ORIGINAL RESEARCH

Epithelial Deletion of Sulf2 Exacerbates Bleomycin-Induced Lung Injury, Inflammation, and Mortality

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Abstract

Epithelial injury has been proposed to be the initiating factor in the pathogenesis of idiopathic pulmonary fibrosis (IPF). We have shown previously that heparan sulfate 6-*O*-endosulfatase (Sulf) 2 is overexpressed in the hyperplastic type II alveolar epithelial cells (AECs) in the IPF lungs. By removing 6-*O*-sulfates from specific heparan sulfate intrachain sites, Sulf2 modulates the functions of many growth factors and cytokines. In this study, we hypothesized that Sulf2 plays a regulatory role in alveolar epithelial injury and repair, using the murine bleomycin model. Consistent with our findings in human IPF lungs, bleomycin treatment in mice resulted in up-regulation of Sulf2 mRNA in whole-lung extracts and overexpression of Sulf2 protein in type II AECs on lung tissue sections. Sulf2 protein was detectable in bronchoalveolar lavage fluid at baseline, and its level was significantly increased after bleomycin exposure. To study the role of Sulf2 in alveolar injury and repair *in vivo*, we generated a doxycycline-inducible epithelial-specific Sulf2 conditional knockout (Sulf2 CKO) mouse line. After bleomycin exposure, Sulf2 CKO mice exhibited enhanced neutrophil infiltration in the lung, with elevated levels of total protein, lactate dehydrogenase, and cytokines (granulocyte colony–stimulating factor and interferon-γ-inducible protein 10) in bronchoalveolar lavage fluid compared with wild-type littermates. We further showed that both the p53–p21 DNA damage response and the transforming growth factor-β1 signaling pathway were up-regulated in Sulf2 CKO mice compared with wild-type. Finally, Sulf2 CKO mice suffered increased mortality after bleomycin exposure. In conclusion, Sulf2 expression in type II AECs plays a protective role in epithelial injury, inflammation and mortality.

Keywords: heparan sulfate; Sulf2; p53; bleomycin; type II alveolar epithelial cell

Clinical Relevance

Previously, we have shown that heparan sulfate 6-*O*-endosulfatase 2 (Sulf2) is overexpressed in the hyperplastic type II alveolar epithelial cells in idiopathic pulmonary fibrosis. Using a conditional epithelial-specific Sulf2 knockout mouse model, we show in this study that Sulf2 plays a protective role in epithelial injury, inflammation, and mortality, establishing Sulf2 as an important regulator of lung injury and repair.

Idiopathic pulmonary fibrosis (IPF) is a progressive, debilitating, and ultimately fatal disease with limited therapeutic options (1). The pathogenic mechanisms involved in the initiation and progression of IPF are still poorly understood. The current paradigm suggests that ongoing or recurrent epithelial injury results in deficient regeneration of normal alveolar structure and aberrant repair processes, leading to fibroblast activation and excessive extracellular matrix (ECM) production in the lung (2, 3). In response to repetitive injury and in attempts to repair the damaged epithelium, type II alveolar epithelial cells (AECs) in IPF undergo a hyperplastic and metaplastic transformation with reactivation of fetal gene programs, including the Wnt/β-catenin and Sonic Hedgehog signaling pathways (4–6).

After injury, type II AECs act as the facultative progenitors in the alveoli, with the capability to both replace themselves as well as differentiate into type I AECs. The importance of type II AECs in alveolar repair and regeneration is underscored by

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animal studies. In the murine bleomycin-induced lung injury and fibrosis model, intratracheal delivery of a purified population of syngeneic type II AECs was sufficient to block fibrotic lung remodeling (7), whereas targeted ablation of type II AECs resulted in abnormal repair with extensive lung fibrosis (8). In conditions where extensive alveolar injury obliterates type II AECs in the alveoli, progenitors located at the bronchoalveolar duct junctions have been shown to differentiate into type II AECs for the repair of damaged alveolar epithelium (9, 10).

Our laboratory has reported previously that heparan sulfate (HS) 6-*O*-endosulfatase 2 (Sulf2) is overexpressed and specifically localized to the hyperplastic type II AECs in the IPF lungs (11). HS is the glycosaminoglycan side chains of the HS proteoglycans, major components of the cell surface, basement membrane, and the ECM (12). Through binding with numerous growth factors and cytokines, the HS side chains mediate many of the biological functions of the HS proteoglycans (13). Sulf2 is one of the two HS-specific Sulfs capable of modifying HS 6-*O*-sulfation status outside of the cells (14). By removing 6-*O*-sulfates from specific HS intrachain sites at the cell surface and/or in the ECM, the Sulfs alter the interaction between HS and HS-binding proteins, and thus HS-dependent cell signaling events and ECM remodeling. For example, canonical Wnt ligands show high-affinity binding to 6-*O*-sulfated HS, which prevents its functional interactions with Frizzled receptor; by removing 6-*O*-sulfates, the Sulfs have been shown to enhance Wnt signaling by restoring active Wnt–Frizzled interaction (15, 16). In contrast, Sulf activity inhibits fibroblast growth factor (FGF)-2 signaling by removing the 6-*O*-sulfate moiety necessary for high-affinity FGF2–HS–FGF receptor ternary complex formation (17, 18). HS 6-*O*-sulfation is also important for transforming growth factor (TGF)-β1–HS interaction, and our laboratory has shown previously that enhancing HS 6-*O*-sulfation by small interfering RNA-mediated silencing of Sulf2 up-regulates TGF-β1 signaling in type II AECs (11).

Sulf2 has been shown to be pro-oncogenic in a multitude of human malignancies, including lung cancer (14). Sulf2 is up-regulated in non–small-cell lung carcinomas, and Sulf2 expression promotes transformed behavior of lung cancer cells *in vitro* and their tumorigenicity *in vivo* (19). The role of Sulf2 in other lung diseases, including IPF, has not been previously studied. The aim of this study was to examine the regulatory role of Sulf2 in the process of lung injury and repair using the murine bleomycin model.

Materials and Methods

Generation of Inducible Epithelial-Specific Sulf Knockout Mice

All animal protocols were prepared in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Louisiana State University Health Sciences Center.

All mice used were in the C57BL/6 background. $Sulf1^{f/f}Sulf2^{f/f}$ mice were kindly provided by Dr. Xingbin Ai (Brigham and Women's Hospital, Boston, MA) (15) and crossed with transgenic mice expressing surfactant protein C (SFTPC)–reverse tetracycline transactivator (rtTA) and tetracycline operator cre-recombinase (tetO-cre) transgenes (Jackson Laboratory, Bar Harbor, ME). Genotyping was performed as described previously (15). The specificity and efficiency of doxycycline (dox)-inducible cre expression was confirmed by crossing these mice with ROSA:lxp-stp-lxp:tdTomato reporter mice (Jackson Laboratory) and by immunostaining directly for cre (*see* Figure E1 in the online supplement). Sulf conditional knockout (CKO) mice and wild-type (WT) littermates (8–10 wk of age) were fed dox-containing diet (Harlan Laboratory, Indianapolis, IN) for 3 weeks before bleomycin administration, and were maintained on dox-containing diet throughout the experiments. Only male mice were used in the *in vivo* bleomycin experiments, whereas both males and females were used for type II AEC isolation.

