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Deficiency of developmental endothelial locus-1 (Del-1) aggravates bleomycin-induced pulmonary fibrosis in mice

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ABSTRACT

Pulmonary fibrosis is a lung disease wherein lung parenchyma is gradually and irreversibly replaced with collagen. The molecular pathogenesis of pulmonary fibrosis is not fully understood and the only effective treatment available is lung transplantation. To test if Del-1, an endogenous anti-inflammatory molecule, may be implicated in the development of pulmonary fibrosis, we induced pulmonary fibrosis in wild type (WT) and Del-1$^{-/-}$ mice by intratracheal administration of bleomycin. Del-1 expression in the lung was decreased in the WT mice treated with bleomycin compared to control mice. In addition, bleomycin-induced pulmonary fibrosis increased collagen deposition and TGF-β production in the lung of Del-1$^{-/-}$ mice. Finally, Del-1$^{-/-}$ mice treated with bleomycin displayed higher weight loss and greater mortality than did WT mice identically treated. These findings suggest that Del-1 may negatively regulate development of pulmonary fibrosis. Further delineation of a role for Del-1 in the development of pulmonary fibrosis will broaden our understanding of the molecular pathogenesis of this disease and hopefully help develop potential therapeutics.

1. Introduction

Pulmonary fibrosis is a lung disease, wherein normal lung parenchyma is gradually and irreversibly replaced with fibrous connective tissue [1]. This condition disrupts gas exchange, elicits respiratory failure, and ultimately leads to death [2]. Pulmonary fibrosis has been reported to develop as a secondary effect of viral infection, radiotherapy, chemotherapy, inhalation of environmental and occupational pollutants, and chronic inflammatory diseases [3]. However, the disease etiology is unknown in most cases, which is called idiopathic pulmonary fibrosis.

Molecular mechanisms underlying pulmonary fibrosis are still elusive, yet proinflammatory and profibrotic mediators were shown to be involved in the initiation and maintenance of pulmonary fibrosis [3,4]. For example, irritants stimulate lung epithelial cells and macrophages, which then promote the production of reactive oxygen species (ROS), interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) [5–8]. IL-1β recruits and activates neutrophils, and enhances production of transforming growth factor beta (TGF-β), which is a key profibrotic cytokine that induces proliferation and differentiation of fibroblasts into myofibroblasts [9]. TGF-β transdifferentiates lung epithelial cells into myofibroblasts by epithelial-mesenchymal transition (EMT) [10,11]. Myofibroblasts secrete extracellular matrix (ECM) components including collagen. Deposition of collagen in the lung is a characteristic feature of pulmonary fibrosis [11].

Despite extensive research on pulmonary fibrosis, little has been determined about its therapeutic modalities. Corticosteroids applied to patients with some types of pulmonary fibrosis have met with limited success [12]. Lung transplantation has been the only effective therapeutics for progressive pulmonary fibrosis to date [13]. Determination of the molecular mechanisms underlying pulmonary fibrosis will facilitate the development of novel therapeutic modalities.

Del-1 is an endogenous protein that inhibits adhesion of leukocytes to vascular endothelial cells, and thus exerts an anti-inflammatory function. Del-1 is highly expressed in the lung and brain, specifically in endothelial cells and some macrophages [14]. Del-1 interferes with interaction of the leukocyte integrin LFA-1, which is a major adhesion molecule that promotes leukocyte adhesion and migration, with the counter receptor on the endothelial cells, and thereby suppresses leukocyte migration [14]. In addition, downregulation of Del-1 expression in response to inflammation stimuli and Del-1 deficiency renders the cells or mice more vulnerable to inflammation assault [14,15]. Del-1 has been reported to be

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implicated in the regulation of angiogenesis, apoptosis, cell adhesion and migration, and inflammation [16–19].

As Del-1 inhibits migration of inflammatory cells and is highly expressed in the lung, we set out to determine a potential role for Del-1 in the pathogenesis of pulmonary fibrosis.

