The molecular mechanisms of pulmonary fibrosis are poorly understood. Previous reports indicate that activation of TGF-β is essential for the development of pulmonary fibrosis. Here, we report that the proapoptotic Bcl-2 family member Bid is required for the development of pulmonary fibrosis after the intratracheal instillation of bleomycin. Mice lacking Bid exhibited significantly less pulmonary fibrosis in response to bleomycin compared with WT mice. The attenuation in pulmonary fibrosis was observed despite similar levels of inflammation, lung injury, and active TGF-β1 in bronchoalveolar lavage fluid 5 days after the administration of bleomycin in mice lacking Bid and in WT controls. Bleomycin induced similar levels cell death in vitro in alveolar epithelial cells isolated from WT and bid−/− mice. By contrast, alveolar epithelial cells from bid−/− mice were resistant to TGF-β1-induced cell death. These results indicate that Bcl-2 family members are critical regulators for the development of pulmonary fibrosis downstream of TGF-β1 activation.

### Results

**Bid-Null Mice Are Protected from Bleomycin-Induced Pulmonary Fibrosis.** Previous studies have suggested that preventing epithelial cell death can prevent pulmonary fibrosis induced by the intratracheal instillation of bleomycin (17–19). However, none of the previous strategies of preventing cell death inhibited specific intracellular death pathways. To address the role of the mitochondrial-dependent cell death pathway in the regulation of pulmonary fibrosis, bleomycin was instilled intratracheally into WT and bid−/− mice. By 21 days, WT mice exposed to bleomycin displayed profound fibrosis as assessed by Masson–Trichrome staining (Fig. 1A and B). By contrast, bid−/− mice exposed to bleomycin did not exhibit fibrosis at day 21. Total lung collagen, as assessed by the Sircol assay, was also significantly higher in WT mice than in bid−/− mice (Fig. 1C). Collectively, these results indicate that loss of Bid prevents bleomycin-induced fibrosis.

**Bid Is Not Required for Bleomycin-Induced Lung Injury.** The fibrotic response due to intratracheal instillation of bleomycin is a late response. The initial response to bleomycin is lung injury, which is characterized by flooding of the alveolar space with protein-rich exudates and inflammation (20). To determine whether Bid is required for the early inflammatory response to bleomycin, histological analysis, bronchoalveolar lavage (BAL) fluid cellularity, and protein levels were assessed in WT and bid−/− mice 5 days after exposure to bleomycin. WT and bid−/− mice both displayed histological evidence of inflammation at day 5 (Fig. 2A). WT and bid−/− mice demonstrated a significant increase in inflammatory cells in the BAL fluid (Fig. 2B and C). Protein in the BAL fluid was elevated to a similar degree in the two genotypes (Fig. 2D). To further assess lung injury, we measured the increases in lung permeability to albumin 5 days after treatment with intratracheal bleomycin in WT mice and Bid-null mice. WT and Bid-null mice displayed similar increased levels of lung permeability to albumin (Fig. 2E). Collectively, these

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Abbreviations: BAL, bronchoalveolar lavage; ARDS, acute respiratory distress syndrome.

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the activation of TGF-β1 (21–25). To determine whether loss of Bid regulates the activation of TGF-β1, BAL fluid from WT and bid<sup>−/−</sup> mice was assessed for active TGF-β1 levels 5 days after the instillation of bleomycin or PBS. The level of active TGF-β1 in BAL fluid of PBS-treated mice in both genotypes was low (Fig. 3). Mice treated with bleomycin displayed comparable levels of active TGF-β1 in both genotypes (Fig. 3). Thus, the lack of fibrosis in Bid-null mice is not due to diminished levels of active TGF-β1.

**TGF-β1-Induced Cell Death Requires Bid.** We have previously demonstrated that overexpression of Bcl-X<sub>L</sub> prevents bleomycin-induced cell death in rat alveolar type II cells, indicating the activation of the mitochondrial-dependent death pathway in response to bleomycin (26). To decipher whether the BH3 protein Bid is the upstream regulator of mitochondrial death pathway in response to bleomycin, mouse alveolar epithelial cells from WT and bid<sup>−/−</sup> mice were exposed to bleomycin (10 or 100 milliunits/ml) for 48 h. Alveolar epithelial cells from WT and bid<sup>−/−</sup> mice displayed similar levels of cell death in response to bleomycin (Fig. 4A). Because Bid is not required for bleomycin-induced alveolar epithelial cell death, we decided to examine the role of Bid in TGF-β1-induced alveolar epithelial cell death. TGF-β1 has been shown to induce cell death in multiple epithelial cells, including alveolar epithelial cells (27). TGF-β1 induced cell death in alveolar epithelial cells isolated from WT mice. However, TGF-β1-induced cell death was not observed in alveolar epithelial cells from bid<sup>−/−</sup> mice (Fig. 4A). To test in vivo that loss of Bid does not prevent bleomycin-induced apoptosis, we measured TUNEL-positive cells at day 5. WT and Bid-null mice both displayed similar levels of TUNEL-positive cells (Fig. 4B). These results indicate that Bid is not required for the initial cell death induced by bleomycin but is required for the later TGF-β1-induced cell death.