Bleomycin Administration

Mice were anesthetized with ketamine/xylazine (150/10 mg/kg, intraperitoneally) and intubated with a 22-G catheter (EXEL International, Los Angeles, CA) using the Biolite mouse intubation kit (BioTex, Houston, TX). Bleomycin (Sigma, St. Louis, MO) at 0.3–1 U/kg in PBS was administered into the lung through the 22-G catheter.

Tissue Harvest and Analysis of Bronchoalveolar Lavage Fluid

After animals were killed with ketamine/xylazine, left lungs were tied off and snap frozen in liquid nitrogen for RNA and protein extraction. Right lungs were lavaged three times with 0.6 ml of PBS plus 2 mM EDTA. Supernatant from first lavage was used for measurement of bronchoalveolar lavage fluid (BALF) total protein (BCA Assay; Pierce Biotechnology, Rockford, IL), lactate dehydrogenase (Cytotoxicity Assay; Promega, Madison, WI), cytokine levels (Multiplex Assay; EMD Millipore, Billerica, MA), and Sulf2 by Western blotting (15 μl/lane), as previously described (11). Cells from all three lavages were combined for BAL differential cell count (Diff-Quik; Siemens, Newark, DE). After BALF collection, right lungs were perfused with Z-Fix (Anatech, Battle Creek, MI) through a tracheal cannula at a pressure of 25 cm H_2O for 12 minutes followed by fixation in Z-Fix for at least 24 hours before processing for paraffin embedding and sectioning.

Immunohistochemistry

Immunostaining for Sulf2 (2B4, 1:200; Novus Biologicals, Littleton, CO), pro-surfactant protein C (SPC) (1:1,000; EMD Millipore) and p21 (1:100; BD Biosciences, San Jose, CA) were performed as described previously (11) using diaminobenzidine (brown) or VIP (purple) as the enzyme substrates (Vector Laboratories, Burlingame, CA).

Type II AEC Isolation

Type II AECs were isolated using the dispase/agarose protocol as previously described (11), with negative selection for CD45, CD16/32, and CD31, followed by positive selection for epithelial cell adhesion molecule (BD Biosciences) using the magnetic cell separation system (Miltenyi Biotec, San Diego, CA).

Statistical Analysis

Data are expressed as mean (±SEM). Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). A P value less than 0.05 was considered statistically significant.