2. Materials and methods

2.1. Animals and reagents

C57BL/6 mice were purchased from The Jackson Laboratory. Del-1$^{-/-}$ mice were kindly provided by Prof. T. Chavakis (Dresden University, Germany) [14]. Animal studies were approved by the Asan Institute for Life Sciences Institutional Animal Care and Use Committee (Project number: 2012-14-190). Bleomycin sulfate was purchased from MB cell (Los Angeles, CA). Perfusion buffer, fixation buffer, and OCT compound were from Electron Microscopy Sciences (EMS; Hatfield, PA). Masson's Trichrome stain kit and, Hematoxylin and Eosin were obtained from Sigma–Aldrich. Sircol collagen assay kit was from Biocolor (Carrickfergus, County Antrim, UK). Human/Mouse TGF-β1 ELISA ReadySET Go was from eBioscience (San Diego, CA).

2.2. Bleomycin-induced pulmonary fibrosis

Six- to ten-week old WT and Del-1$^{-/-}$ mice (body weight: 20–24 g) were anesthetized by intramuscular injection with 20 μl of a mixture of Zoletil 50 (Virbac) and Rompun in a ratio of 3:1. Thirty microliters of PBS or bleomycin (BLM) (1, 5 or 7 U/kg) was injected into the trachea. High doses of bleomycin (7 U/kg) were used for body weight and survival test, and intermediate doses, for histological examination. At 7, 14, or 21 days post-bleomycin administration (dpa) the mice were sacrificed, and bronchoalveolar lavage (BAL) fluid and lung tissues were isolated for TGF-β ELISA assay and RT-PCR, respectively. For histological evaluation, the mice were transcardially perfused with perfusion buffer (EMS), and the lung tissues were fixed with fixation buffer (EMS) and embedded in OCT compound (EMS).

2.3. Real time RT-PCR

Total RNA was isolated from the lung tissues or cells using Trizol (Invitrogen/Life Technologies) and cDNA was synthesized using the ImProm-II reverse transcriptase kit (Promega). The cDNA was amplified using LightCycler 480 SYBR Green 1 Master and a LightCycler 480 machine (Roche, Mannheim, Germany). The primer sequences used are as follows. Del-1 forward primer: 5′-CTT GGT AGC AGC CTG GCT TT-3′; Del-1 reverse primer: 5′-GCC TTC TGG ACA CTC ACA GG-3′; 18s forward primer: 5′-CGC GGT TCT ATT TTG TTG GT-3′; and 18s reverse primer: 5′-AGT CGG CAT CGT TTA TGG TC-3′. The following PCR conditions were used: 95 °C for 15 min; 50 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C; and 95 °C for 15 min. Melting curve analyses were performed on all PCR products to ensure that specific PCR products were generated. Del-1 mRNA levels were normalized to 18s mRNA levels, analyzed using a comparative C_T method [20], and then expressed as the relative fold change.

2.4. Measurement of collagen production

Mice were transcardially perfused and the lung tissues were isolated, fixed, immersed in 15% then 30% sucrose, embedded in OCT compound, frozen and cut into 10 μm thick sections. The sections were stained using Masson's trichrome stain kit (Sigma–Aldrich), according to the manufacture's protocol. In brief, the section slides were washed with distilled water three times for 5 min each, transferred to a Bouin's solution, incubated at 56 °C for 15 min, and then washed with running tap water until the yellow solution faded away. The samples were incubated in Beibrich scarlet acid fuchsin for 15 min, rinsed with distilled water, incubated in a mixture of phosphomolybdic:phosphotungstic solution:distilled water in a ratio of 1:2:2 (v:v:v) for 5 min, transferred to aniline blue solution for 5 min, and then rinsed with distilled water. The samples were incubated in 1% acetic acid solution for 3 min, dehydrated in 95% ethanol for 4 min, 100% ethanol for 3 min twice, and xylene for 5 min twice, and then applied with a mounting medium. The samples were observed under a light microscope (Leica DM 1000) at 10× magnification. Collagen concentration in the lung was measured using Sircol collagen assay kit (Biocolor), according to the manufacturer's protocol. In brief, mice were transcardially perfused with PBS, and the lung tissues were isolated. The whole lung was immersed in 500 μl of pepsin-acid (0.1 mg pepsin in 1 ml of 0.5 M acetic acid) in a microcentrifuge tube, homogenized, and incubated overnight at 4 °C in a shaker. The homogenate was centrifuged at 3000 rpm at 4 °C for 10 min, and the supernatant was transferred to a fresh tube. This centrifugation and collection of supernatant was repeated until the supernatant became clear. The clear supernatant was added with the acid neutralizing reagent (100 μl/tube), mixed by inverting several times, added to the Collagen Isolation & Concentration Reagent (200 μl/tube), mixed by inverting, and then incubated overnight at 4 °C. Collagen samples were prepared by diluting with distilled water in a ratio of 1:4 (v:v). This solution was then centrifuged at 13,000 rpm at 4 °C for 10 min to remove supernatant. The invisible pellet was stained with dye (200 μl/tube), incubated at room temperature for 30 min in a shaker, centrifuged at 13,000 rpm at 4 °C for 10 min to remove supernatant, added with ice cold salt (500 μl/tube), gently mixed by inverting, and then centrifuged at 13,000 rpm at 4 °C for 10 min to remove supernatant. The pellets were treated with alkali reagent (250 μl/tube) and vortexed. The samples were then loaded on a 96 well plate (100 μl/tube) and read at 550 nm using a microplate reader (SpectraMax 190).