**Loss of Bid Does Not Affect TGF-β1-Induced Fibroblast Proliferation.** The lack of fibrosis in the Bid-null mice could also be due to a defect in fibroblast proliferation in response to TGF-β1. To test whether Bid affects fibroblast proliferation, fibroblasts from WT and bid<sup>−/−</sup> mice were subjected to TGF-β1 under low-serum conditions. TGF-β1 induced fibroblasts from WT and bid<sup>−/−</sup> mice to proliferate at similar rates, indicating that the Bid-null mice do not have a defect in fibroblast proliferation (Fig. 5A). We further examined whether Bid-null fibroblasts have an impaired response to death stimuli. Bid-null fibroblasts were not resistant to the classical intrinsic apoptotic stimulus staurosporine (Fig. 5B). As expected, the Bid-null fibroblasts were fairly resistant to the classical extrinsic apoptotic stimulus TNFα plus cycloheximide (28). Collectively, these results indicate that lack of fibrosis observed in Bid-null mice is not due to dysregulation of fibroblast survival or proliferation.

**Discussion**

Recent studies have implicated apoptosis of alveolar epithelial cells as a potential initiating mechanism in the development of pulmonary injury and fibrosis. Pharmacologic inhibition of the apoptotic pathways with caspase inhibitors has been shown to prevent bleomycin-induced fibrosis (6, 18). Activation of caspases by the administration of FasL, which activates the receptor-dependent, apoptotic pathway, can induce pulmonary fibrosis (17). Furthermore, a variety of strategies that have been shown to prevent bleomycin-induced pulmonary fibrosis, for example the overexpression of heme oxygenase-1 (HO-1) or loss of angiotensin receptor AT1a gene, might exert their protective effects by preventing alveolar epithelial cell death (29, 30). The intracellular pathway(s) that result in the development of alveolar epithelial cell death and pulmonary fibrosis have not been elucidated. In the present study, we demonstrate that mice

results indicate that loss of Bid does not alter the initial lung injury due to bleomycin.

**Active TGF-β1 Levels in BAL Fluid Are Not Affected by Bid.** The fibrotic response to bleomycin has been shown to be mediated by
lacking the proapoptotic protein Bid are protected from bleomycin-induced pulmonary fibrosis. Bid is a BH3 protein that can activate the proapoptotic Bcl-2 family members Bax or Bak to initiate the release of cytochrome c into the cytosol to trigger caspase activation (9, 15, 16). Our findings demonstrate a requirement for the mitochondrial-dependent cell death pathway in the development of pulmonary fibrosis.

Protection of bid<sup>−/−</sup> mice from pulmonary fibrosis was not explained by a decrease in the pulmonary inflammatory response to bleomycin. In fact, Bid-null mice displayed more inflammatory cells, particularly neutrophils, in the BAL fluid 5 days after the administration of bleomycin. Similarly, BAL levels of protein, lung permeability, and histologic evidence of lung injury were slightly worse in bid<sup>−/−</sup> animals. The higher levels of inflammation may result from impaired apoptosis of inflammatory cells in bid<sup>−/−</sup> mice. It is formally possible that the elevated levels of inflammation in Bid-null mice might be protective against the development of fibrosis. Future studies using bone marrow chimeras will have to address whether the increase in inflammation observed in the bid-null mice is protective against the development of pulmonary fibrosis. However, our results are consistent with previous published reports indicating that the inflammatory response can be dissociated from the fibrotic response. For example, mice lacking the integrin αvβ6 display an enhanced inflammatory response to bleomycin compared with WT controls but failed to develop pulmonary fibrosis after the

Fig. 2. Bid is not required for bleomycin-induced lung injury. (A) WT and bid<sup>−/−</sup> mice both display histologic evidence of inflammation 5 days after the intratracheal instillation of bleomycin (0.045 units). Representative images are shown at ×200 magnification (hematoxylin/eosin stain). Shown are cell counts with differential (B and C), protein in BAL fluid (D), and lung permeability to FITC-labeled albumin (E) from WT and bid<sup>−/−</sup> mice at 5 days after the intratracheal instillation of bleomycin (0.045 units) or PBS. Data are expressed as means (±SEM) from four different animals. *, P < 0.05 relative to PBS-treated mice.
administration of bleomycin (21). These results suggest that lung inflammation is a marker for the acute lung injury rather than the cause of the ensuing fibrosis. Taken together, these findings indicate that Bid is required for the late fibrosis but not the early lung injury after the intratracheal instillation of bleomycin.