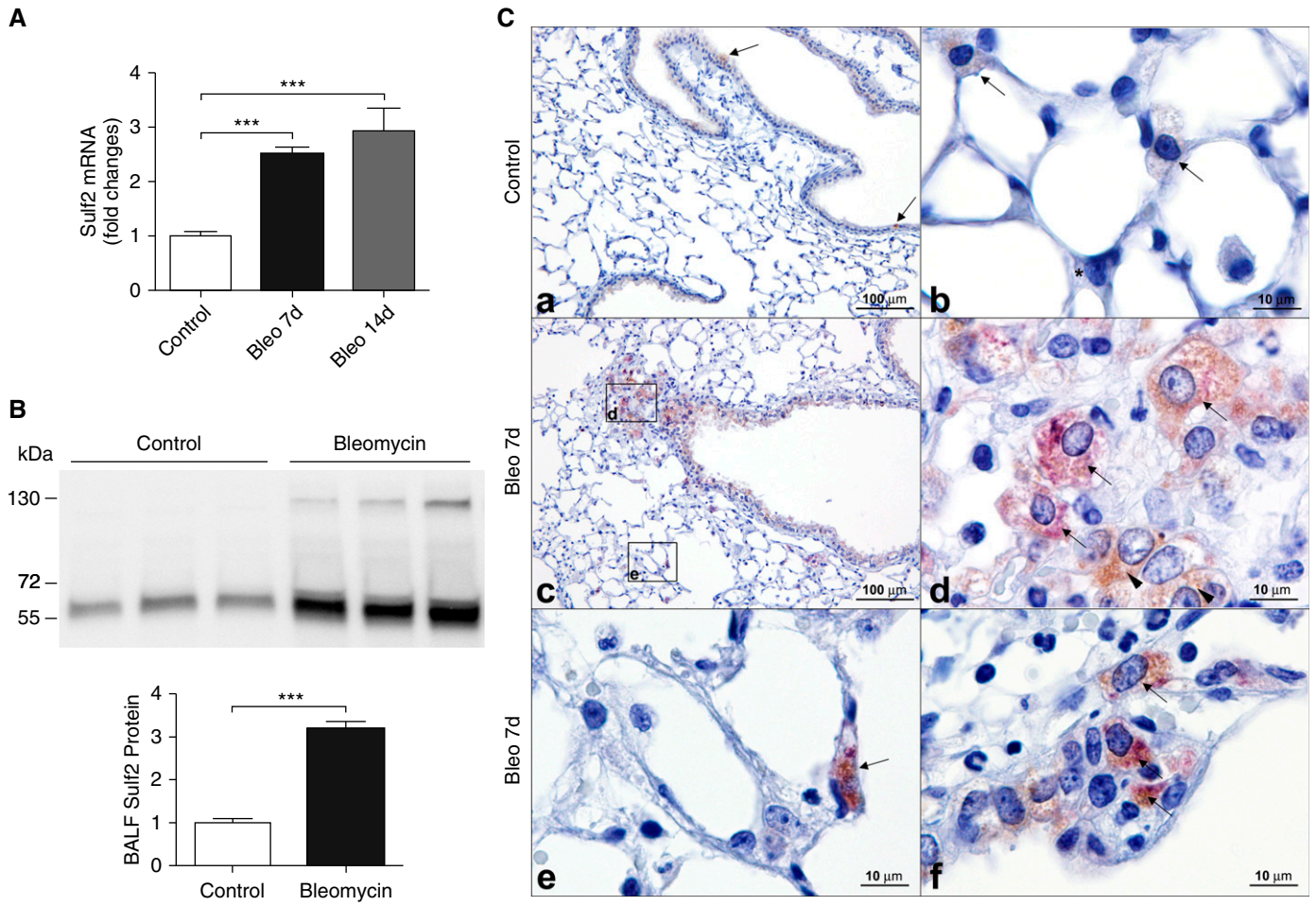

Figure 1. Overexpression of heparan sulfate 6-*O*-endosulfatase 2 (Sulf2) in bleomycin-induced lung injury in mice. (*A*) Sulf2 mRNA expression in total lung extracts from control (PBS, $n = 6$) and bleomycin (bleo)-treated mice (bleo 7 d, 0.7 U/kg, $n = 8$; bleo 14 d, 0.5 U/kg, $n = 4$) were analyzed by quantitative RT-PCR (qRT-PCR). Data were normalized to the expression of housekeeping gene, Tbp (TATA box binding protein). (*B*) Sulf2 protein in bronchoalveolar lavage fluid (BALF) from control and bleo-treated mice (0.7 U/kg at 7 d) were analyzed by Western blotting. ***$P < 0.001$. (*C*) Immunolocalization of Sulf2 in control and bleo-treated mice. (*a* and *b*) Immunostaining for Sulf2 (diaminobenzidine, *brown*) in control mice; *arrows*, Sulf2-positive bronchial epithelial cells or type II alveolar epithelial cells (AECs); *asterisk*, a Sulf2-negative type II AEC. (*c*–*f*) Double immunostaining for Sulf2 (DAB, *brown*) and pro-surfactant protein C (SPC) (*purple*) in bleo-treated mice mice; *arrows*, Sulf2 and pro-SPC double-positive cells; *arrowheads*, Sulf2 single-positive cells. (*d* and *e*) Enlarged views of *insets* in *c*. (*f*) Another example of Sulf2 and pro-SPC double-positive cells at the bronchoalveolar duct junction. *Scale bars*: 10 μm and 100 μm.

Additional detailed methods are described in the online supplement.

Results

Overexpression of Sulf2 in Bleomycin-Induced Lung Injury in Mice

In our published report, we showed that treatment of primary murine type II AECs *in vitro* with bleomycin induces Sulf2 expression at both mRNA and protein levels (11). Bleomycin, a chemotherapeutic agent used in the treatment of multiple human malignancies, is the most commonly used agent to induce lung injury and fibrosis in animal models (20). By inducing DNA strand breaks (21) and oxidative injury (22), bleomycin exposure leads to epithelial injury and inflammation that peaks at 7 days, and ultimately fibrosis at 14–21 days (20). We first examined Sulf2 mRNA expression in the murine lungs at 7 and 14 days after bleomycin exposure. As shown in Figure 1A, similar to human IPF, Sulf2 mRNA was increased in total lung extracts from mice treated with bleomycin at both time points.

Immunohistochemistry revealed that, at baseline, Sulf2 protein expression (brown) was low (Figure 1C, *b*, *arrows*) or undetectable (Figure 1C, *b*, *asterisk*) in type II AECs. High Sulf2 protein expression was only observed in select bronchial epithelial cells (Figure 1C, *a*, *arrows*), consistent with a previous report showing high Sulf2 mRNA expression in the bronchial epithelium in normal adult murine lung (23). After bleomycin exposure, intense Sulf2 staining (*brown*) was observed in surviving type II AECs (pro-SPC positive, *purple*) in badly damaged areas (Figure 1C, *e*, *arrow*) as well as in type II AECs in the bronchoalveolar duct junctions (Figure 1C, *d* and *f*, *arrows*). The Sulf2 and pro-SPC double-positive cells likely represent the newly generated type II AECs from the progenitors located in

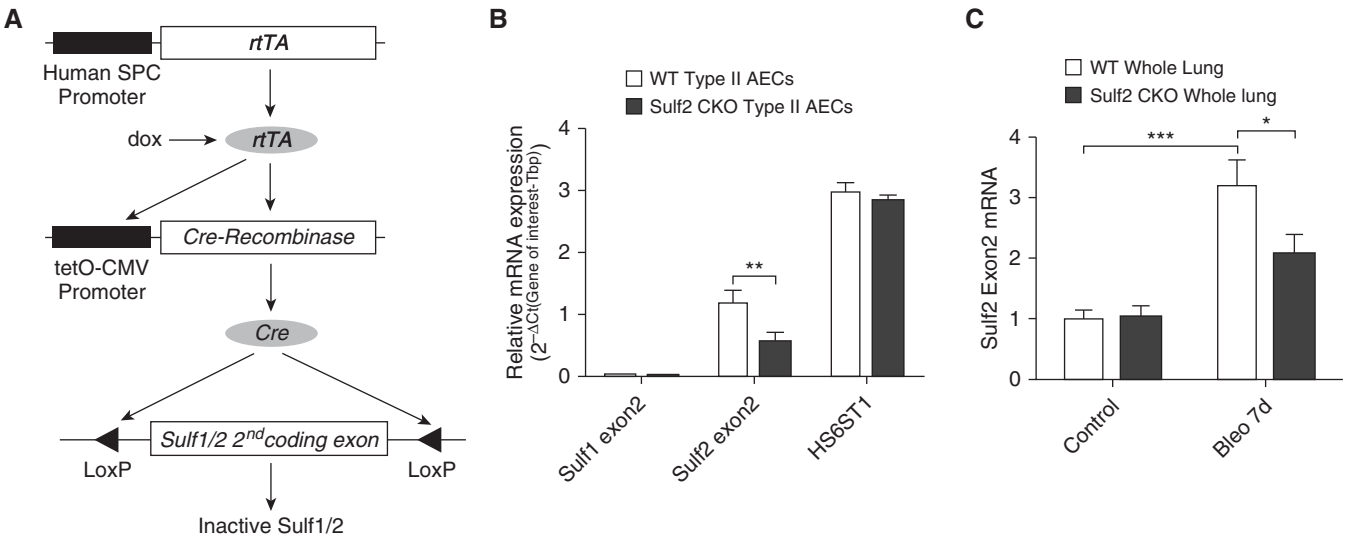

Figure 2. Generation of inducible epithelial-specific Sulf2 conditional knockout (CKO) mice. (*A*) Model. (*B*) Expression of Sulf1, Sulf2, and HS6ST1 in primary type II AECs from wild-type (WT; $n = 7$) and Sulf2 CKO mice ($n = 5$) after doxycycline (dox) administration. Relative mRNA expression in comparison to housekeeping gene, Tbp, is shown. The purity of type II AEC isolation was 80–90% based on pro-SPC expression (data not shown). (*C*) Expression of Sulf2 in WT and Sulf2 CKO mice after control (PBS) and bleo exposure. Relative mRNA expression normalized to housekeeping gene, Tbp, is shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. CMV, cytomegalovirus; *Cre*, cre-recombinase; LoxP, LoxP sites; rtTA, reverse tetracycline transactivator; tetO, tetracycline operator.

these regions after injury (9, 10). Indeed, a subset of these cells was also positive for the proliferation marker, Ki67 (Figure E2). Sulf2 staining observed was mainly intracellular, which assisted in identifying Sulf2-producing cells in the lung. As the Sulfs are associated with the cell surface through ionic interactions between the hydrophilic domains of the Sulfs and the cell surface HS chains (24, 25), it is likely that cell surface– as well as ECM-localized Sulf2 has been removed during the immunostaining procedure. The specificity of Sulf2 staining was demonstrated by substituting primary antibody with nonimmune mouse IgG at equivalent titer, and no staining was observed (data not shown).

