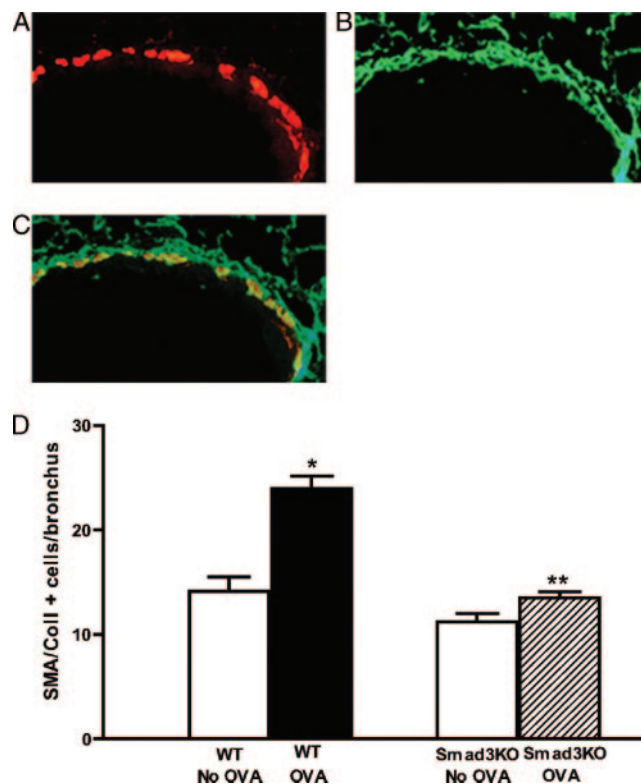


FIGURE 4. Peribronchial myofibroblast accumulation in WT and Smad 3-deficient mice. *A–C*, Representative photomicrographs of lung sections immunostained for the detection of peribronchial cells coexpressing α -SMA and Col-1. Peribronchial cells expressing either α -SMA (immunofluorescent red in *A*) or Col-1 (immunofluorescent green in *B*) can be detected. Cells that express both α -SMA and Col-1 immunofluorescent yellow (*C*). *D*, Quantitation of peribronchial myofibroblasts. Mice repetitively challenged with OVA had a significant increase in levels of peribronchial cells expressing both α -SMA and Col-1 compared with WT non-OVA-challenged mice (*, WT OVA vs WT no-OVA; $p = 0.01$). Smad 3-deficient mice repetitively challenged with OVA had a significantly reduced number of peribronchial cells coexpressing Col-1 and α -SMA compared with WT OVA-challenged mice (**, Smad3 KO OVA vs WT OVA; $p = 0.01$).

Reduction of peribronchial collagen deposition in Smad 3-deficient compared with WT mice

Lung collagen. WT mice challenged repetitively with OVA had a significant increase in the levels of lung collagen compared with non-OVA-challenged WT mice ($577.1 \pm 71.7 \mu\text{g}$ vs $1214.0 \pm 179.6 \mu\text{g}$ collagen/lung; $p = 0.02$). Smad 3-deficient mice similarly challenged with OVA had significantly reduced levels of lung collagen compared with WT mice (435.8 ± 55.8 vs $1214.0 \pm 179.6 \mu\text{g}$ collagen/lung, $p = 0.002$) (Fig. 2A).

Peribronchial trichrome stain. Changes in levels of peribronchial fibrosis were also confirmed by measuring the area of trichrome staining. Repetitive OVA challenge in WT mice induced a significant increase in the area of peribronchial trichrome staining (Fig. 3, *A* and *B*) compared with non-OVA-challenged mice (0.44 ± 0.04 vs $1.34 \pm 0.06 \mu\text{m}^2/\mu\text{m}$ circumference of bronchiole, WT OVA vs WT no-OVA; $p = 0.001$) (Fig. 3E). This increase in trichrome staining area was significantly attenuated in Smad 3-deficient mice repetitively challenged with OVA (Fig. 3, *D* and *E*) compared with WT mice challenged with OVA (0.69 ± 0.05 vs $1.34 \pm 0.06 \mu\text{m}^2/\mu\text{m}$ circumference of bronchiole, Smad 3 KO plus OVA vs WT plus OVA; $p = 0.001$) (Fig. 3E).