Bleomycin induced lung injury, and fibrosis is mediated by the activation of TGF-β. TGF-β in the lung is normally secreted as an inactive complex that is bound to a latency-associated peptide (LAP), a protein that is formed by cleavage of the amino terminus of the TGF-β gene product. LAP prevents binding of mature TGF-β with its receptors. Binding of LAP to αvβ6 integrin alters the conformation of the complex to activate TGF-β (21). Accordingly, mice deficient in the αvβ6 integrin, which fail to activate TGF-β, were completely protected from bleomycin-induced lung fibrosis. The overexpression of active TGF-β in mice leads to profound fibrosis, and inhibition of TGF-β by antibodies abrogates bleomycin-induced fibrosis (22–24). Furthermore, blocking intracellular signaling pathways activated by TGF-β, for example activin receptor-like kinase 5 (ALK5) or Smad3, prevents activated TGF-β-induced fibrosis (25, 31). The fact that Bid-null mice exhibit similar level of active TGF-β compared with WT mice suggests that loss of Bid prevents TGF-β from inducing downstream affects. The initial bleomycin-induced apoptosis in epithelial cells might result in the activation of TGF-β that is independent of Bid. Indeed, the degree of early apoptosis after bleomycin administration was similar in WT and bid−/− animals. There yet might be another BH3 protein that is required for the initial apoptosis elicited by intratracheal instillation of bleomycin that results in activation of TGF-β.

TGF-β is classically a mitogen for lung fibroblasts but has recently been shown to induce mouse alveolar epithelial cell death through the early growth response gene Egr-1 and caspase activation (27). Our present data indicate that loss of Bid does not alter fibroblast proliferation in response to TGF-β. However, our data do demonstrate that Bid is required for TGF-β-induced cell death in alveolar epithelial cells. Based on these observations, we propose a model where the presence of TGF-β...
sustains a low level of epithelial apoptosis to allow a permissive environment for fibroblast invasion and proliferation (32). The mechanism underlying TGF-β1 activation of Bid to trigger mitochondrial-dependent apoptosis in alveolar epithelial cells remains unknown. One possibility would be the engagement of Fas by the type II TGF-β1 receptor. However, in the C57BL/6 strain of mice, the Fas-dependent pathway is dispensable for bleomycin-induced fibrosis (30, 33).

In summary, our data provide two important insights into the mechanisms underlying bleomycin-induced pulmonary fibrosis. First, we have identified that the mitochondrial-dependent cell death pathway mediated by the proapoptotic Bcl-2 family member BID is required for the development of bleomycin-induced pulmonary fibrosis. Second, our results dissociate the early inflammation and lung injury in response to the administration of bleomycin from the fibrotic response. Although the bleomycin-induced model of pulmonary fibrosis is used as an animal model of idiopathic pulmonary fibrosis, it also shares common features with the acute respiratory distress syndrome (ARDS). ARDS has an early phase that is characterized by inflammation and the accumulation of fluid and protein in the alveolar space (34). The latter phase of ARDS develops in a subset of patients and is characterized by fibroproliferation. We have recently shown that active TGF-β1 is present in BAL fluid from ARDS patients (35). The observation that Bid-null mice are protected from fibrosis but not lung injury suggests that strategies to inhibit the mitochondrial-dependent cell death pathway downstream of the activation of TGF-β1 might prevent fibrosis in patients with acute lung injury. This would suggest that there is a window of opportunity to prevent ARDS-associated fibroproliferation.

Materials and Methods

Mice and Bleomycin Administration. The bid−/− mice were backcrossed to C57BL/6 for 12 generations and were kindly provided by the late S. Korsmeyer (28). WT C57BL/6 mice were purchased from The Jackson Laboratory. Age- and sex-matched 8- to 10-week-old mice were used in all experiments, WT mice and bid−/− mice were treated with a single intratracheal injection of 50 µl of PBS or bleomycin (0.045 units in 50 µl of PBS; Sigma-Aldrich). All experiments were approved by the Northwestern University Animal Care and Use Committee.