Analysis of BALF with Western blotting showed that the C-terminal 50-kD subunit of Sulf2 was detectable in BALF at baseline, which was significantly increased after bleomycin exposure (Figure 1B). The full-length Sulf2 at 125 kD was also detectable in BALF from bleomycin-treated mice. The presence of Sulf2 in the BALF at baseline and its increase after bleomycin exposure indicate that Sulf2 may catalyze 6-*O*-desulfation of HS chains of not only the type II AECs, but also other cell types in the alveoli, including type I AECs, as well as the infiltrating inflammatory cells after injury.

Generation of Inducible Epithelial-Specific Sulf2 Knockout Mice

To study the role of Sulf2 in epithelial injury and repair *in vivo*, we generated a mouse line with dox-inducible epithelial-specific deletion of Sulfs using the human SP-C (SFTPC)/rtTA/$(tetO)_7$-cytomegalovirus-Cre system and mice with floxed Sulf alleles (Figure 2A). In this model, introduction of dox led to deletion of the second coding exons of both Sulf1 and Sulf2 genes in the human SPC promoter active epithelial cells in the lung (Figure E1). The second coding exons in both Sulf1 and Sulf2 genes encode for essential amino acid sequences within the enzymatic domains of the Sulfs; thus, deletion of this exon results in the production of truncated inactive enzymes (26). The inducible epithelial-specific Sulf CKO mice had the following genotype: $Sulf1^{f/f}Sulf2^{f/f}$ SFTPC-$rtTA^{+/-}$TetO-$cre^{+/-}$. The WT littermates lacked one or both of the transgenes: $Sulf1^{f/f}Sulf2^{f/f}$SFTPC-$rtTA^{+/-}$TetO-$cre^{-/-}$, $Sulf1^{f/f}Sulf2^{f/f}$SFTPC-$rtTA^{-/-}$TetO-$cre^{+/-}$, or $Sulf1^{f/f}Sulf2^{f/f}$ SFTPC-$rtTA^{-/-}$TetO-$cre^{-/-}$. Although both Sulfs were deleted in our model, analysis of isolated type II AECs showed that murine type II AECs express mainly Sulf2 (mRNA level of Sulf1 is ~100-fold lower than that of Sulf2; Figure 2B). Treatment with dox (3 wk on dox-containing diet) led to significant reduction of full-length Sulf2 mRNA (using primers specific for exon 2) in type II AECs isolated from Sulf CKO mice compared with WT littermates (Figure 2B). Thus, our model was essentially a Sulf2 CKO model (hereafter, Sulf2 CKO). Importantly, bleomycin-induced Sulf2 expression was significantly reduced in Sulf2 CKO mice compared with WT (Figure 2C). Deletion of Sulf2 exon 2 in epithelial cells cannot be detected when whole-lung RNA was analyzed at baseline (Figure 2C, control condition).

The expression of HS6ST1, the major HS 6-*O*-sulfotransferase expressed by type II AECs, was not altered by Sulf deletion (Figure 2B). Conditional deletion of the Sulfs did not alter gene expression examined in this study, either in the whole lung or in isolated type II AECs at baseline (Figures 4–6), and Sulf2 CKO mice appeared grossly indistinguishable from WT littermates. On lung tissue sections, however, we did observe some morphological changes (Figure E3). In 50% of Sulf2 CKO mice ($n = 6$) fed on dox-containing diet for 6–7 weeks, enlarged air spaces were observed in portions of the lung, which was not observed in any of the WT littermates ($n = 6$). The above finding was not entirely surprising, as systemic Sulf2 KO mice showed similar lung phenotype (27).

Epithelial Deletion of Sulf2 Exacerbates Bleomycin-Induced Lung Injury and Inflammation

We examined bleomycin-induced lung injury and inflammation in WT and Sulf2 CKO mice. At 7 days after bleomycin exposure, significantly enhanced inflammation was observed in Sulf2 CKO mice compared with WT littermates (Figure 3). Hematoxylin and eosin staining revealed that epithelial deletion of Sulf2 resulted in enhanced inflammatory cell infiltration in the lung parenchyma and greater destruction of alveolar structures (Figure 3A; higher-magnification images are provided in Figure E4). Analysis of inflammatory cell profile in the BALF revealed significantly increased neutrophils in BALF from Sulf2 CKO mice compared with WT (1.61×10^6 from Sulf2 CKO mice compared with 4.88×10^5 from WT mice; Figure 3B). In addition, 62 and 162% increase in total protein and lactate dehydrogenase levels, respectively, were observed in BALF from Sulf2 CKO compared with WT after bleomycin exposure (Figure 3C), indicating enhanced cytotoxicity. Analysis of BALF cytokine expression revealed increased levels of granulocyte colony–stimulating factor (G-CSF) and CXCL10 (interferon-γ-inducible protein 10) from Sulf2 CKO mice (Figure 3C). The increased level of G-CSF was consistent with the increased neutrophil cell count in BALF from Sulf2 CKO (Figure 3B). CXCL10 has been shown to orchestrate neutrophil migration into the lung in response to a variety of insults (28, 29), and likely performs similar roles in the bleomycin model. IL-6 was induced in both WT and Sulf2 CKO mice after bleomycin exposure, with a trend of greater increase in Sulf2 CKO mice. The murine IL-8 homolog, key chemokine (KC), a neutrophil chemoattractant, was

