Caveolin-1 Deficiency Protects from Pulmonary Fibrosis by Modulating Epithelial Cell Senescence in Mice

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Idiopathic pulmonary fibrosis is associated with a decreased expression of caveolin-1 (*cav-1*), yet its role remains unclear. To investigate the role of *cav-1*, we induced pulmonary fibrosis in wild-type (WT) and *cav-1*-deficient (*cav-1*^{-/-}) mice using intratracheal instillation of bleomycin. Contrary to expectations, significantly less collagen deposition was measured in tissue from *cav-1*^{-/-} mice than in their WT counterparts, consistent with reduced mRNA expression of *procollagen1a2* and *procollagen3a1*. Moreover, *cav-1*^{-/-} mice demonstrated 77% less α -smooth muscle actin staining, suggesting reduced mesenchymal cell activation. Levels of pulmonary injury, assessed by tenascin-C mRNA expression and CD44v10 detection, were significantly increased at Day 21 after injury in WT mice, an effect significantly attenuated in *cav-1*^{-/-} mice. The apparent protective effect against bleomycin-induced fibrosis in *cav-1*^{-/-} mice was attributed to reduce cellular senescence and apoptosis in *cav-1*^{-/-} epithelial cells during the early phase of lung injury. Reduced matrix metalloproteinase (MMP)-2 and MMP-9 expressions indicated a low profile of senescence-associated secretory phenotype (SASP) in the bleomycin-injured *cav-1*^{-/-} mice. However, IL-6 and macrophage inflammatory protein 2 were increased in WT and *cav-1*^{-/-} mice after bleomycin challenge, suggesting that bleomycin-induced inflammatory response substantiated the SASP pool. Thus, loss of *cav-1* attenuates early injury response to bleomycin by limiting stress-induced cellular senescence/apoptosis in epithelial cells. In contrast, decreased *cav-1* expression promotes fibroblast activation and collagen deposition, effects that may be relevant in later stages of reparative response. Hence, therapeutic strategies to modulate the expression of *cav-1* should take into account cell-specific effects in the regenerative responses of the lung epithelium to injury.

Keywords: caveolin-1; lung injury; fibrosis; cellular senescence; apoptosis

Idiopathic pulmonary fibrosis (IPF) is an interstitial lung pathological disease and is associated with severe respiratory consequences resulting in increased mortality (1). Besides familial inheritance and progressive aging, additional factors contribute to the development of IPF; however, they remain unknown. The pathobiology of IPF is associated with usual interstitial pneumonia and chronic inflammation (1). Studies conducted

toward understanding the mechanism of onset and progression of the disease using experimental animals have suggested a positive correlation between epithelial injury along with apoptosis and development of pulmonary fibrosis (2). In addition, the occurrence of epithelial cell senescence has been reported in IPF, resulting in an aberrant interaction of epithelial cells with mesenchymal cells (3). Furthermore, telomere shortening and DNA damage through activation of p21/p53 is linked to the presence of senescent phenotypes in fibrotic lungs (3–5).

Recently, we have shown that genotoxic stress induced by hydrogen peroxide and bleomycin leads to alveolar epithelial cell senescence in a p16/pRB-dependent manner, thereby establishing the fact that age-associated lung pathologies such as chronic obstructive pulmonary disease and IPF may involve cellular senescence as a major contributor to increased pathological consequences and chronic inflammation (6). A plethora of proinflammatory cytokines, chemokines, proteases, growth factors, and matrix metalloproteinases (MMPs) constitute senescence-associated secretory phenotype (SASP), making senescence a paradoxical phenomenon leading to tumor development (7).

A membrane-associated scaffolding protein of the caveolae, caveolin-1 (*cav-1*), has been widely studied regarding senescence under *in vitro* and *in vivo* conditions (8–10). It is ubiquitously expressed in differentiated cells such as lung epithelial type I cells, endothelial cells, and fibroblasts (11–13). In the context of tumor development, increased expression of *cav-1* blocks mitogenic signaling during differentiation, *cav-1* expression is decreased during transformation, and its expression is increased in aggressive tumor development (14). Together, these observations suggest that *cav-1* serves as an antagonist to this exaggerated inflammatory mediator production and tumor progression via activation of tumor suppressor protein p53.