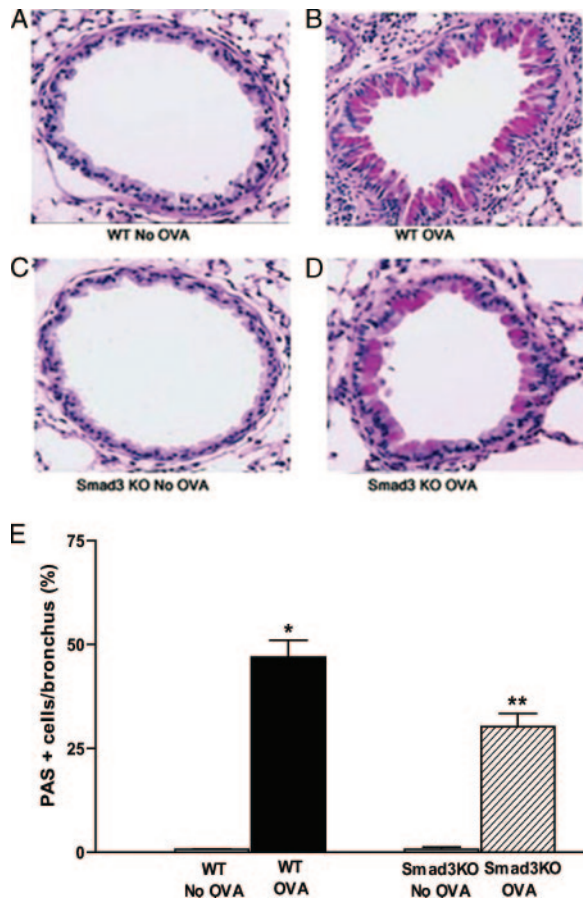


FIGURE 5. Airway mucus production in WT and Smad 3-deficient mice. *A–D*, Representative photomicrographs of PAS-stained lung sections. Non-OVA-challenged WT (*A*) and Smad 3-deficient mice (*C*) exhibited minimal numbers of PAS-positive epithelial cells (dark reddish color). In contrast, repetitive OVA challenge in WT mice induced a greater number of PAS-positive cells (*B*), which was significantly reduced in Smad 3-deficient mice repetitively challenged with OVA (*D*). *E*, Quantitation of percentage of PAS-positive cells/bronchus in WT and Smad 3-deficient mice. Repetitive OVA challenge in WT mice induced a significant increase in the percentage of airway epithelium that stained positive with PAS compared with non-OVA-challenged WT mice (*, WT OVA vs WT no-OVA; $p = 0.001$). The percentage of airway epithelium staining positively with PAS in repetitively OVA-challenged Smad 3-deficient mice was significantly less than that of repetitively OVA-challenged WT mice (**, Smad3 KO OVA vs WT OVA; $p = 0.01$).

Smad 3-deficient mice have reduced thickness of the smooth muscle layer compared with WT mice

Repetitive OVA challenge induced a significant increase in the thickness of the peribronchial smooth muscle layer of WT mice (2.7 ± 0.05 vs 4.0 ± 0.07 μ m, WT no-OVA vs WT OVA; $p = 0.001$). When Smad 3-deficient mice were similarly challenged with OVA, the thickness of their smooth muscle layer was significantly reduced compared with WT mice (3.5 ± 0.6 vs 4.0 ± 0.07 μ m; Smad 3 KO plus OVA vs WT plus OVA; $p = 0.01$) (Fig. 2*B*).