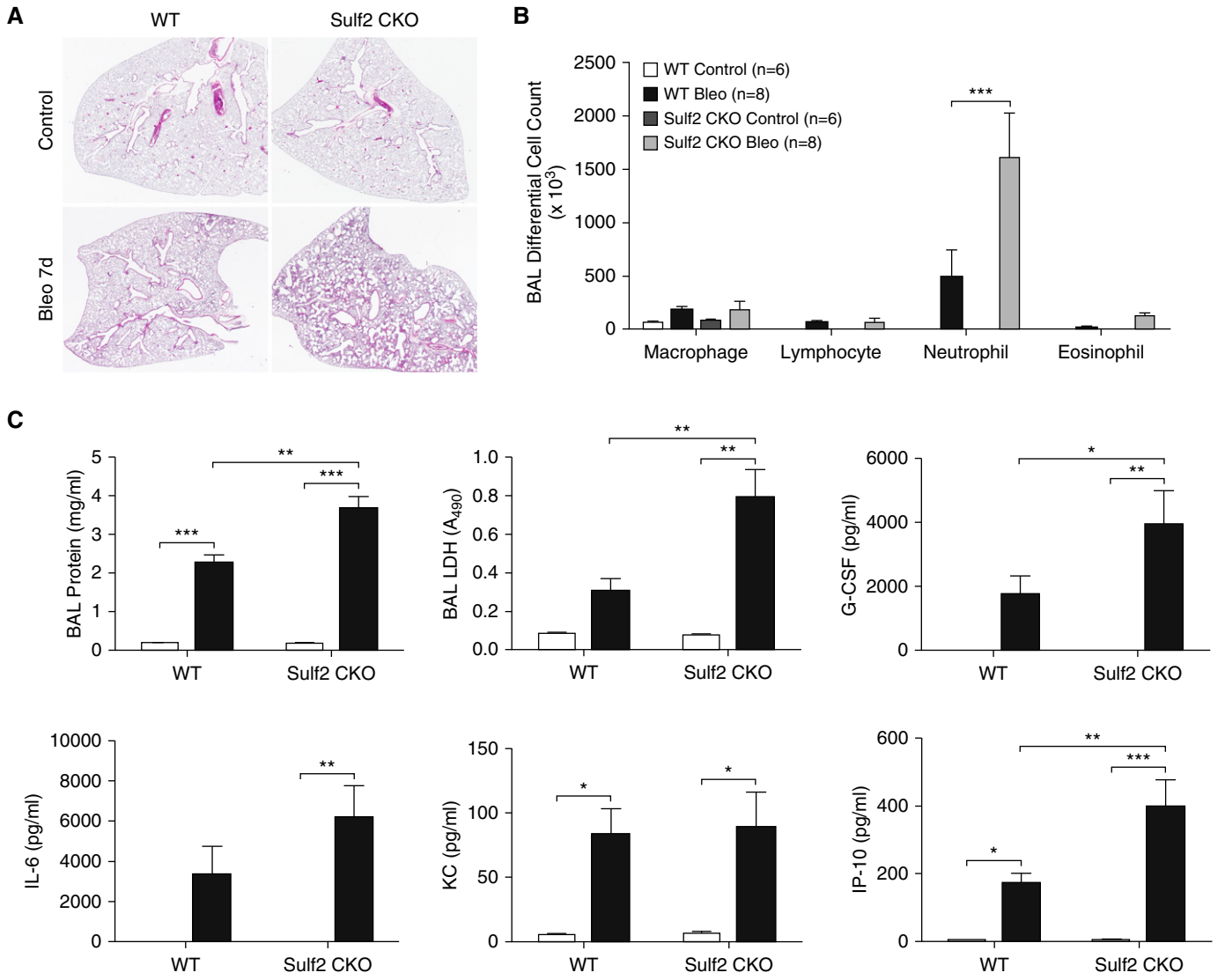

Figure 3. Sulf2 CKO mice exhibit enhanced injury and inflammation after bleo exposure. (*A*) Hematoxylin and eosin staining of lung sections from control and bleo (0.7 U/kg at 7 d)-treated WT and Sulf2 CKO mice. (*B*) BAL differential cell count from control and bleo-treated WT and Sulf2 CKO mice. (*C*) BAL total protein, lactate dehydrogenase (LDH) and cytokine levels from control and bleo-treated WT and Sulf2 CKO mice. *Open bars*, control; *solid bars*, bleo-treated mice (0.7 U/kg, 7 d). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. G-CSF, granulocyte colony-stimulating factor; IP-10, interferon-γ–inducible protein 10; KC, key chemokine.

increased similarly in both WT and Sulf CKO mice, indicating that the enhanced neutrophil infiltration in Sulf2 CKO mice was not due to the expression of KC. TNF-α and IL-1β were not significantly induced in the bleomycin model at the 7-day time point in either WT or Sulf2 CKO mice (data not shown).

Epithelial Deletion of Sulf2 Exacerbates Bleomycin-Induced p53–p21 DNA Damage Response

The p53 signaling pathway plays a central role in cellular response to DNA damage induced by bleomycin in the lung (30, 31). Thus, we examined the expression of p53 and two downstream effectors of p53, p21 and Bcl2-associated X protein (Bax), in WT and Sulf2 CKO mice after bleomycin exposure. The cyclin-dependent kinase inhibitor, p21, is a critical downstream effector of p53, and activates the cell cycle checkpoint arrest (G1 arrest) in response to DNA breaks, serving as a protective mechanism to allow the repair of the damaged DNA (32, 33); in cells with irreparably damaged DNA, however, p53 promotes apoptosis through the induction of Bax (34), an endogenous antagonist of B cell leukemia/lymphoma 2 (35). Our results show that both the total and the phosphorylated form (at serine 15) of p53 were significantly elevated in Sulf2 CKO mice (Figure 4A), with up-regulation of downstream effector p21, but not Bax, at the mRNA level in the lung (Figure 4B). Immunohistochemistry confirmed increased p21-positive cells in Sulf2 CKO mice after bleomycin exposure compared with WT (Figure 4C, *bottom panels*), consistent with p21 mRNA expression (Figure 4B). p21-positive cells were not found in either WT or Sulf2 CKO mice treated with PBS control (Figure 4C, *top panels*).

We further examined apoptosis by Western blotting for full-length and cleaved caspase-3 (active form) and by immunostaining for cleaved caspase-3 on lung tissue sections (Figure E5). Although the inactive full-length caspase 3 expression was increased in both WT and Sulf2 CKO mice after bleomycin exposure, the cleaved active form was undetectable in any of the samples. Consistent with this finding, cleaved caspase-3–positive cells were scarce in both WT and Sulf2 CKO mice after bleomycin exposure, and there was no difference between the two groups (Figure E5B).