In contrast, experimental studies have shown a loss of *cav-1* in a radiation-induced model of pulmonary fibrosis in rats and in bleomycin-induced fibrosis in mice (15, 16). Expression of *cav-1* is reduced in patients with IPF; more importantly, down-regulation is observed in fibroblasts and epithelial cells (13). The significance of down-regulation of *cav-1* in these other cell types to fibrogenesis and to the repair responses to lung injury is unclear. Recent studies have shown that a peptide corresponding to the scaffolding domain of *cav-1* (CSD) inhibits collagen production in normal lung fibroblasts. In addition, systemic administration of the CSD peptide into bleomycin-treated mice can reverse the fibrotic process by blocking epithelial cell apoptosis, inflammatory cell infiltration, and matrix deposition (17). Furthermore, *cav-1* is an essential regulator of fibroblast proliferation and extracellular matrix deposition in IPF and systemic sclerosis (17, 18).

The lungs of *cav-1*^{-/-} mice are known to have abnormalities such as thickened alveolar septa from uncontrolled endothelial cell proliferation and reduced airway function at an early age from increased collagen deposition (19, 20). *cav-1* deficiency in fibroblasts has been shown to dramatically inhibit premature senescence induced by oxidants contained in cigarette smoke (21).

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It has also been shown that *cav-1* is required for oxidative stress-induced premature senescence in fibroblasts (22).

Based on the current paradigms of a role for *cav-1* in inhibiting profibrogenic signaling, the response of the *cav-1*^{-/-} mice would be expected to augment bleomycin-induced fibrosis. However, due to the fact that *cav-1* enhances senescent phenotypes in lung epithelium, we hypothesized that the absence of *cav-1* may promote resistance to senescence in lung epithelial cells upon bleomycin challenge and the development of increased fibrotic lesions (10, 21, 22). Thus, we investigated the role of *cav-1* in bleomycin-induced lung fibrosis by comparing susceptibility to lung fibrosis in *cav-1*^{-/-} versus WT mice. Our results indicated that *cav-1* deficiency protected the animals from fibrotic injury caused by bleomycin and was positively correlated with a decreased level of senescence. *Cav-1* deficiency also resulted in decreased production of contributors of SASP, MMP-2 and MMP-9; conversely, release of proinflammatory cytokines IL-6 and macrophage inflammatory protein (MIP)-2 was not compromised in *cav-1*^{-/-} mice and was comparable to the levels of their WT counterparts (7, 23).

MATERIALS AND METHODS

Animals and Induction of Pulmonary Fibrosis

Animal experiments were conducted using a protocol approved by the Institutional Animal Care and Use Committee of the University of Hawaii. WT C57BL/6J (WT) and Cavtm1Mls/J (*cav-1*^{-/-}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The genetic background for the *cav-1*^{-/-} mice is a mixture of C57BL/6J and 129Sv/SvEv. The resulting chimeric animals were then back-crossed into C57BL/6J background no more than six times due to a lower fertility. To induce pulmonary fibrosis, WT and *cav-1*^{-/-} mice were administered with 1.3 U/kg body weight of bleomycin intratracheally. Control animals from both cohorts received PBS intratracheally. Animals were killed at the indicated time points. Lungs and blood samples were collected. Dynamic compliance of the lungs was analyzed as previously described by Jourdan-Le Saux and colleagues (19) as a measure of lung mechanics using a Flexivent instrument (Scireq Inc., Montreal, Quebec, Canada) (21, 22).

Assessment of Pulmonary Fibrosis Development and Lung Injury

The extent of fibrosis indices in the lungs of bleomycin-challenged mice were assessed with established markers such as collagen and α -smooth muscle actin (α -SMA) expression using immunohistochemical staining. The level of expression of the α -2 chain of type I procollagen (*procollA2*) and the α 1 chain of type III procollagen (*procoll3A1*) was detected by quantitative RT-PCR using commercially available TaqMan probes (Applied Biosystems, Foster City, CA). Total collagen was assessed by the Sircol Collagen Assay kit (Biodye Science, Westbury, NY) according to the manufacturer's instructions.

For the assessment of lung injury and repair, levels of CD44v10 were analyzed by immunohistochemistry (IHC), and expression of tenascin-C (*tn*) was analyzed at mRNA level. Using the specific probes available commercially, expression levels of *cav-1* and *actin* were also analyzed at mRNA levels to understand their relationship with the development of fibrosis.

Analyses of Senescence and Apoptosis

Lung cell senescence was measured by senescence-associated β -gal activity as described by Debacq-Chainiaux and colleagues (24). The levels of senescence markers p16 and pRB and the heterochromatin-associated protein, macrohistone 2A (MH2A) were analyzed by immunohistochemical and immunofluorescent staining (6, 25). Quantification of apoptotic index in whole-lung paraffin-embedded sections was performed using terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) in the *in situ* cell death detection kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. Quantification of apoptosis in epithelial cells was performed by flow cytometric analysis

using an Annexin V/PI labeling kit (BD Pharmingen, San Diego, CA) and E-cadherin labeling (Cell Signaling, Danvers, MA) (26). Analysis was done on the isolated total lung cell population from WT and *cav-1*^{-/-} mice at 2, 4, and 6 days after bleomycin challenge. Results are represented as percent total and E-cadherin-gated cells population showing annexin-V positivity and PI negativity to derive the actual number of cells undergoing apoptosis as opposed to the total apoptotic cells quantified by TUNEL assay.

