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Am J Physiol Lung Cell Mol Physiol 300:L887-L897, 2011. First published 25 March 2011; doi:10.1152/ajplung.00397.2010

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TGFβ signaling in lung epithelium regulates bleomycin-induced alveolar injury and fibroblast recruitment

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Submitted 4 November 2010; accepted in final form 22 March 2011

Degryse AL, Tanjore H, Xu XC, Polosukhin VV, Jones BR, Boomershine CS, Ortiz C, Sherrill TP, McMahon FB, Gleaves LA, Blackwell TS, Lawson WE. TGFβ signaling in lung epithelium regulates bleomycin-induced alveolar injury and fibroblast recruitment. *Am J Physiol Lung Cell Mol Physiol* 300: L887–L897, 2011. First published March 25, 2011; doi:10.1152/ajplung.00397.2010.—The response of alveolar epithelial cells (AECs) to lung injury plays a central role in the pathogenesis of pulmonary fibrosis, but the mechanisms by which AECs regulate fibrotic processes are not well defined. We aimed to elucidate how transforming growth factor-β (TGFβ) signaling in lung epithelium impacts lung fibrosis in the intratracheal bleomycin model. Mice with selective deficiency of TGFβ receptor 2 (TGFβR2) in lung epithelium were generated and crossed to cell fate reporter mice that express β-galactosidase (β-gal) in cells of lung epithelial lineage. Mice were given intratracheal bleomycin (0.08 U), and the following parameters were assessed: AEC death by terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling assay, inflammation by total and differential cell counts from bronchoalveolar lavage, fibrosis by scoring of trichrome-stained lung sections, and total lung collagen content. Mice with lung epithelial deficiency of TGFβR2 had improved AEC survival, despite greater lung inflammation, after bleomycin administration. At 3 wk after bleomycin administration, mice with epithelial TGFβR2 deficiency showed a significantly attenuated fibrotic response in the lungs, as determined by semiquantitatve scoring and total collagen content. The reduction in lung fibrosis in these mice was associated with a marked decrease in the lung fibroblast population, both total lung fibroblasts and epithelial-to-mesenchymal transition-derived ($S100A4^+/\beta\text{-gal}^+$) fibroblasts. Attenuation of TGFβ signaling in lung epithelium provides protection from bleomycin-induced fibrosis, indicating a critical role for the epithelium in transducing the profibrotic effects of this cytokine.

epithelial mesenchymal transition; transforming growth factor-β; S100A4; pulmonary fibrosis

IDIOPATHIC PULMONARY FIBROSIS (IPF) remains a progressive and relentless restrictive lung disease for which there is no cure or effective therapy short of lung transplantation (1, 15). Current thoughts on the pathogenesis of disease in IPF include prominent roles for alveolar epithelial cell (AEC) injury and fibroblast activation. Over the past two decades, the role of the fibroblast in this disease process has been extensively studied, and it is clear that these cells are responsible for the collagen and other extracellular matrix deposition that occurs in IPF. Recently, the role of the AEC population in this disease process has also been prominently addressed and defined.

Multiple lines of evidence implicate the AEC as a pivotal cell type in the fibrotic response in IPF (42). In IPF biopsies, epithelial abnormalities are common and include hyperplastic AECs lining areas of honeycombing (39). Mutations in surfactant protein (SP) C (SP-C) and SP-A linked to IPF highlight the importance of type II AECs in disease pathogenesis (12, 48, 54). AEC apoptosis has been implicated in lung fibrosis on the basis of human disease and animal models (49). Furthermore, AECs produce key profibrotic cytokines, including transforming growth factor-β (TGFβ), connective tissue growth factor, and platelet-derived growth factor (2, 22, 38). However, the role of the alveolar epithelium in modulating the fibrotic response requires further investigation.

In addition to epithelial cells, TGFβ is produced by many other cells in the lung, including fibroblasts and inflammatory cells. TGFβ pathway signaling occurs when TGFβ binds at the cell surface to the major TGFβ receptor (TGFβR2), which then recruits TGFβR1 to form a heterodimer (14). The kinase activity of TGFβR2 phosphorylates TGFβR1, which in turn leads to activation of smad2 and smad3 signaling (17, 36, 57). Once in the nucleus, smad2 and smad3 (in conjunction with smad4) can activate transcription of multiple genes (3, 21). Deficiency of TGFβR2 disrupts the TGFβ signaling pathway (4, 9).

TGFβ is a key profibrotic cytokine in humans and animals. Transgenic or adenoviral vector-mediated overexpression of TGFβ in the lungs results in fibrosis in mouse models (25, 32, 44, 55). TGFβ promotes fibroblast proliferation and activation and is one of the most important stimulators of extracellular matrix production (43). In addition to stimulating fibroblasts, TGFβ signaling can affect epithelial cells by enhancing cell death (32) and inducing epithelial-mesenchymal transition (EMT) (23, 47, 56). In these studies, we sought to dissect the pivotal cellular targets for TGFβ signaling in the lungs by evaluating the role and importance of AECs in mediating the profibrotic effects of TGFβ. To address this issue, we developed a transgenic murine model in which TGFβR2 is specifically deleted in lung epithelium. While mice with lung epithelial cell deficiency of TGFβR2 have slightly greater alveolar space than controls and otherwise grow normally into adulthood, these mice are protected from bleomycin-induced AEC injury and fibrosis. Together, our studies define a critical role for lung epithelium in transducing the profibrotic effects of TGFβ.

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MATERIALS AND METHODS

Transgenic mice. Transgenic mice from the C57BL/6J background weighed 20–30 g and were ≥8 wk old at the beginning of the study. Transgenic mice expressing Cre recombinase under control of the 3.7-kb human SP-C promoter (SPC.Cre) were obtained from Dr. Brigid Hogan (Duke University, Durham, NC). R26Rosa.Stop.lacZ reporter mice were obtained from Jackson Laboratories (Bar Harbor, ME). With these mice, the R26Rosa endogenous promoter drives expression of a construct that consists of a loxP-flanked STOP cassette upstream of *lacZ* [whose gene product is β-galactosidase (β-gal)] and a polyadenylation sequence (46). SPC.Cre mice were mated to R26Rosa.Stop.lacZ reporter mice, resulting in SPC.Cre.R26Rosa.Stop.lacZ mice, which serve as a lung epithelium cell fate reporter system, as described previously (13, 47). Transgenic mice in which the major receptor for TGFβ (TGFβR2) is flanked by loxP sites were obtained from Dr. Hal Moses (Vanderbilt University), and these TGFβR2$^{fl/fl}$ mice were crossed to the cell fate reporter mice described above, yielding the triple-transgenic model SPC.Cre.TGFβR2$^{fl/fl}$.R26Rosa.Stop.lacZ. Mice were housed in the central animal care facility at Vanderbilt University Medical Center and given food and water ad libitum. The experimental protocol was reviewed and approved by the Institutional Animal Care and Utilization Committee at Vanderbilt University.

Bleomycin model. Bleomycin (Teva Parenteral Medicines, Irvine, CA) was prepared and administered by an intratracheal intubation procedure at a dose of 0.08 U in a total volume of 100 μl of sterile saline, as previously described (13). At designated times after bleomycin administration, mice were euthanized by exposure to carbon dioxide, and lungs were harvested for histological preparations and frozen tissue, or bronchoalveolar lavage (BAL) was performed as described below and elsewhere (13, 30, 31, 47).