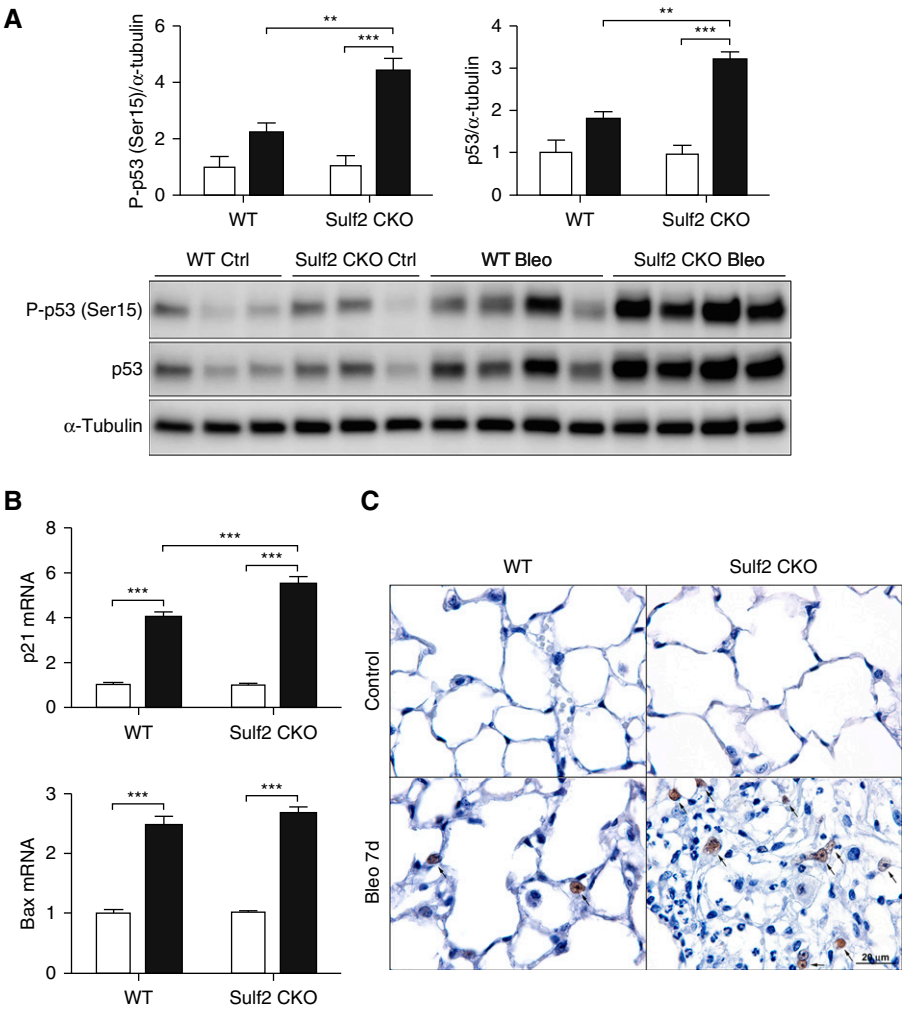

Figure 4. Sulf2 CKO mice exhibit enhanced p53–p21 signaling after bleo exposure. (*A*) Expression of total and phosphorylated (at serine 15) p53 was examined by Western blotting. (*B*) The mRNA expression of p21 and Bcl2-associated X protein (Bax) were examined by qRT-PCR and normalized to housekeeping gene, Tbp. *Open bars*, control; *solid bars*, bleo-treated mice (0.7 U/kg, 7 d). $^{**}P < 0.01$; $^{***}P < 0.001$. (*C*) Immunostaining of p21 on lung tissue sections from control and bleo-treated WT and Sulf2 CKO mice. *Arrows*, p21 positive cells (DAB, *brown*). *Scale bar*: 20 μm. Ctrl, control.

Epithelial Deletion of Sulf2 Up-Regulates TGF-β1 Signaling after Bleomycin Exposure

Total TGF-β1 levels in the BALF were examined by ELISA, which revealed significantly higher TGF-β1 in the BALF from Sulf2 CKO mice compared with WT mice after bleomycin exposure (Figure 5A). This increased TGF-β1 level was associated with enhanced Smad2 phosphorylation (activation) (Figure 5B) and up-regulation of collagen I mRNA expression (Figure 5C) in the lungs of Sulf2 CKO mice compared with WT mice. On lung tissue sections, Mason's trichrome staining revealed enhanced collagen deposition around the major vasculature in the Sulf2 CKO mice (Figure 5D). Significant collagen deposition was not detected in the alveoli region at the 7-day time point after bleomycin administration in either WT or Sulf2 CKO mice.

As Sulf2 has been shown to regulate Wnt signaling, we examined the expression of Wnt ligand Wnt5a (36), Wnt target gene, cyclin D1 (37), as well as total and active β-catenin. As shown in Figure E6, we did not observe significant differences between WT and Sulf2 CKO mice in any of the above Wnt signaling components at 7 days after bleomycin exposure.

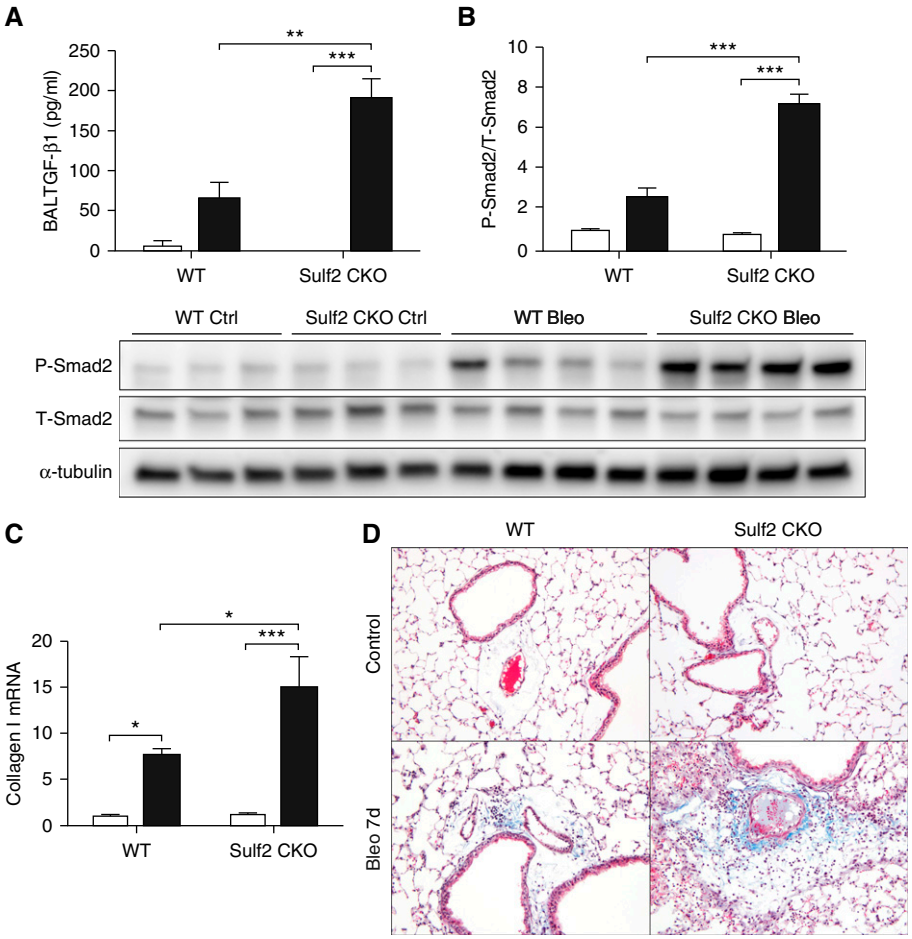

Figure 5. Sulf2 CKO mice exhibit enhanced transforming growth factor (TGF)-β1 signaling after bleo exposure. (*A*) BAL total TGF-β1 levels were examined by ELISA. (*B*) Expression of total and phosphorylated Smad2 was examined by Western blotting. (*C*) The mRNA expression of collagen I was examined by qRT-PCR and normalized to housekeeping gene, Tbp. *Open* bars, control; *solid* bars, bleo-treated mice (0.7 U/kg, 7 d). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (*D*) Mason's trichrome staining on lung tissue sections revealed enhanced collagen deposition around vasculatures in Sulf2 CKO mice after bleo exposure.