Statistical Analysis

Student's *t* test and two-way ANOVA were used to determine statistical significance between groups. Data entry, management, and statistical analysis were performed using Prism software (GraphPad Software, San Diego, CA).

RESULTS

Effects of Bleomycin Injury on Body Weight and Lung Compliance in *cav-1*^{-/-} Mice

Bleomycin-treated WT mice were more symptomatic of the fibrotic disease process than the *cav-1*^{-/-} mice. There was 50% attrition rate in the WT population over the 21-day observation period, with the majority of deaths occurring within Days 10 to 15, compared with a 100% survival rate in the *cav-1*^{-/-} mice (see Figure E1A in the online supplement). WT mice showed a steady approximately 20% decrease in body weight by Day 12 (Figure E1B) before slowly recovering toward their original weight by Day 20. The bleomycin-treated *cav-1*^{-/-} mice showed an initial rapid 10% decrease in body weight by Day 2, preceding a steady increase in weight to 96% of original at Day 11 and 100% of original at Day 21. There was no significant difference in lung compliance between *cav-1*^{-/-} and WT mice treated with PBS (Figure E1C). However, in bleomycin-injured groups, WT mice at Day 11 demonstrated a 46% loss in compliance compared with only a 26% loss for *cav-1*^{-/-} animals. Together, these data indicate that *cav-1* deficiency protects the mice against bleomycin-induced loss of body weight and lung compliance.

Development of Bleomycin-Induced Pulmonary Fibrosis Is Attenuated in *cav-1*^{-/-} Mice

Blind histological scoring of paraffin sections stained for collagen type I indicated that WT mice had a progressive and statistically significant increase in collagen deposition from Day 6 through Day 21 (Table 1). In contrast, in *cav-1*^{-/-} mice,

TABLE 1. IMMUNOHISTOCHEMISTRY WAS USED TO ASSESS EXPRESSION OF COLLAGEN TYPE I AND α SMOOTH MUSCLE ACTIN

Traits	Day 6	Day 11	Day 21
WT-COL1	1.6 $\pm$ 0.3	7.7 $\pm$ 0.4**	11.4 $\pm$ 1***
<i>cav-1</i> ^{-/-} -COL1	1 $\pm$ 0.4	3.3 $\pm$ 0.4 ⁺	1.6 $\pm$ 0.3***
WT- α -SMA	0	2.5 $\pm$ 0.3***	2.7 $\pm$ 0.3***
<i>cav-1</i> ^{-/-} - α -SMA	0	1.6 $\pm$ 0.3	0.9 $\pm$ 0.1***

Definition of abbreviations: α -SMA = α smooth muscle actin (dilution 1800; Dunn, Labortechnik GmbH, Asbach, Germany); *cav-1* = caveolin-1; COL1 = collagen type 1 (dilution 120; Quartett, Berlin, Germany); WT = wild type.

The COL1 score was expressed as: immunopositive area in % (10% = 1; 100% = 10) $\times$ staining intensity (0 = negative; 1 = weakly positive; 2 = strongly positive). The α -SMA score was expressed as: 0–5% myofibroblast positive = 1, 6–20% myofibroblast positive area = 2, and >20% myofibroblast positive area = 3. Histological scoring of deposition are given as mean $\pm$ SD. There was a time-dependent significant increase in COL1 and α -SMA in WT mice. However, in *cav-1*^{-/-} mice there was no significant increase in COL1 deposition and only a significant increase for α -SMA at Day 11. A minimum of five sections per group were analyzed; significance was assessed by two-tailed *t* test.

Asterisks denote significance within groups (***P* < 0.01; ****P* < 0.001); plus symbols denote significance between strains at matched pairs (⁺*P* < 0.05; ****P* < 0.001).