Histology and microscopy. For tissue harvesting, the lungs were perfused with normal saline from the right to the left ventricle of the heart. The right hilum was identified, tied off, and surgically removed; the lobes were flash-frozen immediately in liquid nitrogen and stored at −70°C. The trachea was isolated, and with use of a blunt-tipped needle and syringe, the remaining left lung was inflated with 10% neutral buffered formalin by a 25-cm pressure column. The trachea was tied off, and the lung was removed for fixation overnight in formalin and then embedded in paraffin. Sections (5 μm) were cut for hematoxylin-eosin and trichrome blue staining, as well as for immunohistochemistry studies. For cell fate mapping, frozen sections were processed as previously described (47). Briefly, lungs were perfused with normal saline and then inflated with 4% paraformaldehyde by a 25-cm pressure column. The trachea was tied off, and the lungs were kept in 4% paraformaldehyde for 2 h at 4°C and then transferred into a 20% sucrose solution for 24 h. Then the lungs were flash-frozen in liquid nitrogen and transferred to a −70°C freezer until processed on a cryostat for frozen tissue sectioning. Light and fluorescence microscopy was performed using an inverted research microscope (model IX81, Olympus, Tokyo, Japan) configured with a biological disk scanning unit (model IX2, Olympus).

Lung lavage and cell counts. BAL was performed as described previously (13, 30). After euthanasia, three 800-μl lavages of sterile saline were performed using a 20-g blunt-tipped needle inserted into the trachea. Samples were centrifuged at 400 *g* for 10 min, and the supernatant was discarded. Cell counts were performed manually under light microscopy using a hemocytometer. A Cytospin 2 (Shandon Southern Products) was used to load ~30,000 cells from each specimen onto slides. These slide preparations were stained using a modified Wright stain and viewed under light microscopy for differential white blood cell counts.

Type II AEC isolation. Type II AECs were isolated from adult transgenic mice, as described elsewhere (31, 47). Isolation of type II AECs with the cell fate-mapping construct has been >95% Xgal-positive, indicative of epithelial cell lineage, and >90% pro-SP-C-positive by immunohistochemistry, indicative of type II AECs. After isolation, type II AECs were cultured in DMEM for use in experiments. In one set of experiments, AECs were placed in culture medium; after 24 h, TGFβ1 (10 ng/ml; R & D Systems, Minneapolis, MN) was added to the culture medium for 2 h, and the cells were fixed with formalin, as previously described (31), for immunofluorescence.

PCR array studies. Type II AECs were isolated from mice 2 wk after bleomycin administration, and AECs from two mice were pooled for a single sample. These isolated type II AECs were immediately processed for RNA isolation using an RNeasy Mini Kit according to the manufacturer's recommendations (Qiagen, Valencia, CA) and then converted to cDNA using a RT^2 First Strand Kit as directed by the manufacturer (SABiosciences, Frederick, MD). For these experiments, a 96-well RT^2 Prolifer PCR Array focused on the mouse TGFβ/bone morphogenetic protein (BMP) pathway was performed according to the manufacturer's instructions (SABiosciences), with loading of 10 ng of cDNA per well using a StepOne Plus real-time PCR machine (Applied Biosystems, Carlsbad, CA). For these experiments, three samples were run for each group, with the mean level of expression of an individual gene reported as level of expression from the targeted group (SPC.Cre.TGFβR2$^{fl/fl}$.R26Rosa.Stop.lacZ) relative to the control group (TGFβR2$^{fl/fl}$.R26Rosa.Stop.lacZ). Statistical analysis was performed using manufacturer-provided software (SABiosciences), with $P < 0.05$ considered significant.

Immunofluorescence staining. Immunofluorescence was performed on frozen lung sections as previously described (13, 47). Frozen lung tissue blocks were cut into 5-μm sections and fixed in 0.2% paraformaldehyde in PIPES buffer [0.1 M PIPES (Sigma, St. Louis, MO), 2 mM $MgCl_2$, and 5 mM EGTA]. After the sections were blocked with 3% BSA, they were incubated with primary antibodies against S100A4 (nonbiotinylated rabbit polyclonal antibody; obtained from Dr. Eric Neilson, Vanderbilt University) and β-gal (chicken polyclonal antibody; Abcam, Cambridge, MA) at 4°C overnight. After the sections were washed with phosphate-buffered saline, they were stained with fluorescent secondary antibodies (Jackson Immunoresearch, West Grove, PA). Immunofluorescence was also performed on isolated type II AECs for TGFβR2 (goat polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA) and phosphorylated smad2 (rabbit polyclonal antibody; Santa Cruz Biotechnology) in conjunction with fluorescent secondary antibodies (Jackson Immunoresearch). For tissue and cell preparations, nuclear staining was done with 4′,6-diamidino-2-phenylindole using Vectashield mounting medium (Vector Laboratories).

TUNEL and lactate dehydrogenase cytotoxicity assays. Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assays on lung sections were performed as previously described (13) using a commercially available kit in accordance with the manufacturer's directions (In Situ Cell Death Detection Kit, Roche Molecular Biochemicals, Indianapolis, IN). Counterstains for preparations were performed with hematoxylin. Isolated type II AECs from untreated mice were placed in culture medium and, 24 h later, exposed to bleomycin (0.1 U in 2 ml) in the culture medium for 24 h; then media supernatant and cell lysates were collected for determination of cytotoxicity using a lactate dehydrogenase (LDH)-based assay according to the manufacturer's instructions (Cytox 96 Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI). For these studies, percent cytotoxicity was calculated as LDH in supernatant divided by total LDH (supernatant + lysate). Samples were run in triplicate, with the mean representing the score for an individual mouse type II AEC isolation.

LacZ staining. Xgal substrate staining on lung tissues and cells was performed as previously described (13, 47). Frozen lung tissue sections were fixed in LacZ fixative solution containing 0.2% glutaraldehyde, 5 mM EGTA (pH 7.3), and 100 mM $MgCl_2$ in 0.1 M $NaPO_4$ (pH 7.3) for 15 min at 4°C, washed three times in phosphate-buffered saline, and incubated at 37°C in 1 mg/ml Xgal (Sigma), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.2% Nonidet P40, and 0.1% sodium deoxycholate in phosphate-

buffered saline for 16 h. Sections were counterstained with eosin and mounted with Permount (Sigma).

Semiquantitative scoring. Lung fibrosis on histological specimens was quantified by an investigator blinded to the group using a semiquantitative score, as previously described (13, 30). Briefly, slides were evaluated on 10 sequential, nonoverlapping fields (×300 magnification) of lung parenchyma from each specimen. Lung fibrosis was evaluated on trichrome-stained lung sections using a 0–4 point scale, with a score of 0 for normal lung architecture, 1 for increased thickness of some (≤50%) interalveolar septa, 2 for thickening of >50% of interalveolar septa without formation of fibrotic lesions, 3 for thickening of the interalveolar septa with formation of isolated fibrotic lesions, and 4 for formation of multiple fibrotic lesions with total or subtotal distortion of parenchymal architecture. The mean score for the 10 fields represented the score for each individual specimen. For evaluation of TUNEL staining, the percentage of cells with TUNEL-positive nuclei on 10 sequential, nonoverlapping high-power fields from each specimen was recorded. The mean percentage of TUNEL-positive cells on 10 sequential fields represented the score for each individual specimen. For quantitation of immunofluorescence-positive cells in tissue preparations, green fluorescent, red fluorescent, and dual-fluorescent cells were counted on 10 nonoverlapping high-power fields at ×600 magnification. The average number, as well as mean percentage, represented the score for each individual specimen.