Type II AECs from Sulf2 CKO Mice Express Increased Levels of Inflammatory Cytokines and p53 Target Genes after Bleomycin Exposure

We further examined the expression of inflammatory cytokines and DNA damage response genes in freshly isolated type II AECs from WT and Sulf2 CKO mice after control or bleomycin exposure *in vivo*. To ensure the isolation of sufficient numbers of type II AECs, the bleomycin dosage was reduced to 0.3 U/kg with type II AECs isolated at 4–5 days after bleomycin administration. Our data indicate that type II AECs were a significant source of inflammatory cytokines, including G-CSF, IP-10, IL-6, and KC (Figures 6A–6D). In the absence of Sulf2, G-CSF and IP-10 mRNA levels were significantly increased after bleomycin exposure (Figures 6A and 6B). Bleomycin exposure also led to increased expression of IL-6 and KC in type II AECs from Sulf2 CKO mice compared with WT mice; however, this did not reach statistical significance, due to the large variability within the Sulf2 CKO group (Figures 6C and 6D). Consistent with the data from total lung extracts (Figure 4), p21 expression in type II AECs from Sulf2 CKO mice after bleomycin exposure was significantly increased compared with those from WT mice (Figure 6E). It is worth noting that both the magnitude of bleomycin-induced p21 expression and the difference in p21 expression between WT and Sulf2 CKO mice were much greater in isolated type II AECs compared with total lung extracts. Bleomycin induced 4- and 5.5-fold increase in p21 mRNA expression in whole-lung extracts from WT and Sulf2 CKO mice (37.5% increase over WT), respectively, compared with 12- and 25-fold increase in isolated type II AECs from WT and Sulf2 CKO mice (108% increase over WT). These results indicate that type II AECs were the primary target of bleomycin-induced injury, and data from isolated type II AECs more accurately reflected the effect of Sulf2 deletion. In contrast to the whole-lung extract (Figure 4B), we did observe a modest, but significant, increase in Bax expression in type II AECs isolated from bleomycin-challenged Sulf2 CKO mice compared with WT mice.

The expression of TGF-β1 was not significantly increased at the mRNA level in the type II AECs isolated from either WT or Sulf2 CKO mice after bleomycin exposure (data not shown); thus, the source of increased TGF-β1 in the BALF from Sulf2 CKO mice (Figure 5A) was likely the inflammatory cells.

Epithelial Deletion of Sulf2 Exacerbates Bleomycin-Induced Mortality

Table 1 lists the mortality rates of WT and Sulf2 CKO mice treated with bleomycin at different dosages at the 7- and 14-day time points. Although the bleomycin dosage was reduced to 0.3 U/kg in an attempt to study the effect of Sulf2 deletion on lung fibrosis, the majority of Sulf2 CKO mice died before 14 days after bleomycin administration. In fact, most of the Sulf2 CKO mice died around 7–9 days after bleomycin exposure (Figure 7), at or immediately after the peak of lung inflammation.

Discussion

The Sulfs are extracellular sulfatases that have emerged as critical regulators of HS activity through their ability to catalyze specific 6-*O*-desulfation of HS polysaccharide chains, and consequently HS-dependent signaling events and biological processes. Depending on the cell types, the stimuli, and the specific biological processes involved, the functions of Sulf1 and Sulf2 can be either redundant (38), similar (11, 39), or unique for a particular

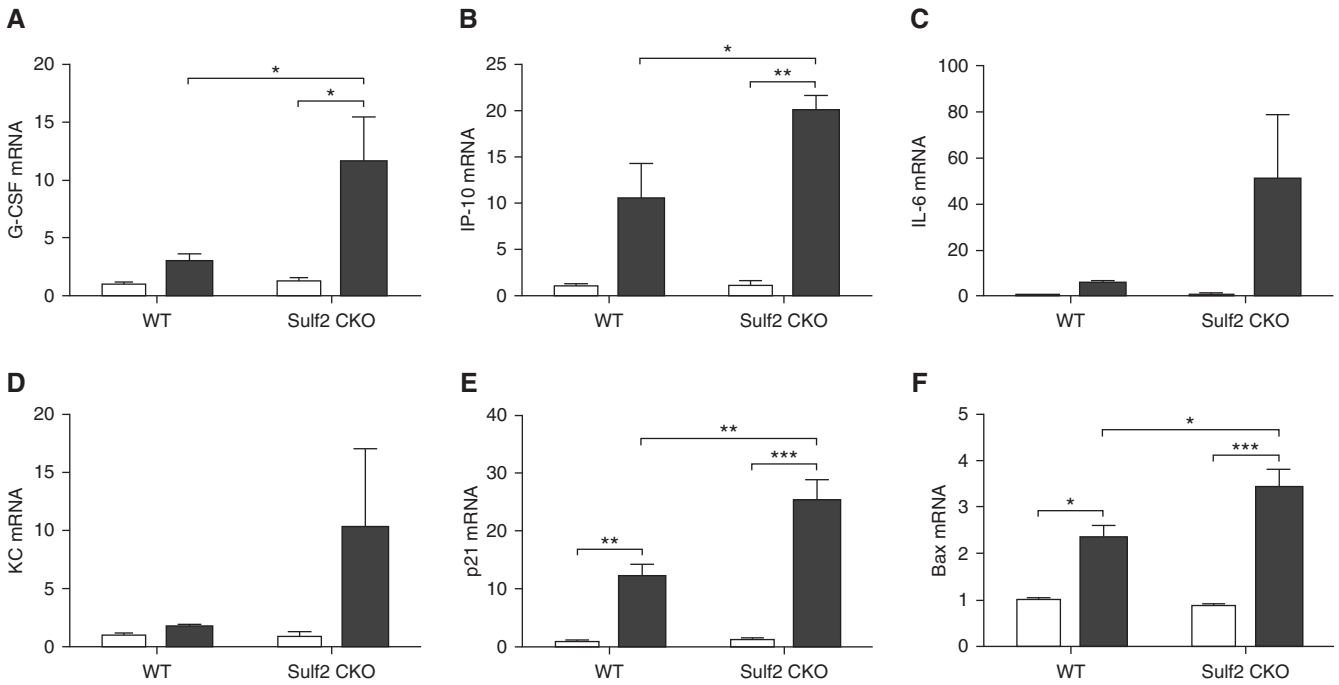

Figure 6. Type II AECs from Sulf2 CKO mice express increased levels of inflammatory cytokines and DNA damage response genes after bleo exposure. The mRNA expression of G-CSF (*A*), IP-10 (*B*), IL-6 (*C*), KC (*D*), p21 (*E*), and Bax (*F*) was analyzed in freshly isolated type II AECs from control (WT, $n = 4$; Sulf2 CKO, $n = 4$) or bleo-challenged mice (WT, $n = 4$; Sulf2 CKO, $n = 4$). *Open bars*, control; *solid bars*, bleo-treated mice (0.3 U/kg, 4–5 d). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

isoform. For example, our laboratory has shown that TGF-β1 induces the expression of Sulf1 and Sulf2, which, in turn, function as negative-feedback regulators of TGF-β1 (11, 39). The induction of the Sulfs by TGF-β1 and its biological implications, however, is cell-type specific. In A549 cells, a type II AEC cell line with Sulf2 as the predominant Sulf isoform, TGF-β1 induces Sulf2 expression, which, in turn, negatively regulates TGF-β1–induced plasmin activator inhibitor-1 and matrix metalloproteinase-9 expression (11). In lung fibroblasts where both Sulf1 and Sulf2 are expressed, TGF-β1 specifically induces Sulf1, which functions to prevent exaggerated fibrotic response after TGF-β1 stimulation (39). The function of the Sulfs in DNA damage response and tissue regeneration, however, appears to be unique to Sulf2, independent of cell type. In both type II AECs (11) and lung fibroblasts (data not shown), bleomycin treatment results in the induction of Sulf2, but not Sulf1.