2.5. Hematoxylin and eosin (H&E) staining

Lung sections were prepared as described above. The sections were washed with distilled water three times each for 5 min, applied with hematoxylin (Sigma) for 1.5 min, washed with distilled water for 5 min, incubated with 0.3% acid alcohol (a mixture of 60 ml distilled water, 140 ml ethanol, and 0.6 ml hydrochloric acid) for 5 min, and subsequently washed with tap water for 5 min. The samples were treated with eosin (Sigma) for 1.5 min, subsequently with 70%, 80%, 90%, 95%, and 100% ethanol each for 1 min, and then with xylene twice each for 1 min. The sections were combined with a mounting medium and observed under a light microscope (Leica DM 1000) at 20× magnification.

2.6. ELISA for TGF-β

BAL fluid was centrifuged and the supernatant was evaluated for active TGF-β using a Human/mouse TGF-β1 ELISA Ready-SET Go kit (eBioscience; San Diego, CA), according to the manufacturer's protocol. In brief, the plate was coated with capture antibody at 4 °C overnight, washed with PBST (0.1% Tween20 in PBS) five times, blocked with 1X assay buffer, incubated at room temperature for 1 h, and washed with 0.1% PBST five times. The plate was then loaded with samples and standards, incubated at room temperature for 2 h or at 4 °C overnight, washed with 0.1% PBST five times, incubated in a mixture of biotin-conjugated antibody, avidin-conjugated horseradish peroxidase and assay buffer at

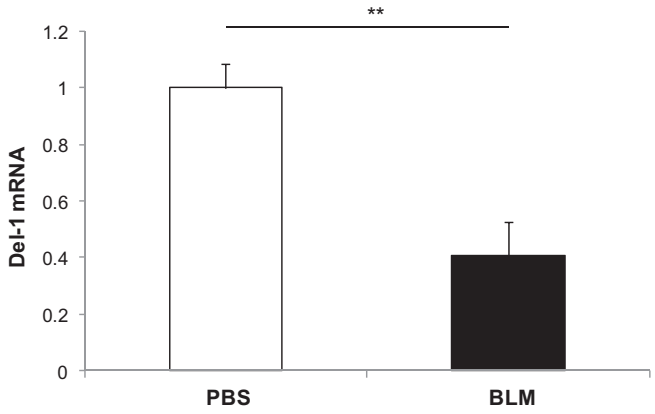

Fig. 1. Del-1 expression decreases in the lung of mice treated with bleomycin. PBS or bleomycin (BLM; 1 U/kg) intratracheally administered into BL6 WT mice. The lung tissues were isolated at 21 days post-administration (dpa) and Del-1 mRNA was analyzed by real time RT-PCR. Values are ±SE ($n = 3$ for WT and $n = 4$ for Del-1$^{-/-}$ mice). **, $p < 0.01$.

room temperature for 1 h, and washed with 0.1% PBST five times. The samples were treated with tetramethylbenzidine solution and then read at 650 nm using a microplate reader (SpectraMax 190).

2.7. Statistical analysis

Data were compared using the Student's *t*-test. $p < 0.05$ was considered significant.