Lung collagen content determination. Frozen tissue samples from the right upper and right middle lung lobes were combined into a single sample and hydrolyzed in 6 N HCl, and hydroxyproline content was quantitated using a microplate assay based on the Ehrlich reaction, as previously described (7). Lung collagen content was calculated from these results, as hydroxyproline accounts for ~13.3% of collagen by weight.

Morphometry measurements. To analyze lung morphometry, alveolar diameter and alveolar perimeter were measured in hematoxylin-eosin-stained lung tissue sections using a computerized image analyzer system (Image-Pro Express, Media Cybernetics, Silver Spring, MD). Ten nonoverlapping digital images were prepared starting from the apex of each tissue section (×40 objective). Alveolar diameter was calculated as an average of four measurements taken at 45° angles for each alveolus in the field. Alveolar perimeter was measured as the length of the inner edge of each alveolus in the field. All measurements were made by a pathologist blinded to study group and reported in micrometers.

Statistics. Statistical analyses were performed using GraphPad InStat (GraphPad Software, San Diego, CA). Differences among groups were assessed using one-way ANOVA or Kruskal-Wallis rank ANOVA. Differences between pairs were assessed using a Student's *t*-test or a Mann-Whitney test. Survival differences were evaluated using a Fisher's exact test. Results are presented as means ± SE. $P < 0.05$ was considered significant.

RESULTS

Mice with selective deletion of TGFβR2 in lung epithelium are developmentally normal. For these studies, we developed a transgenic murine model in which TGFβR2 can be selectively deleted in lung epithelium. SPC.Cre mice were crossed to Rosa.Stop.lacZ mice, as we reported previously (47). Because of SP-C expression during lung development, all lung epithelial cells from the trachea to the alveolus have β-gal expression in these mice, allowing for lung epithelial cell fate-mapping studies. Next, double-transgenic mice were crossed to TGFβR2$^{fl/fl}$ mice. When bred to homozygosity for the TGFβR2$^{fl/fl}$ construct, mice with the SPC.Cre construct have deficiency of TGFβR2 in lung epithelium. A schematic for the triple-transgenic model is shown in Fig. 1*A*. To verify the targeted deletion of TGFβR2, we evaluated β-gal expression in lung tissue sections from SPC.Cre.TGFβR2$^{fl/fl}$.Rosa.Stop.lacZ mice as an indicator of Cre recombination (Fig. 1*B*). Xgal staining was identified in airway and alveolar epithelium. In addition, type II AECs isolated from these mice also stained for Xgal (Fig. 1, *C* and *D*), but not for TGFβR2 (Fig. 1, *E* and *F*). When these primary type II AECs were exposed to TGFβ in vitro, phosphorylated smad2 immunofluorescence was readily detected in AECs from the nonfloxed littermate controls, whereas type II AECs with deficiency of TGFβR2 failed to induce phosphorylated smad2 (Fig. 1, *G* and *H*). These studies demonstrate selective deficiency of TGFβR2 in lung epithelium while maintaining cell fate-mapping capabilities.

Pups containing the SPC.Cre.TGFβR2$^{fl/fl}$.Rosa.Stop.lacZ constructs appeared normal at birth and through adulthood, had no evidence of respiratory distress, and reproduced normally. On gross histological examination, lung architecture appeared normal (Fig. 2, *A* and *B*), but when morphometric modalities were employed, alveolar size was slightly enlarged in mice with lung epithelial cell deficiency of TGFβR2 compared with wild-type littermates on the basis of measurements of alveolar diameter and alveolar perimeter (Fig. 2, *C* and *D*). Since there was no evidence of an alveolar destructive process, this finding was interpreted as alveolar space enlargement, rather than true emphysema. Except for this morphometric difference, there was no other evidence that lung epithelial cell deficiency of TGFβR2 affected other aspects of development, survival, or overall condition of these mice.

To further characterize the integrity of the model, we sought to examine the downstream differential effects of AEC-specific deletion of TGFβR2 on TGFβ-dependent mediator production. We isolated type II AECs from SPC.Cre.TGFβR2$^{fl/fl}$.Rosa.Stop.lacZ mice and littermate controls at 2 wk after bleomycin administration, at which time lung TGFβ levels are elevated (18). After AEC isolation, we performed a targeted TGFβ/BMP-specific 96-well array (SABiosciences) to evaluate expression of TGFβ/BMP-dependent genes. As summarized in Table 1, expression of a number of TGFβ-regulated genes (17 of 84) was reduced in AECs isolated from SPC.Cre.TGFβR2$^{fl/fl}$.Rosa.Stop.lacZ mice compared with littermate controls. These results demonstrate that this model of cell-specific deletion of TGFβR2 blocks TGFβ-dependent gene expression in AECs.

Mice with deficiency of TGFβR2 in lung epithelium have lower mortality, less AEC death, and greater lung inflammation following intratracheal bleomycin administration. To investigate the impact of epithelial TGFβ signaling on lung injury and remodeling, we treated mice with lung epithelial cell TGFβR2 deficiency and littermate controls with intratracheal bleomycin. After administration of 0.08 U of bleomycin, all mice with lung epithelial cell TGFβR2 deficiency survived, while the wild-type controls experienced 36% mortality by *week 3* ($P < 0.05$, by Fisher's exact test).

AEC apoptosis has been prominently implicated in pulmonary fibrosis. To determine if attenuation of TGFβ signaling in lung epithelium impacted AEC apoptosis following bleomycin, lungs were harvested at baseline and at 1 wk after bleomycin administration, and sections were analyzed by TUNEL assay. In untreated animals, no difference was noted between groups, as TUNEL-positive cells were very rare at baseline in the lungs of mice with lung epithelial cell TGFβR2 deficiency and littermate controls. At 1 wk after bleomycin administration,