Figure 1. Effect of caveolin-1 (*cav-1*) deficiency on the amount of collagen deposition and gene expression with time. (A) Representative histological sections from the whole left pulmonary lobe were taken from each animal and stained with picrosirius red. To minimize variation, samples were processed under identical conditions. Levels of picrosirius red staining were quantified with ImagePro software. Values given are mean $\pm$ SEM, and significance was measured by one-way ANOVA ($n = 5$ controls and minimum of 10 per treatment). (B) Soluble collagen content measured in the salt extracts of lung tissues using Sircol assay ($n = 3$ for each group) show a gradual increase and a significant increase on Day 21 in collagen levels in the wild-type (WT) mice as compared with the *cav-1*-deficient (*cav-1*^{-/-}) mice after bleomycin challenge. Values given are mean $\pm$ SEM, and significance was measured by one-way ANOVA. (C) RT-PCR expression levels for mRNA from *procol1a2* and *procol3a1* (D). Values are arbitrary units $\pm$ SEM (two-tailed Student's *t* test; $n = 5$ individual specimens in triplicate). Asterisks denote significance within group (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$); + denotes significance between the traits at matched pairs ($P < 0.05$).

we measured no significant increase in collagen deposition at any of the time points evaluated. For α -SMA levels, WT mice showed a progressive, statistically significant increase in the presence of α -SMA from Day 6 to that measured at Days 11–21. Conversely, *cav-1*^{-/-} mice showed a lack of response at Day 6 followed by a significant increase in α -SMA at Day 11 that returned to baseline levels by Day 21 (Table 1). Analysis of picrosirius red-stained tissue sections (Figure 1A) confirmed the data obtained through blind scoring of collagen type 1 deposition. In WT mice, the level of picrosirius red staining in lung parenchyma increased through Day 21. This increase was significant at all three measured time points compared with PBS-treated mice. However, in *cav-1*^{-/-} mice, there was no significant increase in collagen deposition. Reminiscent of the scored samples, we observed an initial significant increase from PBS mice at Day 11, which was lost by Day 21. As previously described (19), we measured significantly more collagen in the lungs of PBS-treated *cav-1*^{-/-} mice than in the lungs of their WT counterparts, indicating a higher background level of collagen in the *cav-1*^{-/-} mice. Thereafter, WT mice showed significantly more collagen than *cav-1*^{-/-} mice, whose collagen deposition had returned to baseline levels by Day 21 (Figure 1A). In addition, quantification of collagen was performed by SIRCOL assay and confirmed the reduced levels of collagen in bleomycin-challenged *cav-1*^{-/-} mice (0.18 ± 0.05 in the WT versus 0.04 ± 0.002 in *cav-1*^{-/-} mice on Day 21 after bleomycin treatment) (Figure 1B).

Furthermore, to calculate whether the increase in collagen deposition correlated with a change in gene expression, we analyzed levels of *procol1a2* and *procol3a1* mRNA extracted from whole lung tissue (Figures 1C and 1D). Although not statistically significant, at Days 6 and 11 we detected a general increasing trend in *procol1a2* mRNA expression in WT mice. This progressed into a statistically significant doubling (1.9 ± 1.4) of *procol1a2* at Day 21 compared with PBS-treated mice. There was also an insignificant increase in *procol3a1* expression at Day 21 in *cav-1*^{-/-} mice, where we measured a 1.5 ± 0.5 -fold increase above averaged controls, compared with a 2.0 ± 0.8 -fold increase in WT mice. These data suggested a correlation between the increased collagen depositions measured in bleomycin-treated WT mice and increased gene expression for *procol1a2* and *procol3a1*.

Reduced Injury and Repair Response in the Lungs of *cav-1*^{-/-} Mice after Bleomycin Challenge

We measured expression levels of the matrix protein *tnc* mRNA as a marker of tissue repair (27–29). *Tnc* showed a marked increase in WT mice from Day 6 through Day 21 after bleomycin challenge. In addition, at Day 21, levels of *tnc* found in WT mice were significantly higher than those found in *cav-1*^{-/-} mice. A significant but smaller increase in *tnc* was measured in *cav-1*^{-/-} mice through Day 21 (Figure 2A). These data suggested the possibility that repair mechanisms are not triggered

Figure 2. *cav-1*^{-/-} mice exhibit reduced tissue damage and epithelial injury after bleomycin treatment. (A) RT-PCR for analysis of expression levels of tenascin-C *tnc* mRNA. Values are arbitrary units $\pm$ SEM (two-tailed Student's *t* test; *n* = 5 individual specimens in triplicate). Asterisks denote significance within strain (**P* < 0.05; ***P* < 0.01; ****P* < 0.001); ++ denotes significance between strains at matched pairs (*P* < 0.01). (B) Paraffin sections of mouse lung tissues from WT and *cav-1*^{-/-} animals. Representative immunostaining for CD44v10 (undiluted supernatant; kind gift from Dr. U. Günthert, Basel, Switzerland) in untreated (A: WT; B: *cav-1*^{-/-} mice) and bleomycin-treated (C: 21 d WT; D: 21 d *cav-1*^{-/-} mice). Scale bars in A and B = 50 μ m; scale bars in C and D = 100 μ m. (C) *cav-1* expression in WT mouse lungs over time after bleomycin challenge. *cav-1* and β -actin expression at mRNA level were determined by qRT-PCR. In WT mice treated with bleomycin, *cav-1* expression decreases with time as fibrosis develops. Values are mean $\pm$ SEM (*n* $\geq$ 4). ****P* < 0.001.