We reported previously that Sulf2 is specifically up-regulated in the hyperplastic type II AECs in the IPF lungs (11). In the current study, we show that the predominant Sulf isoform expressed by type II AECs in the alveoli is Sulf2, and Sulf2 expression is up-regulated in lung epithelium after bleomycin-induced lung injury *in vivo* (Figure 1). Furthermore, we show that epithelial deletion of Sulf2 results in exacerbated injury, inflammation, and mortality after bleomycin exposure (Figures 3–7). Our data suggest that Sulf2 regulates the process of lung injury and repair at multiple levels. First, Sulf2 regulates bleomycin-induced DNA damage response in type II AECs; in the absence of Sulf2, p53–p21 signaling pathway was significantly up-regulated (Figures 4 and 6). Second, Sulf2 expression regulates cytokine expression in type II AECs; in the absence of Sulf2, type II AECs express significantly higher levels of G-CSF and IP-10 after bleomycin exposure (Figure 6), contributing to enhanced neutrophil infiltration and potentiating epithelial damage in Sulf2 CKO mice compared with WT littermates (Figure 3). Lastly, our data suggest that Sulf2 may be required for the regeneration of type II AECs from progenitor cells located at the bronchoalveolar duct junctions, as a subset of Sulf2-positive cells are also positive for proliferation marker, Ki67 (Figure E2). Sulf2 and Ki67

Table 1. Inducible Epithelial-Specific Sulf2 Conditional Knockout Mice Suffer Increased Mortality after Bleomycin Exposure

Endpoint	Bleomycin Dosage (*U/kg*)	Mortality Rate % (*n*)	
		WT	Sulf2 CKO
7 d	**1.0**	57.1 (7)	55.6 (9)
	0.7	11.1 (9)	20 (10)
14 d	**0.5**	33.3 (6)	100 (5)
	0.3	16.7 (6)	87.5 (8)

Definition of abbreviations: CKO, conditional knockout; Sulf2, heparan sulfate 6-*O*-endosulfatase 2; WT, wild type.

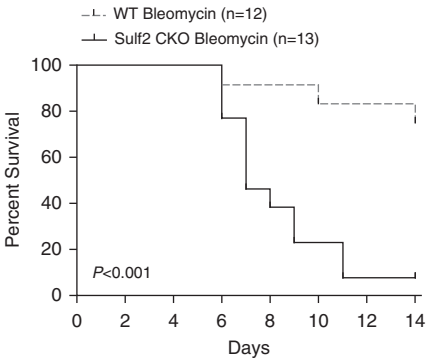

Figure 7. Sulf2 CKO mice suffer increased mortality after bleo exposure. Survival curves of WT and Sulf2 CKO mice treated with bleo at 0.3 and 0.5 U/kg (combined) were analyzed by log-rank (Mantel–Cox) test.

double-positive type II AECs were also found in the injured alveoli after bleomycin challenge (Figure E2).

Sulf2 has been shown to be a direct transcription target of p53 (40, 41), and Sulf2 induction by a number of stimuli, including chemotherapy, γ irradiation, and hypoxia, is mediated by p53 (41–43). Our study suggests that bleomycin-induced Sulf2, likely mediated by p53 as well, in turn regulates p53 signaling. In the absence of Sulf2, both the total and phosphorylated (at serine-15) p53 are elevated with up-regulation of the downstream effector, p21. Although the induction of p21 is a protective response allowing the cells to repair the damaged DNA, type II AECs were arrested from re-entering the cell cycle to regenerate the damaged alveolar epithelium. On the other hand, overexpression of Sulf2 may allow cell cycle progression in the presence of damaged DNA, and the up-regulation of Sulf2 in lung cancer cells supports this hypothesis. It is currently unclear how Sulf2 expression and the subsequent changes in HS 6-*O*-sulfation regulate p53–p21 signaling or cell cycle progression.

The role of the Sulfs in inflammatory responses has not been addressed previously, although a few studies in the literature support such a role. Sulf2 expression is highly up-regulated in inflammatory bowel diseases (44), and inflammatory cytokine TNF-α has been shown to induce Sulf1 expression in human lung fibroblasts (45). In addition, a whole-genome transcriptome array study showed that silencing of Sulf2 in lung cancer cell lines activates genes involved in immune responses (46). Our study supports a protective role of Sulf2 in lung inflammation, as epithelial deletion of Sulf2 leads to increased expression of inflammatory cytokines and increased neutrophil infiltration in the lung (Figures 3 and 6). As a secreted enzyme present in the BALF (Figure 1B), Sulf2 could further modulate the interaction between HS and HS-binding cytokines within the alveolar microenvironment. Future studies should evaluate whether recombinant Sulf2 enzyme could be used as an effective antiinflammatory agent.

Our study suggests that the overexpression of Sulf2 in IPF is a compensatory response as the type II AECs attempt to regenerate the injured epithelium, and Sulf2 expression likely contributes to the hyperplastic and hypertrophic features of the type II AECs in IPF. The question is, then, why the overexpression of Sulf2 did not lead to regeneration of functional alveolar units in IPF. The answer could lie, in part, in the failed differentiation of type II AECs into type I AECs in the diseased lungs. It was shown 30 years ago that the basement membrane underneath type I AECs is more sulfated than that underneath type II AECs (47), and, in fact, the type I AECs do not express Sulf2 (Figure 1C, *b*). It is plausible that expression of Sulf2 helps maintain type II AECs in a low-sulfated state, and down-regulation or silencing of Sulf2 may be required for the differentiation of type II AECs into type I AECs during lung regeneration.

Our study also suggests that Sulf2 expression by type II AECs is important in lung homeostasis, as Sulf2 CKO mice exhibit emphysematous changes with enlarged air spaces in the lung. Similar phenotype was previously observed in Sulf2 systemic KO mice (27), as well as in mice with multiple sulfatase deficiency (48). Future work is warranted to examine the role of Sulf2 or HS 6-*O*-sulfation in maintaining normal lung structure and function. ■

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