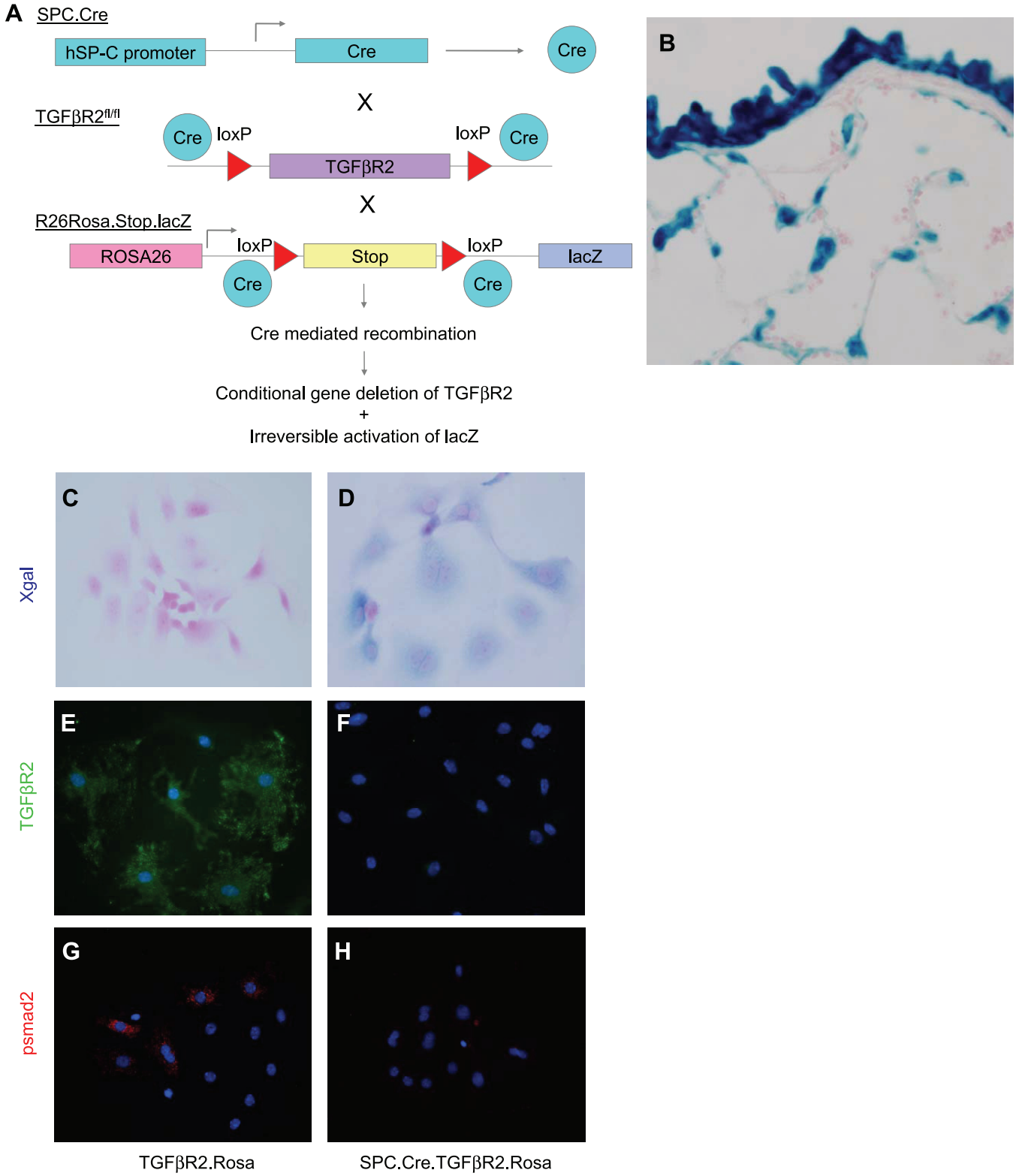

Fig. 1. Development of transgenic model for deficiency of transforming growth factor-β (TGFβ) receptor 2 (TGFβR2) in lung epithelium. *A*: schematic illustrating how the combination of the 3 individual transgenic mice results in the desired model. *B*: Xgal staining in a lung section from an untreated adult SPC.Cre.TGFβR2$^{fl/fl}$.R26Rosa.Stop.lacZ mouse. Magnification ×600. *C* and *D*: Xgal staining of type II alveolar epithelial cells (AECs) isolated from transgenic mice with TGFβR2 deficiency in lung epithelium (SPC.Cre.TGFβR2.Rosa) demonstrated positive blue staining (*D*) as opposed to type II AECs from littermate controls (TGFβR2.Rosa), which were not Xgal-positive (*C*). *E* and *F*: immunofluorescence (green) for TGFβR2 demonstrated that TGFβR2 expression was absent in type II AECs isolated from mice with TGFβR2 deleted from lung epithelium (*F*) but was readily observed in type II AECs isolated from littermate controls (*E*). *G* and *H*: isolated type II AECs were exposed to TGFβ (10 ng/ml), and immunofluorescence (red) was performed for phosphorylated smad2 (psmad2). There was evidence of pmad2 expression in many of the AECs from mice with intact lung epithelial cell TGFβR2, while psmad2 expression was markedly attenuated in AECs from mice with TGFβR2 deficiency in lung epithelium.

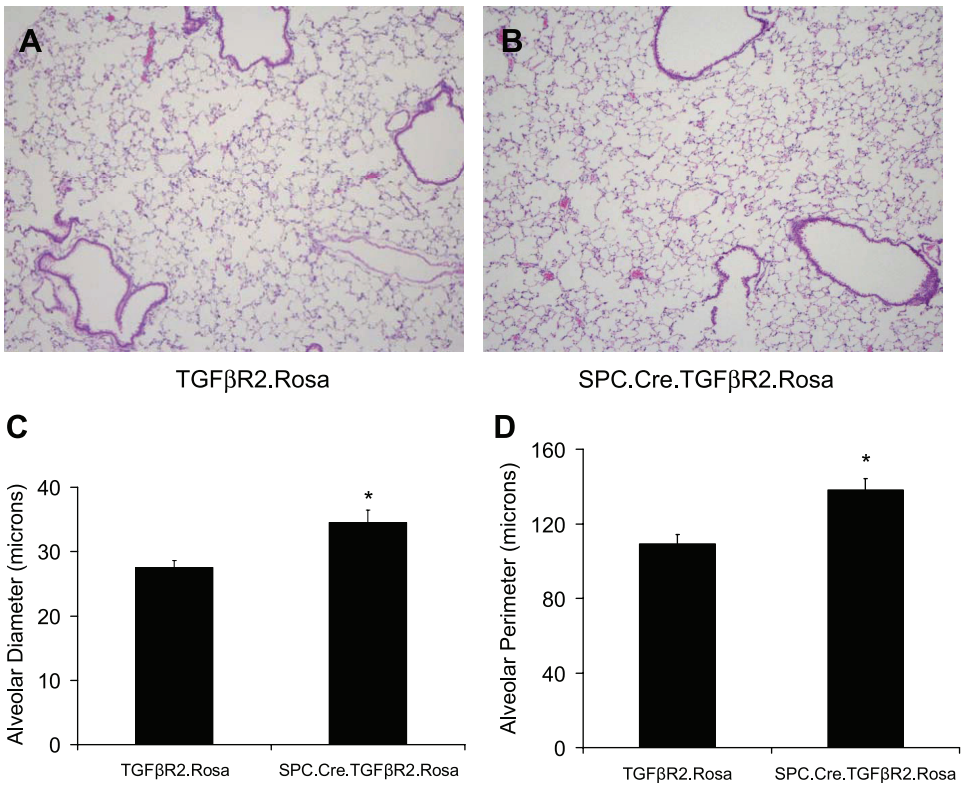

Fig. 2. Mice with deficiency of TGFβR2 in lung epithelium had normal-appearing lung architecture on gross examination of histology but, by morphometric measurements, had slightly enlarged alveolar spaces compared with controls. *A* and *B*: hematoxylin-eosin-stained sections of lung from a mouse with intact TGFβR2 in lung epithelium and a mouse with TGFβR2 deficiency in lung epithelium. Magnification ×100. *C* and *D*: in mice with TGFβR2 deficiency in lung epithelium, mean alveolar diameter and mean alveolar perimeter were slightly increased relative to littermate controls ($n = 3$ in each group). *$P < 0.05$ vs. TGFβR2.Rosa.

mice with TGFβR2 deficiency in lung epithelium had markedly fewer TUNEL-positive epithelial cells than littermate controls (Fig. 3, *A–C*), illustrating that TGFβ signaling in lung epithelium contributes to AEC death in the bleomycin model. When type II AECs were isolated from the transgenic mice and exposed to bleomycin in vitro for 24 h, a trend toward greater AEC death was noted in mice with intact AEC expression of TGFβR2 than in AECs with deficiency of TGFβR2, as determined by LDH cytotoxicity assay (Fig. 3*D*).