3. Results

3.1. Del-1 expression decreases in the lung tissues from mice with bleomycin-induced pulmonary fibrosis

As Del-1 is an anti-inflammatory molecule that is constitutively expressed in the lung, we reasoned that Del-1 may be implicated in the development of pulmonary fibrosis. To test this hypothesis, we first induced pulmonary fibrosis by intratracheal administration of bleomycin into BL6 mice and harvested the lungs at 14 days post-administration (dpa). RNA was extracted from the lungs and then analyzed by RT-PCR. Lung tissues harvested from bleomycin-treated mice showed decreased Del-1 expression as compared to control lung tissue levels, suggesting that Del-1 might be implicated in the development of pulmonary fibrosis (Fig. 1).

3.2. Del-1 deficiency promotes the synthesis and secretion of collagen

Collagen deposition in the lung has been determined to be the most important pathologic feature of pulmonary fibrosis. Hence, we next sought to assess collagen deposition in the lung of WT and Del-1$^{-/-}$ mice treated with bleomycin. To this end, we administered bleomycin into WT or Del-1$^{-/-}$ mice and assessed collagen deposition in the lung by histological and biochemical assays at 21 dpa, when collagen deposition peaks. First, Masson's trichrome staining of the lung revealed that Del-1$^{-/-}$ mice exhibited more extensive deposition of collagen fibers compared to lung of WT mice (Fig. 2A). These results indicate more pulmonary production of collagen in Del-1$^{-/-}$ mice than WT mice. Second, we used Sircol

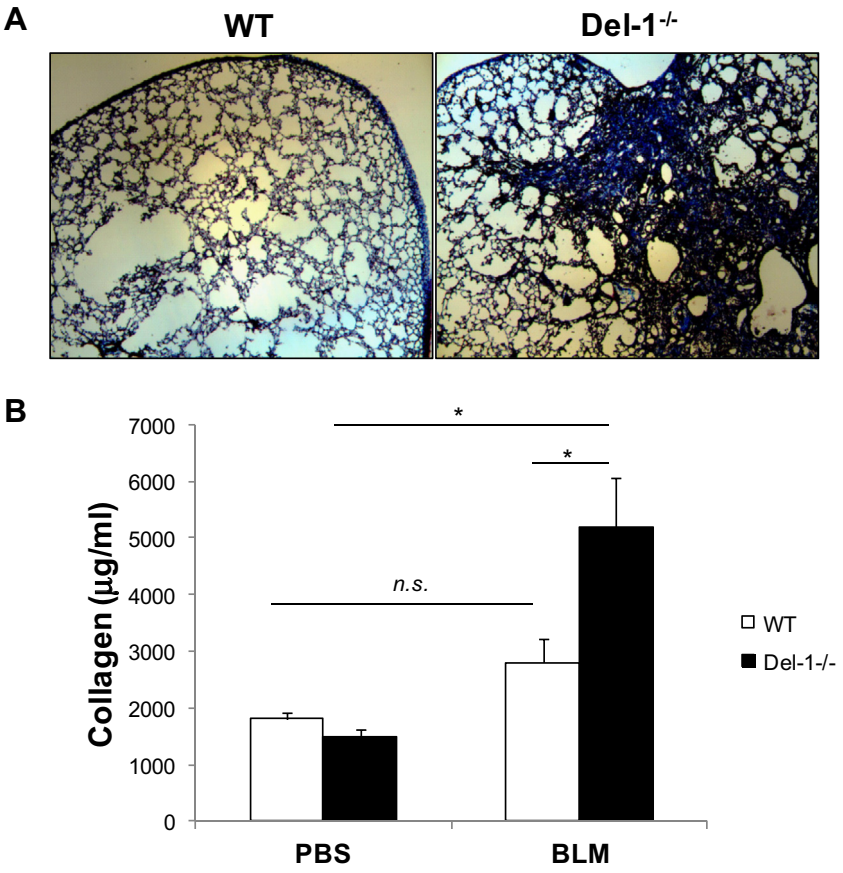

Fig. 2. Del-1 deficiency increases collagen production in the lung of mice treated with bleomycin. (A) Bleomycin (BLM; 5 U/kg) was administered into WT and Del-1$^{-/-}$ mice and lung tissues were isolated at 21 dpa. The lungs were serially sectioned and then processed for Masson's trichrome staining. (B) PBS or bleomycin (1 U/kg) was intratracheally administered into WT and Del-1$^{-/-}$ mice and lung tissues were isolated at 21 dpa. The lung homogenates were subject to Sircol collagen assay and collagen concentration was measured. Values are means ± SE ($n = 3$–5 mice per group). *, $p < 0.05$; *n.s.* non-significant.