Smad 3- and allergen-induced accumulation of peribronchial myofibroblasts

Because TGF- β is hypothesized to induce myofibroblast differentiation, we performed double-label immunohistochemistry experiments to quantitate the number of peribronchial myofibroblasts (i.e., cells coexpressing both α -SMA and collagen-1 (Col-1)) in Smad 3-deficient mice. Repetitive OVA challenge in WT mice

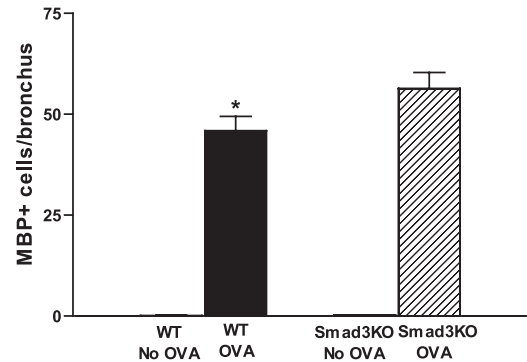


FIGURE 6. Peribronchial eosinophilic inflammation in WT and Smad 3-deficient mice. WT mice challenged with OVA had a significant increase in the number of peribronchial MBP-positive cells (*, WT OVA vs WT no-OVA; $p = 0.001$). There was no significant reduction in the number of MBP-positive cells in Smad 3-deficient mice challenged with OVA compared with WT mice challenged with OVA (Smad 3 KO vs WT OVA; $p = \text{NS}$).

induced a significant increase in α -SMA-positive/Col-1-positive peribronchial cells compared with non-OVA-challenged mice (14.1 ± 1.3 vs 23.9 ± 1.2 , WT no-OVA vs WT OVA; $p = 0.01$), and this increase in double-positive cells was significantly attenuated in similarly challenged Smad 3-deficient mice (13.4 ± 0.6 vs 23.9 ± 1.2 , Smad 3 KO plus OVA vs WT plus OVA; $p = 0.01$) (Fig. 4).

Smad 3-deficient mice have reduced airway mucus expression

To study the effects of Smad 3 on airway mucus expression, we quantified the number of epithelial cells staining positive for PAS. Repetitive OVA challenge in WT mice induced a significant increase in the percentage of airway epithelium that stained positive with PAS (Fig. 5, *A* and *B*) compared with non-OVA-challenged mice (0 ± 0 vs $46.9 \pm 4.0\%$ PAS-positive cells/bronchus, WT no-OVA vs WT OVA; $p = 0.001$) (Fig. 5*E*). When compared with OVA-challenged WT mice, OVA-challenged Smad 3-deficient mice developed a significantly reduced percentage of airway epithelium that stained positive with PAS (Fig. 5, *C* and *D*) (30.3 ± 3.1 vs $46.9 \pm 4.0\%$ PAS-positive cells/bronchus, Smad 3 KO plus OVA vs WT plus OVA; $p = 0.01$) (Fig. 5*E*).

Smad 3-deficient and WT mice have similar levels of peribronchial eosinophilic inflammation

To study the effects of Smad 3 on airway eosinophilic inflammation, the number of peribronchial MBP-positive cells was counted. Repetitive OVA challenge in WT mice induced a significant peribronchial accumulation of MBP-positive cells (eosinophils), which is largely absent in non-OVA-challenged mice (0.1 ± 0.1 vs 45.8 ± 3.6 MBP-positive cells/bronchus WT no-OVA vs WT OVA; $p = 0.001$) (Fig. 6). Similarly, OVA-challenged Smad 3-deficient mice also developed peribronchial accumulation of MBP-positive cells that was not significantly different from that of OVA challenged WT mice (56.3 ± 3.9 vs 45.8 ± 3.6 MBP-positive cells/bronchus, Smad 3 KO plus OVA vs WT plus OVA; $p = \text{NS}$) (Fig. 6).