Because of its known anti-inflammatory effects, we next wanted to determine if interrupting TGFβ signaling in lung epithelium would impact lung inflammation after bleomycin administration. For these studies, BAL was performed at baseline and 2 wk after bleomycin administration, and cell counts and differentials were performed. In the untreated state, total BAL leukocytes, macrophages, neutrophils, and lymphocytes were similar between mice with lung epithelial cell TGFβR2 deficiency and littermate controls (Fig. 4). At

Table 1. *Downregulated genes in type II AECs from mice 2 wk after bleomycin with lung epithelial TGFβR2 deficiency and controls*

Gene Symbol	Gene Name	Expression Relative to Control	95% CI	*P* Value
Amhr2	Anti-Mullerian hormone type 2 receptor	0.35	0.20–0.50	0.030478
Chrd	Chordin	0.36	0.19–0.53	0.033344
Col1a2	Collagen, type 1, α_2	0.45	0.27–0.63	0.032059
Eng	Endoglin	0.58	0.45–0.71	0.015407
Gdf3	Growth differentiation factor 3	0.36	0.17–0.55	0.021924
Gdf6	Growth differentiation factor 6	0.35	0.19–0.51	0.039985
Gdf7	Growth differentiation factor 7	0.44	0.20–0.68	0.031946
Gsc	Goosecoid homeobox	0.35	0.21–0.49	0.003772
Inha	Inhibin-α	0.37	0.20–0.54	0.021689
Inhbb	Inhibin-β_B	0.59	0.49–0.69	0.003695
Lefty1	Left-right determination factor 1	0.38	0.21–0.55	0.029466
Ltbp4	Latent TGFβ binding protein 4	0.48	0.27–0.69	0.049865
Nodal	Nodal	0.38	0.21–0.55	0.011002
Nog	Noggin	0.40	0.23–0.57	0.007869
Nr0b1	Nuclear receptor subfamily 0, group B, member 1	0.13	0.001–0.28	0.023193
Tdgf1	Teratocarcinoma-derived growth factor 1	0.47	0.33–0.61	0.010635
Tgfb3	TGFβ3	0.41	0.23–0.59	0.022743

Gene symbol and gene name were based on *Mus musculus* nomenclature. Expression relative to control, determined by transforming growth factor (TGF) β/bone morphogenetic protein PCR array, is calculated as level of expression for alveolar epithelial cells (AECs) with deficiency of TGFβ receptor 2 (TGFβR2) compared with AECs with intact TGFβR2 (SPC.Cre.TGFβR2.Rosa.lacZ/TGFβR2.Rosa.lacZ). CI, confidence interval.

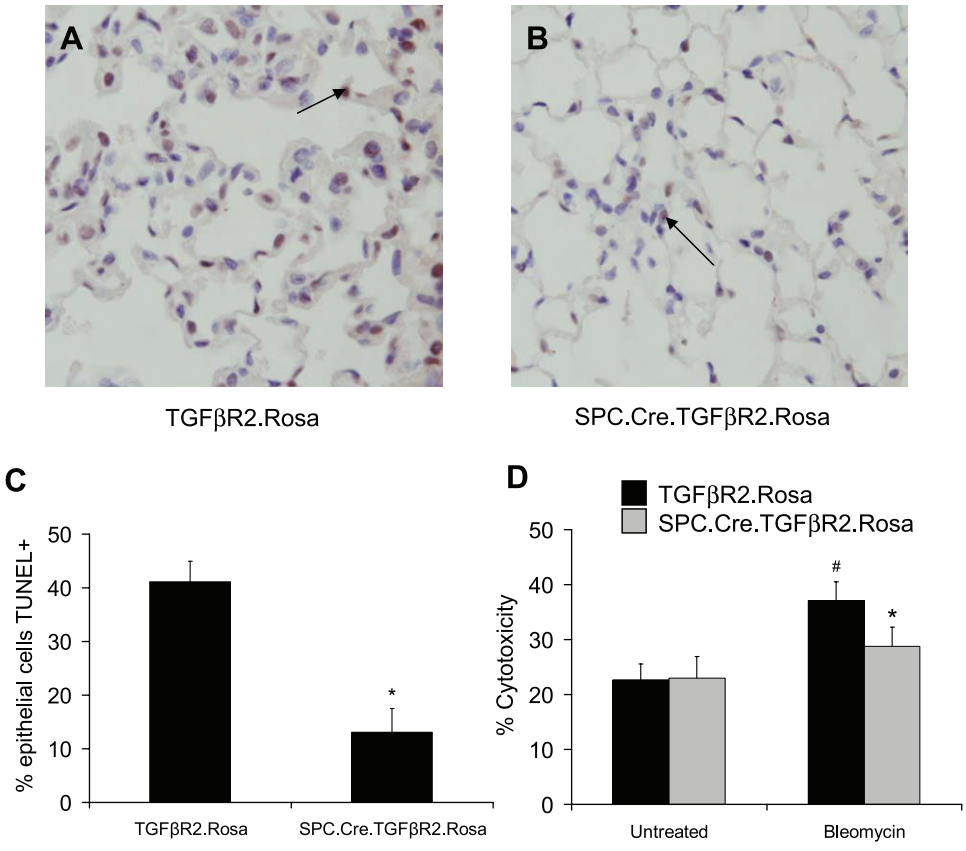

Fig. 3. Mice with deficiency of TGFβR2 in lung epithelium had less AEC death following bleomycin. Fewer terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL)-positive epithelial cells were observed in mice with deficiency of TGFβR2 in lung epithelium at 1 wk after bleomycin administration. *A* and *B*: representative lung sections from a mouse with intact TGFβR2 expression in lung epithelium (*A*) and a mouse with deficiency of TGFβR2 in lung epithelium (*B*). Arrows point to representative TUNEL$^+$ cells. Magnification ×600. *C*: when quantitated per high-power field, a lower percentage of cells were TUNEL$^+$ in mice with TGFβR2 deficiency in lung epithelium ($n = 5$ mice per group). *$P < 0.01$ vs. TGFβR2.Rosa. *D*: type II AECs were isolated from SPC.Cre.TGFβR2$^{fl/fl}$.R26Rosa.Stop.lacZ mice and littermate controls and then exposed to bleomycin (0.1 U in 2 ml of culture medium) in vitro for 24 h. Cell death was evaluated by lactate dehydrogenase cytotoxicity assay. No statistical difference in cell death was noted between AECs in the untreated state or after exposure to bleomycin ($n = 3$ mice in each group). #$P < 0.05$, untreated vs. bleomycin-treated AECs from control mice. *$P = 0.1$ between both groups of AECs treated with bleomycin.

2 wk after bleomycin administration, total BAL leukocytes and macrophages were similar in SPC.Cre.TGFβR2$^{fl/fl}$.Rosa.Stop.lacZ mice and littermate controls (Fig. 4, *A* and *B*). However, total BAL neutrophils and lymphocytes were greater in mice with lung epithelial cell deficiency of TGFβR2 than in littermates with intact TGFβR2 expression (Fig. 4, *C* and *D*). Taken together, these studies show that TGFβ signaling in AECs has opposing effects on epithelial