in *cav-1*^{-/-} mice after bleomycin challenge. To analyze the level of epithelial injury, we assessed levels of CD44v10 by IHC. CD44v10 is a marker of alveolar epithelial type II cells that also stains the bronchial epithelium in mouse lungs. During fibrogenesis, epithelial alterations can be monitored with epithelial isoforms of CD44 (30). In most cases, alveolar epithelial type II cell morphology changes toward an (intermediate) alveolar epithelial type I cell-like cell type. In bleomycin-injured WT mouse lungs, CD44v10 staining was prominent, compared with that of PBS-treated WT controls, which showed no change over time. However, *cav-1*^{-/-} mice showed mild positivity of CD44v10 upon bleomycin challenge, whereas no apparent difference was observed in the PBS-*cav-1*^{-/-} controls. More importantly, the positive correlation observed between epithelial injury and repair process in bleomycin-injured WT mouse lungs versus *cav-1*^{-/-} indicated that *cav-1*^{-/-} mice might have experienced less injury and therefore less repair process (Figure 2B). Injury and repair profiles are reduced in bleomycin-challenged *cav-1*^{-/-} mouse lungs.

It has been reported that *cav-1* expression is decreased with the development of pulmonary fibrosis in mice and human IPF (15). To reconcile our findings with these previous published data, we determined that in our animal model, *cav-1* expression levels were significantly decreased by over 2-fold in the lungs of WT mice after bleomycin challenge only starting at Day 11 through Day 21 (Figure 2C). Indeed, at Day 6 after injury, *cav-1* expression was not affected when compared with PBS control samples. This decrease in *cav-1* was a time-dependent process concomitant with the progression of fibrosis.

Absence of *cav-1* Protects against Bleomycin-Induced Epithelial Cell Senescence and Apoptosis

Cellular senescence was evaluated using four well accepted senescence markers: the expression of SA- β gal activity, p16, pRB, and MH2A. The injury and repair processes showed distinct responses in the two bleomycin-challenged animal groups. Therefore, we focused our analyses of epithelial cell senescence at a mid-point on Day 11 after bleomycin challenge. Bleomycin-injured WT lung tissues presented an increase of SA- β gal activity after Day 11 (Figure 3A). In contrast, no evidence of the activation of this enzyme was detected in samples from bleomycin-injured *cav-1*^{-/-} lung tissue. Examination of nuclear localization of pRB by immunofluorescence staining also depicted a similar phenotype (Figure 3B). We then analyzed the profile of epithelial cell senescence on Days 6, 11, and 21 in bleomycin-injured lungs by IHC analysis of the senescence markers p16, pRB, and MH2A. The senescence nuclear localization of p16, pRB, and MH2A was not yet observed on Day 6 after injury on WT and *cav-1*^{-/-} mice but showed a clear cytosolic staining in the alveolar lining and bronchial epithelial cells (Figure E2). On Days 11 and 21, challenged WT lung sections showed prominent nuclear localization of p16, pRB, and MH2A along with alveolar wall thickening and fibroblasts recruitment (Figure 3C and Figure E2). In contrast, in *cav-1*^{-/-} mice, nuclear localization of senescence markers was reduced (Figure 3C and Figure E2).

Because senescence is decreased in the lungs of bleomycin-challenged *cav-1*^{-/-} mice, we assessed the impact of *cav-1* during bleomycin-induced apoptosis. To understand if the apoptotic process was prominent during the early response to the bleomycin-induced injury to the lungs, we performed a TUNEL assay on lung tissue sections at 2, 4, and 6 days after bleomycin challenge. Two days after injury, we counted a near 3-fold increase in TUNEL-positive cells in bleomycin-challenged WT mice (15.6 $\pm$ 1.9%) compared with PBS-treated WT mice (5.3 $\pm$ 1.6%). At Day 4 and Day 6, apoptosis was still increased