AHR in WT vs Smad 3-deficient mice

WT mice challenged with repetitive administration of OVA developed significantly increased AHR to MCh (WT no-OVA vs WT OVA; $p = 0.01$, MCh 48 mg/ml; Table I). Similarly, Smad 3-deficient mice repetitively challenged with OVA developed significantly increased AHR to MCh (Smad 3-deficient no-OVA vs Smad

Table I. Airway resistance in WT vs *Smad 3*-deficient mice^a

	WT	<i>Smad 3</i> ^{-/-}
No OVA	2.8 ± 0.3	2.7 ± 0.3
OVA	4.7 ± 0.4	3.9 ± 0.7

^a Airway resistance (cmH₂O · s/ml) was measured in intubated and ventilated WT or *Smad 3*-deficient mice following nebulization of MCh (48 mg/ml). OVA challenge induced a significant increase in airway resistance (Raw) in WT mice (WT no OVA vs WT OVA; *p* = 0.01, 48 mg/ml MCh). The percent increase in Raw following repetitive OVA challenge was not statistically different in WT compared with *Smad 3*-deficient mice (WT OVA vs *Smad 3*-deficient OVA; *p* = 0.20, 48 mg/ml MCh).

3-deficient OVA; *p* = 0.01, MCh 48 mg/ml; Table I). The percent increase in Raw following repetitive OVA challenge was not statistically different in WT compared with *Smad 3*-deficient mice (WT OVA vs *Smad 3*-deficient OVA; *p* = 0.20, MCh 48 mg/ml; Table I).

Discussion

Our studies demonstrate that *Smad 3* mediates both accumulation of peribronchial myofibroblasts, as well as key structural changes in the airway (fibrosis, thickness of the smooth muscle layer, and mucus expression), but does not significantly mediate changes in eosinophilic airway inflammation or AHR associated with allergen-induced airway remodeling in the mouse.

The importance of *Smad 3* to airway remodeling is underscored by the fact that *Smad 3* is a downstream signaling pathway for both TGF- β and activin A signaling. As we detected increased levels of both TGF- β and activin A in the remodeled airway, either and/or both of these profibrotic mediators could be responsible for activation of *Smad 3* in the remodeled airway. Although TGF- β may signal through non-*Smad* signaling pathways (i.e., MAPKs and PI3K), these *Smad*-independent signaling pathways appear to be insufficient for the induction of fibrosis (25–27). Thus, *Smad 3*-deficient mice could have reduced airway remodeling due to impaired responses to either TGF- β and/or activin A in the remodeled airway. Although *Smad 3*-deficient mice have significantly reduced levels of airway remodeling, a deficiency of *Smad 3* did not influence the levels of peribronchial eosinophilic inflammation, levels of activin A, or the number of TGF- β ⁺ cells in the airway, indicating that the generation of eosinophilic inflammation, TGF- β , and activin A does not appear to be dependent on *Smad 3* signaling. In contrast, in *Smad 3*-deficient mice TGF- β signaling is significantly impaired in fibroblasts as well as in other cell types which express TGF- β receptors.

Our study demonstrates that the absence of the *Smad 3* signaling molecule results in reductions in several features of airway remodeling, including peribronchial extracellular matrix (ECM) deposition (assessed by trichrome staining), smooth muscle layer thickness, mucus production, and the number of myofibroblasts. The mechanism by which *Smad 3* mediates the reduction of the ECM (which is largely composed of collagen) is unclear but may be through direct blockade of the TGF- β /*Smad* induction of collagen synthesis. Studies investigating differential expression of ECM-related genes in human fibroblasts identified a number of collagen gene promoters that were induced by TGF- β 1 and dependent on *Smad 3* (28). Activation of these collagen gene promoters by TGF- β can be blocked by dominant-negative *Smad 3* expression vectors. Therefore, an absence of TGF- β /*Smad* signaling may directly reduce the expression of collagen-producing genes. The reduction of the peribronchial ECM in *Smad 3*-deficient mice may also occur through the reduction in the number of myofibroblasts, which are believed to be a significant cellular source of collagen (29). There is also evidence that *Smad 3* signaling is involved in

the maintenance of the balance between protease and antiprotease activity (30). For example, a deficiency of *Smad 3* leads to an inability of TGF- β to negatively regulate the expression of certain proteases, and this is associated with a decrease in the ECM deposition in *Smad 3*-deficient mice (30).