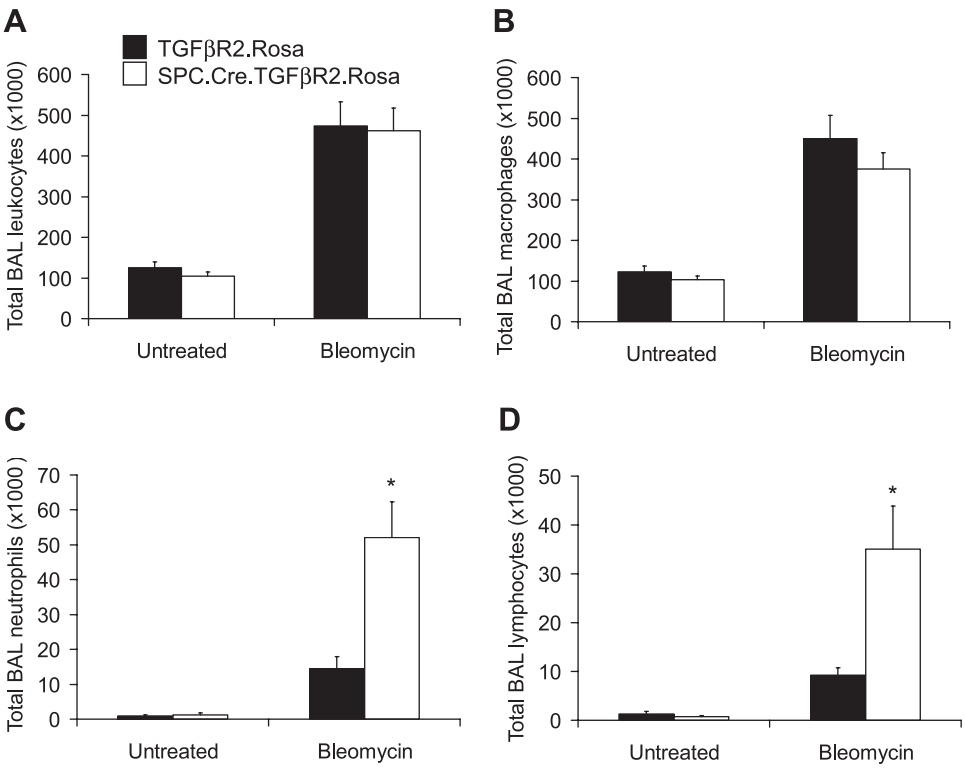

Fig. 4. Lung inflammation was increased in mice with TGFβR2 deficiency in lung epithelium following intratracheal bleomycin administration. At baseline, total bronchoalveolar lavage (BAL) leukocytes, macrophages, neutrophils, and lymphocytes were similar between mice with TGFβR2 deficiency in lung epithelium and controls. After bleomycin administration, mice with lung epithelial cell TGFβR2 deficiency had similar numbers of total BAL leukocytes (*A*), similar numbers of total BAL macrophages (*B*), greater numbers of total BAL neutrophils (*C*), and greater numbers of total BAL lymphocytes (*D*) than mice with intact TGFβR2 in lung epithelium ($n = 3$ untreated mice and 5 bleomycin-treated mice). *$P < 0.05$ vs. TGFβR2.Rosa. For TGFβR2.Rosa and SPC.Cre.TGFβR2.Rosa mice, BAL total leukocytes, macrophages, neutrophils, and lymphocytes increased after bleomycin administration ($P < 0.05$).

cell survival and inflammation in response to bleomycin treatment.

Mice with deficiency of TGFβR2 in lung epithelium have attenuated lung fibrosis and decreased numbers of lung fibroblasts. At 3 wk after bleomycin administration, lungs from SPC.Cre.TGFβR2$^{fl/fl}$.Rosa.Stop.lacZ mice and littermate controls were harvested to evaluate development of lung fibrosis. As noted on trichrome-stained lung sections, mice with lung epithelial cell deletion of TGFβR2 were relatively protected from bleomycin-induced lung fibrosis. SPC.Cre.TGFβR2$^{fl/fl}$.Rosa.Stop.lacZ mice had less architectural distortion and smaller fibrotic areas than littermates (Fig. 5, *A* and *B*), with reduced lung fibrosis by semiquantitative scoring and lung hydroxyproline quantitation (Fig. 5, *C* and *D*).

TGFβ has a significant impact on the lung fibroblast population, including effects on fibroblast recruitment and activation. Recently, it has been shown to regulate EMT in the lungs (56), a process by which epithelial cells can directly contribute to the lung fibroblast population (13, 23, 24, 47). In previous studies, we showed a peak in the number of S100A4$^+$ lung fibroblasts at 2 wk after bleomycin administration (31, 47). For these experiments, we harvested lungs for frozen section from SPC.Cre.TGFβR2$^{fl/fl}$.Rosa.Stop.lacZ and SPC.Cre.Rosa.Stop.lacZ (control) mice, allowing us to maintain cell fate-mapping capabilities in mice with and without lung epithelial cell TGFβR2 deficiency. Immunofluorescence for β-gal (indicative of lung epithelial cell origin) and the fibroblast markers S100A4, vimentin, and α-smooth muscle actin (α-SMA) was performed. Detection of EMT-derived fibroblasts was suboptimal with vimentin and α-SMA. Immunofluorescence for vimentin lacked specificity because of high levels of background staining. With immunofluorescence for α-SMA, EMT-derived α-SMA-positive cells were rare in the lung after bleomycin administration in both groups. In contrast, S100A4 served as a better marker for this evaluation. At baseline, dual β-gal$^+$/S100A4$^+$ cells were very rarely encountered in SPC.Cre.TGFβR2$^{fl/fl}$.Rosa.Stop.lacZ or SPC.Cre.Rosa.Stop.lacZ mice. However, at 2 wk following bleomycin administration, mice with intact lung epithelial cell TGFβR2 expression had more lung fibroblasts (based on S100A4 expression) and more EMT-derived fibroblasts (dual β-gal$^+$/S100A4$^+$ cells) than mice with lung epithelial cell TGFβR2 deficiency (Fig. 6). Taken together, these results demonstrate that lung epithelial cell TGFβ signaling impacts epithelial cell-fibroblast interactions and fibroblast recruitment, modulating the degree of extracellular matrix deposition and regulating the degree of lung fibrosis after bleomycin administration.

DISCUSSION

Over the past two decades, multiple lines of evidence have shown that AECs have a pivotal role in the pathogenesis of pulmonary fibrosis. Furthermore, over this same time period, TGFβ has been clearly implicated as a principal profibrotic cytokine involved in the regulation of lung fibrosis. Here, we define the intersection of lung epithelial control of fibrotic remodeling and TGFβ pathway signaling in the bleomycin model of pulmonary fibrosis. Deficiency of TGFβR2 in lung epithelium was found to be protective from bleomycin-induced lung fibrosis. Furthermore, lung epithelial cell TGFβR2 deficiency was associated with less AEC death, increased lung inflammation, and attenuation of fibroblast numbers, including fibroblasts derived via EMT, in mice treated with intratracheal bleomycin compared with wild-type controls. With this model, TGFβR2 is deleted in the entire lung epithelium, so it is possible that TGFβ signaling in the epithelium at several