Figure 3. Reduced cellular senescence in bleomycin-injured *cav-1*^{-/-} lung tissues. (A) Representative photomicrographs on frozen sections of WT mice challenged with bleomycin for 11 days show SA-β galactosidase activity (*blue staining* shows senescence foci versus the *clear areas*, which are normal). No SA-β gal activity was detected in the *cav-1*^{-/-} mice challenged with bleomycin. (B) Immunofluorescence staining of pRB-positive nuclei (*red*) for the presence of senescent cells versus the normal nuclei stained only with DAPI (*blue*). Original magnification: $\times 200$. A histogram demonstrates the percentage of pRB-positive nuclei per field of view in the lung sections collected from WT and *cav-1*^{-/-} mice treated with PBS or bleomycin for 11 days. (C) Representative paraffin-embedded lung sections immunohistochemically stained for p16, pRB, and MH2A. Immunohistochemistry analyses showed nuclear localization of senescence markers p16, pRB, and MH2A. Hematoxylin was used as a negative counterstain. Original magnification: $\times 400$. Arrow points to positive stain.

in bleomycin-challenged WT mice at $12 \pm 2.3\%$ and $7.8 \pm 2.2\%$, respectively (Figure 4A). There was also a moderate increase in apoptosis at 2 days ($7.0 \pm 1.2\%$) and 4 days ($5.7 \pm 1.5\%$) after bleomycin treatment as compared with that of PBS-treated *cav-1*^{-/-} mice ($2.6 \pm 1.5\%$). By Day 6, the level of apoptosis in bleomycin-challenged *cav-1*^{-/-} mice had returned to baseline levels. At Day 4, approximately 50 to 60% of TUNEL-positive cells also stained positive for the epithelial marker K19 (data not shown) in bleomycin-challenged mice from both WT and *cav-1*^{-/-} groups.

To quantify the rate of apoptosis in epithelial cells from WT and *cav-1*^{-/-} mouse lungs, we performed a flow cytometric Annexin-V/PI labeling assay by sorting the cells with the epithelial cell marker E-cadherin on Day 2, 4, and 6 after bleomycin-induced injury. A significant decrease in E-cadherin-sorted apoptotic cells was measured in the *cav-1*^{-/-} samples ($8.80 \pm 0.92\%$ in WT versus $4.42 \pm 0.75\%$ in *cav-1*^{-/-} mice; $P = 0.002$), with no significant difference between the two strains on Day 2 or 6.

Cav-1^{-/-} Mice Exhibit a Low Profile of SASP

We further examined the secretory phenotype of senescent cells (SASP) in bleomycin-challenged WT and *cav-1*^{-/-} mice by analyzing the levels of two of the most characteristic proteins of SASP, MMP-2 and MMP-9 (6, 7). The marked increase in

MMP-2 levels in the perivascular and alveolar spaces observed in the bleomycin WT lungs sections was attenuated in *cav-1*^{-/-} counterparts at Day 11 after injury (Figure 5A). MMP-9 levels were more concentrated in the peribronchial areas and alveolar spaces in the WT bleomycin lung sections, and there was a reduced staining in the lung sections of *cav-1*^{-/-} mice (Figure 5A). MMP-2 and MMP-9 levels were only increased over time in WT lungs after bleomycin injury (Figure 5B). Tissue IL-6 levels were similarly increased in bleomycin-challenged WT mice ($P \leq 0.001$) and *cav-1*^{-/-} mice ($P = 0.002$). In contrast, no significant difference was observed in MIP-2 levels in the lung homogenates of both strains after challenge with bleomycin (Figure 5C).

DISCUSSION

The lung responds to injury in a stereotypic manner with activation of inflammatory and reparative host processes. The intratracheal instillation of the chemotherapeutic agent bleomycin has been used as a model of studying the responses of the lung to noninfectious lung injury and promoting lung fibrosis (31). In this study, we demonstrate that *cav-1*^{-/-} mice are protected from bleomycin-induced lung injury. Our studies suggest that this protection is related to reduced epithelial cell senescence

Figure 4. Rate of apoptosis is altered in *cav-1*^{-/-} mice during bleomycin-induced lung injury. (A) Representative photomicrographs of the terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) assay performed on lung frozen lung tissue sections from Days 2, 4, and 6 after bleomycin challenge of WT and *cav-1*^{-/-} mice. Note a decreased rate of TUNEL positivity in *cav-1*^{-/-} mice compared with bright nuclear TUNEL positivity in the WT mice, which is reduced over time. To calculate the apoptosis index, the data are expressed as the percentage of cells positively expressing fluorescein versus the total cells (DAPI) present for each field of view. Significant increases in apoptosis were observed at Days 2 and 4 in bleomycin-treated WT mice and in *cav-1*^{-/-} mice. There was significantly less apoptosis in *cav-1*^{-/-} mice than in their WT counterparts. Values are mean $\pm$ SEM (Student's *t* test; *n* = 3 different specimens with three fields of view analyzed per section). Asterisks denote significance within group (*P* < 0.05; ***P* < 0.01; ****P* < 0.001); + denotes significance between the traits at matched pairs (*P* < 0.05). (B) Flow cytometric sorting of E-cadherin-positive apoptotic cells showed a significant shift in the Annexin-V⁺/PI⁻ cell population on Day 4 after bleomycin challenge. The percentage of E-cadherin/AnnexinV⁺/PI⁻ cells is reduced *cav-1*^{-/-} mice.