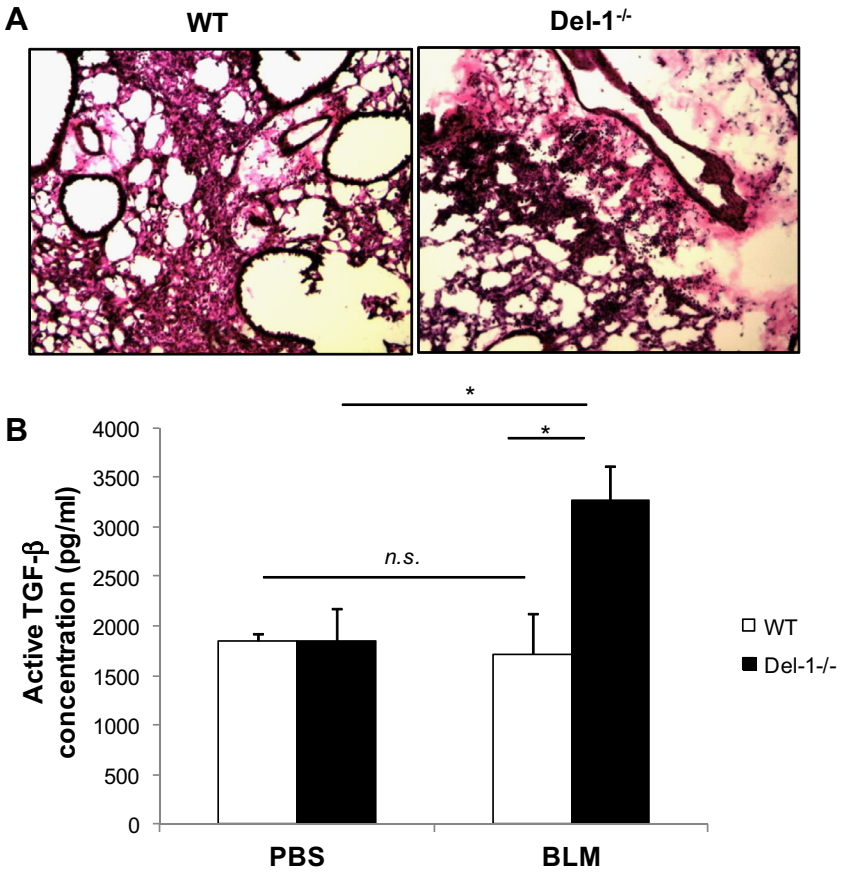

Fig. 3. Del-1 deficiency increases leukocyte infiltration and active TGF-β levels in the lung of mice treated with bleomycin. (A) Bleomycin (BLM; 5 U/kg) was administered into WT and Del-1$^{-/-}$ mice and lung tissues were isolated at 7 dpa. The lungs were serially sectioned and then processed for H&E staining. (B) PBS or bleomycin (1 U/kg) was intratracheally administered into WT and Del-1$^{-/-}$ mice and the BAL fluid was collected at 21 dpa. The levels of active TGF-β were measured by ELISA. Values are means ± SE (n = 3–4 mice per group). *, $p < 0.05$.

assay to measure collagen content in the lung. When left untreated, no significant difference in collagen content was noted between WT and Del-1$^{-/-}$ mice, which suggested equal basal levels of collagen between the two groups (Fig. 2B). Bleomycin administration, however, elicited significant increased pulmonary collagen content in Del$^{-/-}$ mice compared to WT mice (Fig. 2B). Taken together, these findings signify that Del-1 depletion causes increase in collagen deposition in the lung, thereby aggravating pulmonary fibrosis.

3.3. Del-1 deficiency promotes leukocyte infiltration and production of TGF-β in the lungs with bleomycin-induced pulmonary fibrosis

As inflammation precedes pulmonary fibrosis, we checked if there were any difference in the extent of pulmonary inflammation between WT and Del-1$^{-/-}$ mice treated with bleomycin. At 7 dpa, lungs were harvested from WT and Del-1$^{-/-}$ mice, sectioned serially and then stained with hematoxylin and eosin (H&E). Del-1$^{-/-}$ mice showed more inflammatory infiltration in the lung than did WT mice, indicative of enhanced migration of inflammatory cells to the lung in Del-1$^{-/-}$ mice (Fig. 3A).