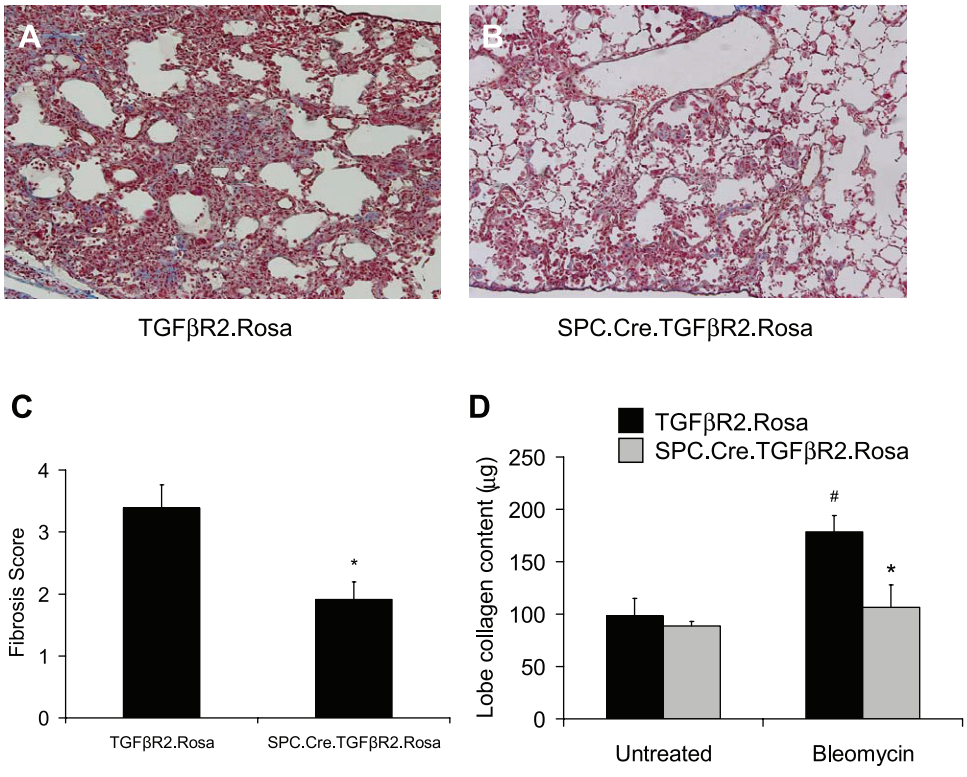

Fig. 5. Lung fibrosis was attenuated in mice with TGFβR2 deficiency in lung epithelium at 3 wk after bleomycin administration. *A* and *B*: representative images of trichrome-stained lung sections from a mouse with intact TGFβR2 expression in lung epithelium and a mouse with TGFβR2 deficiency in lung epithelium. Magnification ×400. *C*: lung fibrosis scored on trichrome blue-stained lung sections reveals a lower score with deficiency of TGFβR2 in lung epithelium (n = 9 TGFβR2.Rosa mice and 12 SPC.Cre.TGFβR2.Rosa mice). *$P < 0.05$ vs. TGFβR2.Rosa. *D*: microplate hydroxyproline assay reveals lower lung lobe (right upper lobe + right middle lobe) collagen content in lungs from mice with deficiency of TGFβR2 in lung epithelium at 3 wk after bleomycin administration (n = 4 untreated and 7 bleomycin-treated mice). *$P < 0.05$ between bleomycin-treated mice. #$P < 0.05$, untreated vs. bleomycin-treated TGFβR2.Rosa.

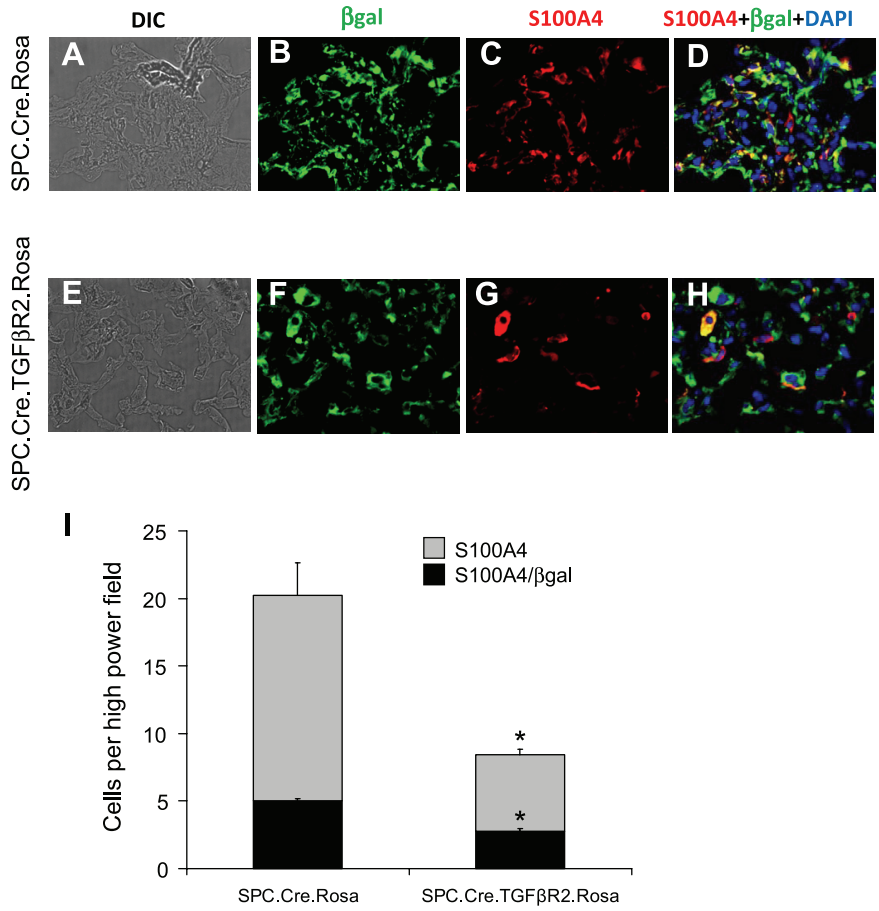

Fig. 6. Fewer total and epithelial-mesenchymal transition (EMT)-derived fibroblasts were observed in mice with TGFβR2 deficiency in lung epithelium at 2 wk after bleomycin administration. Confocal microscopy was performed to detect fluorescent immunostaining for β-gal (green), S100A4 (red), and 4′,6-diamidino-2-phenylindole (DAPI, blue) in lung epithelial cell fate reporter mice with intact TGFβR2 expression (SPC.Cre.Rosa) and TGFβR2 deficiency (SPC.Cre.TGFβR2.Rosa). *A–H*: representative images from lung sections from mice 2 wk after a single intratracheal dose of 0.08 U of bleomycin. Dual-fluorescent (yellow) cells indicate S100A4$^+$ fibroblasts derived from lung epithelial lineage. DIC, differential interference contrast. Magnification ×600. *I*: fewer total S100A4$^+$ cells and dual S100A4$^+$/β-gal$^+$ cells per high-power field (HPF) were observed in mice with TGFβR2 deficiency in lung epithelium ($n = 5$ mice in each group). *$P < 0.05$ vs. SPC.Cre.Rosa.

different levels of the tracheobronchial tree, not just the AEC population, contributes to lung fibrosis.

On the basis of reports from the evaluation of human IPF lung tissue and experimental animal models, TGFβ appears to be the principal profibrotic cytokine involved in the pathogenesis of pulmonary fibrosis. TGFβ expression has been shown to be upregulated in lung biopsies from individuals with IPF, and immunohistochemistry studies have localized TGFβ to areas of fibroblastic foci (6, 10, 22). Furthermore, animal models have greatly delineated the profibrotic nature of TGFβ in experimental lung fibrosis. TGFβ expression is increased in the lungs of mice following exposure to bleomycin, and inhibition of TGFβ pathways attenuates the severity of bleomycin-induced lung fibrosis, as demonstrated by studies using a soluble TGFβ receptor (52) or a TGFβR1 inhibitor (5). Prior studies have demonstrated that epithelial expression of TGFβ induces fibrotic remodeling in the lungs. Delivery of an adenovirus expressing active TGFβ into the lung, an approach first reported by Sime et al. in 1997 (44), results in significant lung fibrosis in rats and mice (25, 44, 55). In addition, Lee et al. (32) showed that expression of active TGFβ by Clara cell 10-positive lung epithelial cells results in lung fibrosis. Both of these modalities overexpress TGFβ in the lung, but their effects are not restricted to the epithelial cell population and have the potential for diverse effects on multiple cell populations. A remaining question has been the critical effector cells for TGFβ signaling in the lungs. Prior to our studies, the most compelling evidence linking TGFβ signaling in the epithelium to fibrosis comes from studies involving integrin-$\alpha_v\beta_6$, which is expressed only on epithelial cells and activates latent TGFβ (20). Mice deficient in the β_6-subunit have impaired activation of TGFβ at the cell surface and are protected from lung fibrosis following bleomycin (37) and radiation (40). While localized TGFβ activation by integrin-$\alpha_v\beta_6$ has the potential to directly activate other cells in the microenvironment, our transgenic model is designed such that targeted attenuation of TGFβ signaling occurs only in lung epithelium. Taken together, these results demonstrate that lung epithelium can participate in lung fibrosis through expression of TGFβ and signaling through TGFβ receptors.