and apoptosis with an associated reduction in the fibrotic response. Pulmonary fibrosis pathobiology is perhaps best described as an aberrant wound-healing response to recurrent or stress-induced alveolar injury. Normal healing requires proliferation and repopulation of denuded alveolar epithelial cells (AECs). The restitution of epithelium integrity is also associated with apoptosis of AECs and fibroblasts (32, 33). Repeated insults, however, lead to aberrant responses where AECs can no longer proliferate and instead excessive apoptosis and replicative senescence is observed. Subsequently, fibroblasts proliferate and become resistant to apoptosis, leading to the development of progressive fibrosis (34, 35). Modeling pulmonary fibrosis, such as IPF, in animals is complicated because the etiology and natural history of the pathology is unclear and because chronic diseases have proven to be difficult to model in animals. None of the animal models currently available mimics the progressive and irreversible nature of this chronic disease (36, 37). Histological and biochemical markers of fibrosis, such as intra-alveolar buds and collagen deposition, are present in bleomycin-treated animals, similar to patients with IPF; however, unlike in human IPF, they are reduced over time in animals. The bleomycin model is the standard of experimental pulmonary fibrosis in animals (31, 36). Previous studies have shown that *cav-1* participates in the regulation of inflammation and fibrosis of pulmonary

fibrosis. Therefore, it would be expected that, in *cav-1*^{-/-} mice, bleomycin would have an increased fibrogenic effect (13, 17). Our study indicates that bleomycin induced much less fibrosis in *cav-1*^{-/-} mice relative to that in WT animals, as assessed by lung collagen content, histopathology, and α -SMA expression in the lungs. Mice deficient in *cav-1* did not develop fibrosis after bleomycin injury, most likely because the cascade of events initiated after injury was not promoted. Along with the absence of epithelial injury as revealed by the CD44v10 marker, the levels of *tnc* and *procol3a1* gene expression were not increased in *cav-1*^{-/-} lung samples. Both of these extracellular matrix proteins are distributed in alveolar septal walls and secondary septal tips in the areas of tissue damage as part of the early response to pulmonary injury induced by bleomycin (19, 27). As the development of fibrosis proceeded, *tnc* mRNA expression decreased, whereas expression of *procol3a1* persisted into the reparative phase. The deficiency in epithelial repair associated with fibrotic lung disorders has been shown to be a strong contributor to a persistent deposition of ECM components including *tnc* (38). Therefore, the expression of *tnc* has been proposed as a unique early-response ECM component and can provide information on sites of active foci of developing fibrotic lesions (27). Newer mechanisms involved during early

Figure 5. A subset of the senescence-associated secretory phenotype profile shows a decreased level in bleomycin-challenged *cav-1*^{-/-} mice. (A) Representative photomicrographs of immunohistochemical analyses of matrix metalloproteinase (MMP)-2 and MMP-9 using deparaffinized lung tissue sections of Day 11 after bleomycin injury in WT and *cav-1*^{-/-} mice. Note the increased perivascular and alveolar staining of the MMPs in the lung sections of bleomycin-challenged mice versus the reduced staining in those of *cav-1*^{-/-} mice. (B) Western blot analysis of MMP-2 and MMP-9 using the lung tissue homogenates from Days 6, 11, and 21 after bleomycin injury show decreased levels in the *cav-1*^{-/-} mice as compared with the WT mice. (C) Levels of IL-6 and MIP-2 as measured by ELISA in the lung homogenates and serum samples obtained from WT and *cav-1*^{-/-} mice challenged with bleomycin and their corresponding PBS-treated controls. Values are represented as an average pg/mg tissue or pg/ml serum $\pm$ SD ($n = 5$).

stages of pathobiology in bleomycin-induced lung injury as well as in pulmonary fibrosis include apoptosis and senescence.