Myofibroblasts, specialized cells considered to be important in the pathogenesis of airway remodeling, have features of both fibroblasts and smooth muscle cells. They have the synthetic machinery of fibroblasts that is used for the synthesis of the ECM, as well as some of the components of the contractile apparatus of myocytes. Our studies indicate that the induction of myofibroblasts seen in the airway remodeling of murine asthma involves *Smad 3* because a deletion of *Smad 3* results in a reduction in the number of peribronchial myofibroblasts. EMSAs and DNA high-affinity precipitation studies have furthermore determined that *Smad 3* binding to promoters containing CAGA motifs or *Smad 3*-binding elements is required for α -SMA expression of fibroblasts (31, 32), providing a mechanistic pathway by which TGF- β , through the direct actions of *Smad 3*, may induce myofibroblast differentiation.

The increase in the peribronchial smooth muscle layer and hyperplasia/hypertrophy of goblet cells, both detected in the remodeled airways of asthmatics, is also significantly attenuated in *Smad 3*-deficient mice. These results with *Smad 3*-deficient mice are similar to those noted with studies using anti-TGF- β Abs in a murine model of airway remodeling (14) and indicate that smooth muscle and goblet cell hyperplasia/hypertrophy may also occur through TGF- β /*Smad 3*-dependent pathways.

The demonstration that *Smad 3* plays an important role in mediating several key features of airway remodeling but does not play a significant role in mediating AHR is counterintuitive as mathematical models of asthma predict that the increased airway wall thickening in remodeled airways would result in disproportionately severe airway narrowing and responsiveness (33). Thus, based on this mathematical model of AHR, the reduced airway wall thickening in *Smad 3*-deficient mice (a consequence of reduced airway remodeling) should result in reduced AHR, a result not observed in *Smad 3*-deficient mice in this study. However, similar to our observations regarding the relationship between airway wall thickening and AHR in *Smad 3*-deficient mice, studies in human asthmatics have also demonstrated that airway wall thickening in asthma is associated with reduced rather than increased airway reactivity to MCh (34). In these human asthma studies, noninvasive high-resolution computerized tomography scanning methods were used to assess airway wall thickening and, surprisingly, demonstrated that airway wall thickening in asthma is associated with reduced rather than increased airway reactivity to MCh (34). One potential explanation suggested by Pare (34) for the discrepancy in results between the mathematical modeling studies in asthma and the computerized tomography scan studies in asthma evaluating airway wall thickness and AHR is that the mathematical modeling studies were primarily based on altered airway geometry and did not fully take into account the potential effect of airway wall thickening on the mechanical properties of the airway, e.g., stiffness of the airway (35). Our studies with *Smad 3*-deficient mice underscore the fact that a gene such as *Smad 3*, which plays a significant role in mediating several important aspects of airway remodeling, may not play an essential role in mediating AHR. Thus, targeting genes that influence airway remodeling may not necessarily influence airway hyperreactivity a key end point in the development of therapeutic interventions in asthma.

As *Smad 3* plays a critical role in the pathogenesis of fibrotic disease, inhibition of *Smad 3* would appear to be a prime target for intervention and attenuation of fibrotic conditions, including airway remodeling in asthma. In vitro studies have demonstrated that

the Smad 3 inhibitor, SIS3, suppresses TGF- β 1-induced type I procollagen up-regulation, as well as inhibits differentiation of human dermal fibroblasts to myofibroblast through inhibition of Smad 3 phosphorylation (36). In this study, we have demonstrated that there is an important requirement for Smad 3 in the development of airway remodeling and myofibroblast accumulation in a murine model of allergic asthma. We have also demonstrated that there are significant increased airway levels of expression of both TGF- β 1 and activin A in the remodeled airway. As both TGF- β 1 and activin A are important mediators of Smad 3 signaling, both these cytokines detected in the remodeled airway may be important in mediating airway remodeling. Therefore, there may be a therapeutic advantage to targeting Smad 3 in preference to targeting either cytokine alone in the treatment of airway remodeling in asthma as targeting Smad 3 would inhibit both TGF- β 1 and activin A signaling. However, targeting Smad 3 does not significantly reduce AHR, suggesting that inhibiting Smad 3 may significantly reduce structural features of airway remodeling but not significantly reduce AHR.

Disclosures

The authors have no financial conflict of interest.

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