Prior studies by other investigators have also targeted deletion of TGFβR2 in different cell populations in the lung. Recently, Hoyles et al. (19) reported that deletion of TGFβR2 in resident lung fibroblasts in the first 2 wk after birth resulted in less lung fibrosis and decreased numbers of fibroblasts and fibrocytes when the mice were given intratracheal bleomycin as adults. When considered in conjunction with our current studies, this demonstrates how TGFβ signaling in different cell populations can impact lung remodeling. In 2008, using a doxycycline-dependent transgenic system to delete TGFβR2 in lung epithelium, Chen and colleagues (8) found postnatal lung developmental abnormalities, including retardation of alveolarization, enlargement of alveolar spaces, and decreased numbers of type I AECs. Recently, Li et al. (33) reported a model similar to ours, in which TGFβR2 was deleted in lung epithelium using the Nkx2.1 promoter, instead of the SP-C promoter,

and also noted enlargement of the alveolar spaces and a relative protection from bleomycin-induced lung fibrosis. In our present study, we also noted that the alveolar spaces of mice with lung epithelial cell deficiency of TGFβR2 were slightly enlarged compared with littermate controls, but there was no evidence of true alveolar destruction, which is seen in emphysema, and, in fact, the mice appeared healthy and grew normally into adulthood. Whether this preexisting difference in alveolar size impacts the response to bleomycin is not known, but it may be a contributing factor.

AEC apoptosis has been implicated as a key component of pulmonary fibrosis in human forms of disease and in animal models (35, 49). In IPF lung biopsies, AEC apoptosis is observed in regions adjacent to areas of heavy myofibroblast activity and collagen deposition (28, 50). Animal studies have shown that bleomycin-induced lung injury is associated with AEC apoptosis (16, 27, 34) and that inhibition of apoptosis attenuates fibrosis (29, 53). Recently, Sisson et al. (45) targeted diphtheria toxin to type II AECs, resulting in increased AEC death and the development of lung fibrosis. We noted that lung epithelial cell deficiency of TGFβR2 was associated with a decrease in AEC death at 1 wk after bleomycin administration, and it is possible that this finding could explain, at least in part, the decreased fibrosis noted with this model. Taken together with the findings of Lee et al. (32) that overexpression of TGFβ induces apoptosis in AECs, our data support the idea that TGFβ-induced AEC apoptosis is key for profibrotic effects of this cytokine.

TGFβ is expressed by all cell types and, depending on the environment and cell type expressing this cytokine, can exact seemingly opposite effects. As such, it has been shown to have prominent anti- and proinflammatory effects (51). However, classic studies in which TGFβ signaling is disrupted imply that it has significant anti-inflammatory properties (43). Mice expressing a null mutation for TGFβ1 have marked inflammation, leading to early lethality (26). Furthermore, mice deficient in integrin-$\alpha_v\beta_6$, which activates latent TGFβ to its active form, develop chronic inflammation in the lungs and skin (20). In the present study, we noted an increase in the number of neutrophils and lymphocytes in BAL from mice with lung epithelial cell TGFβR2 deficiency at 2 wk after bleomycin administration compared with mice with intact TGFβR2 in lung epithelium, illustrating that epithelial cell-specific regulation of TGFβ signaling impacts recruitment of inflammatory cells to the lung; however, the precise mechanism explaining this observation remains unclear. When considered in conjunction with the observation of increased apoptosis of AECs in mice with epithelial TGFβR2 deficiency, our findings demonstrate that the extent of AEC cell death does not necessarily predict the level of the inflammatory response in the lungs after bleomycin administration.

TGFβ has prominent effects on the lung fibroblast population, the effector cells responsible for the deposition of extracellular matrix in lung fibrosis. Multiple in vitro studies have shown that TGFβ induces recruitment and activation of fibroblasts, leading to greater production of collagen, other extracellular matrix components, and other profibrotic cytokines (11, 41). One of the ways that TGFβ may lead to increased lung fibroblast numbers is through EMT. In 2005, Willis et al. (56) demonstrated that TGFβ1 exposure to RLE6TN rat lung epithelial cells could induce EMT in vitro. Subsequently, using a lung epithelial cell fate-mapping model, Kim et al. (23) demonstrated that the delivery of active TGFβ by adenoviral vector resulted in EMT-derived lung fibroblasts in areas of fibrosis. In subsequent studies using cell fate-mapping models, Kim et al. and our laboratory demonstrated that EMT occurs in vivo in the bleomycin model (13, 24, 47). Here, we demonstrate that lung epithelial cell TGFβR2 deficiency is associated with a decrease in the number of lung fibroblasts, both total and derived via EMT. Interestingly, however, these studies demonstrate that EMT can occur even with interruption of the TGFβ signaling cascade, and, in fact, the percentage of EMT-derived S100A4$^+$ fibroblasts was similar in mice with lung epithelial cell TGFβR2 deficiency and cell fate-mapping controls: 32 ± 12% in SPC.Cre.TGFβR2$^{fl/fl}$.Rosa.Stop.lacZ mice and 25 ± 4% in SPC.Cre.Rosa.Stop.lacZ mice (P = NS). Our study results suggest two interesting points regarding fibroblast recruitment: *1*) TGFβ signaling in AECs impacts EMT-derived fibroblasts and recruitment or proliferation of fibroblasts of other origin as well, and *2*) EMT can occur in the absence of TGFβ signaling in AECs. The mechanisms by which EMT occurs in the absence of TGFβ signaling could be an interesting topic for future investigations.

In summary, our studies define a prominent role for epithelial TGFβ signaling in regulating the fibrotic process in the lungs and suggest that specific targeting of this cell population could be beneficial in the development of new therapies for fibrotic lung diseases.

GRANTS

This study was supported by National Institutes of Health (NIH) Grants HL-85406 (W. E. Lawson), HL-85317 and HL-92870 (T. S. Blackwell), HL-87738 (A. L. Degryse), and DK-80219 (C. S. Boomershine); National Center for Research Resources (NCRR) Grant UL1 RR-024975; an American Lung Association Dalsemer Research Grant (W. E. Lawson); an American Thoracic Society/Coalition for Pulmonary Fibrosis Research Grant and the Francis Families Foundation (H. Tanjore); IPFNet Cowlin Career Development Award (A. L. Degryse); and the Department of Veterans Affairs (W. E. Lawson, T. S. Blackwell, and C. S. Boomershine). H. Tanjore is a Parker B. Francis Fellow in Pulmonary Research. This work was also supported in part by Vanderbilt Clinical and Translational Science Award Grant 1 UL1 RR-024975 from the NCRR/NIH.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

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