We measured AEC apoptosis in bleomycin-injured WT mice. In the absence of *cav-1*, the number of TUNEL-positive and E-cad/Annexin V-positive cells was reduced on Day 4. This suggested that *cav-1* might be a regulator of apoptosis. *cav-1* has been weakly linked to apoptosis but has been linked strongly with cellular senescence (14, 22). Cellular senescence is not well understood in lung injury and fibrosis; it is a phenomenon observed in cells that can no longer proliferate. It is the ultimate and irreversible loss of replicative capacity occurring in primary somatic cells (39). The two common factors that link senescence with lung injury and fibrosis are telomere shortening and reactive oxygen species-mediated stress (40, 41). Our data strongly suggested that cellular senescence not only contributes to the chronic inflammation that affects the lungs of patients with chronic obstructive pulmonary disease, as previously reported (42), but may also lead to impaired reepithelialization affecting epithelial repair responses associated with lung injury and fibrosis. The known mechanisms of senescence involve the tumor suppressor proteins p53/p21 and p16/pRB/MH2A, which arrest the cell cycle (43, 44). Similar mechanisms are suggested for progressive aging in the lungs, which include telomere shortening associated with the DNA damage response, resulting in the impaired regeneration of epithelial cells; this is also observed in pulmonary fibrosis diseases (5, 45). Indeed, senescence-associated β -gal activity and the expression of p16, pRB, and MH2A were observed in WT but not in *cav-1*^{-/-} lung tissue after bleomycin treatment. The importance of senescence has also been pointed out by Xu and colleagues, who found that in a senescence-accelerated mouse model, development of fibrosis was more pronounced (46).

MMP-2, MMP-9, and the proinflammatory cytokines IL-6 and MIP-2 levels were also increased in bleomycin-injured WT mice, suggesting a positive correlation between senescence and fibrosis, contributed by the SASP (23, 47). Despite a clear phenotypic difference between the WT and *cav-1*^{-/-} for MMP-2 and MMP-9 expression, IL-6 and MIP-2 levels did not show a significant difference in the lungs of WT and *cav-1*^{-/-} mice after bleomycin injury. As evident from the photomicrographs, *cav-1*^{-/-} mice display abundant lymphocyte infiltration 11 days after bleomycin challenge. In addition, generalized inflammatory status and an abnormal lung parenchymal architecture, which is inherent of *cav-1*^{-/-} mice, without any apparent changes in lung cell function might also explain increased levels of MIP-2 (48). Moreover, the origin of IL-6 and MIP-2 is not exclusively from senescent cells. It is therefore a more delicate set of data to interpret because these data exclusively reflect the senescence status in the injured tissue.

Expression of *cav-1* is reduced in IPF and bleomycin-treated mice and most specifically in epithelial cells, and it is associated with abnormal reepithelialization in lung fibrosis (49). In contrast, bleomycin-induced cellular senescence in epithelial lung cancer A549 cells is associated with the up-regulation of *cav-1* expression (8, 50). In our system using *cav-1*^{-/-} mice, we did not observe the increased fibrosis and collagen expression typically observed in a murine bleomycin model, and, as supported in studies by Linge and colleagues (8), nor did we observe excessive apoptosis and cellular senescence. The contradictory data might be due to the fact that the reduced expression of *cav-1* occurred only 11 days after bleomycin injury in WT animals and was further reduced through Day 21. Moreover, the difference in senescence markers of *cav-1*^{-/-} mice when compared with those of the bleomycin-challenged WT mice were in agreement with the low profiles of *tnf* expression on Day 11 after bleomycin challenge. In the early phase of the response to bleomycin, *cav-1* appeared to be expressed, and eventually increased

Figure 6. Schematic illustration of the role of *cav-1* in the development of pulmonary fibrosis. Immediately after injury, there might be an increased expression of *cav-1*, leading to apoptosis and cellular senescence. Later in the progression of the disease, *cav-1* expression decreased, and it is then associated with collagen deposition.

expression may be reported, as suggested by Linge and colleagues; therefore, we suspect *cav-1* to contribute to apoptosis and cellular senescence in epithelial cells as proposed in Figure 6. In contrast, when fibroblasts are deficient for *cav-1* expression, the fibrotic response is enhanced. In WT mice, the *cav-1* status in epithelial cells is slightly increased in the early phase and promotes senescence in later phases, and *cav-1* status in fibroblasts is reduced and promotes fibrosis (10).

Previous work has suggested that treatment with the CSD peptide or adenoviral construct resulted in reduced inflammatory and profibrotic responses in the bleomycin model (13, 17). Our data tend to urge caution in the use of CSD. Depending on the cause of injury, an absence of *cav-1* at an early stage might indeed be protective. *cav-1* expression is increased in endothelial cells of fibrotic lung and liver, indicating that the role of *cav-1* in the inflammatory responses is still unclear (16). This demonstrates that, in the response to initial injury and cellular response to injury, *cav-1* may balance a positive or negative outcome. Timing, cause, and stage of the disease need to be considered to maximize the beneficial effects of targeting *cav-1* as a therapeutic strategy.

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