Histology. A 20-gauge angiocath was sutured into the trachea, the lungs and heart were removed en bloc, and the lungs were inflated to 25 cm of H2O with PBS and then heated to 60°C in PBS. The lungs were then inflated with 0.8 cc of 4% paraformaldehyde. The heart and lungs were fixed in paraffin, and 5-µm sections were stained with hematoxylin/eosin or Masson’s Trichrome.

BAL Analysis. BAL was performed through a 20-gauge angiocath ligated into the trachea. A 1.0-ml aliquot of PBS was instilled into the lungs and then carefully removed three times. A 200-µl aliquot of the BAL fluid was placed in a cytospin and centrifuged at 500 × g for 5 min. The glass slides were Wright stained and subjected to a blinded manual cell count and differential. The remaining BAL fluid was centrifuged at 130 × g for 5 min, and the supernatant was used for the measurement of BAL protein (Bradford) and cytokine analysis.

Sircoll Assay. Total collagen content was determined by harvesting lungs from mice 21 days after treatment with bleomycin or PBS. Exsanguination was accomplished by a left nephrectomy, followed by perfusion of the right ventricle with 2 ml of cold PBS. Both lungs were removed and homogenized (1 min with a tissue homogenizer followed by 12 strokes in a Dounce homogenizer) in 1 ml of PBS. Aliquots of lung homogenate were then assayed for total lung collagen levels and compared with a standard curve prepared from rat tail collagen by using the Sircoll collagen dye binding assay (BioColor Ltd., Newtownabbey, U.K.) according to the manufacturer’s directions.

Active TGF-β1 Analysis. BAL fluid (above) from animals exposed to either PBS or bleomycin was analyzed for active TGF-β1 levels by using the Emax ImmunoAssay System according to the manufacturer’s directions (Promega). TGF-β1 levels were measured from fresh isolated BAL fluid.

Isolation and Culture of Alveolar Epithelial Cells. Alveolar type II cells were isolated from WT and bid−/− mice. Briefly, the lungs were perfused via the pulmonary artery, lavaged, and digested with elastase (1 mg/ml, Worthington). Those cells were purified by negative immunoselection by using magnetic beads, followed by differential adherence to CD90 pretreated dishes. Cell viability was assessed by trypan blue exclusion (>95%). Cells were suspended in DMEM containing 10% FBS with 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured at an air–liquid interface of 0.4-µm transwell membranes inserted into six-well culture dishes to eliminate fibroblast contamination. Cells were incubated in a humidified atmosphere of 5% CO2/95% air at 37°C and used 4 days after isolation.

Isolation and Culture of Mouse Lung Fibroblasts. Fibroblast cells were isolated from male C57BL/6. Briefly, the lungs were perfused via the pulmonary artery, lavaged, and digested with elastase (1 mg/ml; Worthington) and 0.25% trypsin in the presence of 1 mM EDTA. Lung tissue was dissected from the airways, minced into 2-mm3 pieces, and placed in tissue culture flasks for 15–20 min in a humidified incubator at 37°C under 5% CO2 atmosphere with a minimal volume of minimum essential media (MEM) supplemented with 20% FBS, 50 units/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine. An appropriate volume of media was then added to the flasks, and cells were maintained for 7–10 days or until fibroblasts began to migrate out from the tissue. Identification of fibroblasts was based on the presence of vimentin staining.

TUNEL Assay. End labeling of exposed 3′-OH ends of DNA fragments in paraffin-embedded tissue was done by using the TUNEL AP In Situ Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer’s directions. After staining, 20 fields of alveoli (× 400) were randomly chosen, and TUNEL-positive nuclei were counted.

Assessment of Lung Permeability. Five days after treatment with intratracheal bleomycin (0.045 units in 50 µl of PBS) or PBS, WT and bid−/− mice were anesthetized, and a 20-gauge angiocath was sutured into the trachea. The mice were mechanically ventilated (Minivent; Harvard Apparatus) with a respiratory rate of 100 and a tidal volume of 0.2 ml. A midline laparotomy was performed, and the inferior vena cava was identified into which 0.15 ml of a 16 mg/ml solution of FITC-labeled albumin (Sigma–Aldrich) in 5% BSA was injected. After 10 min, a bronchoalveolar lavage was performed. Relative lung permeability was estimated from the fluorescence in the BAL fluid measured by using a microplate reader (excitation = 488 nm, emission = 530 nm).

Statistical Analysis. Results are expressed as mean ± SEM. Data were analyzed by using one-way analysis of variance (ANOVA). When ANOVA indicated that a significant difference was present, we explored individual difference with the Student t test using Bonferroni correction for multiple comparisons. Statistical significance was determined at 0.05 level. All groups were compared with WT mice treated with PBS.
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