TGF-β has been determined to be a positive regulator of collagen production in pulmonary fibrosis and a key cytokine in the development of pulmonary fibrosis [21]. Therefore, we elected to test TGF-β levels in WT and Del-1$^{-/-}$ mice to ascertain a potential role for this cytokine in promoted collagen production. To this end, bleomycin (1 U/kg) was administered into WT and Del-1$^{-/-}$ mice and bronchoalveolar lavage (BAL) was performed on both groups of mice at 21 dpa. Subsequently, active form of TGF-β was assayed in the BAL fluid by ELISA. Bleomycin-treated WT mice did not show a change in the levels of TGF-β at 21 dpa compared to the PBS-administered control mice (Fig. 3B). This may be caused by the return of increased TGF-β levels in the lung of bleomycin treated mice to basal levels at 21 dpa. Nevertheless, Del-1$^{-/-}$ mice displayed significant increases in the levels of active TGF-β compared to control WT mice (Fig. 3B). These results demonstrated that increase in both inflammatory infiltrate and active TGF-β may account for aggravated pulmonary fibrosis in Del$^{-/-}$ mice.

3.4. Del-1 deficiency increases weight loss and mortality in bleomycin-treated mice

Finally, we sought to assess the consequences of bleomycin-induced pulmonary fibrosis in WT and Del$^{-/-}$ mice. Two primary consequences of pulmonary fibrosis are weight loss and death. We first examined weight loss of WT and Del-1$^{-/-}$ mice with bleomycin-induced pulmonary fibrosis. Intratracheal administration of saline into WT and Del-1$^{-/-}$ mice did not alter body weight (BW). The BW of WT mice was reduced by 10% at 4 dpa of bleomycin (7 U/kg), after which their weight was restored to that of mice just prior to bleomycin administration (Fig. 4A). However, the BW of Del-1$^{-/-}$ mice was reduced by 10% at 2 dpa and kept decreasing until the very end of the experiment at 14 dpa (Fig. 4A). Similar results were noted with a high dose of bleomycin (10 U/kg; data not shown).

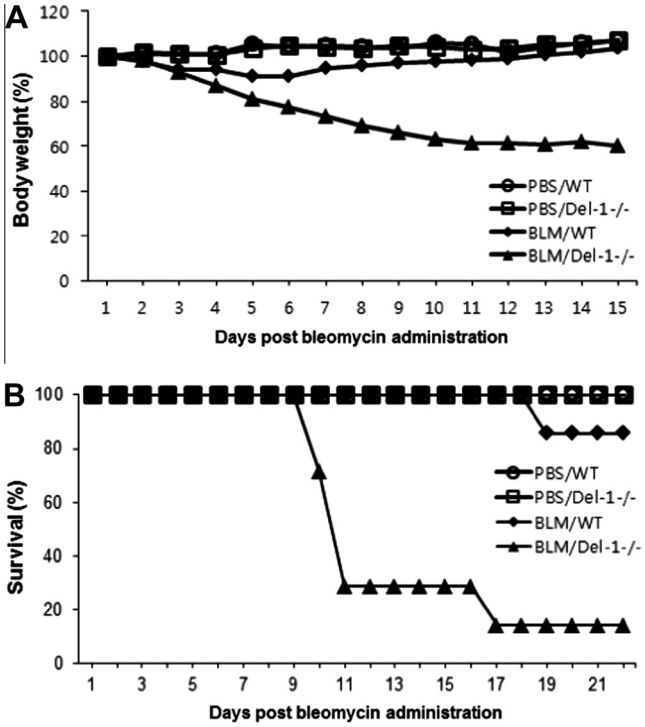

Fig. 4. Del-1 deficiency increases weight loss and mortality in mice treated with bleomycin. PBS or bleomycin (7 U/kg) was administered into WT and Del-1$^{-/-}$ mice, and the body weight (A) and the survival rate (B) were daily measured up to 15–22 dpa. Values are means (n = 3–7 mice per group). Data are representative of three independent experiments.

We next examined survival rates of WT and Del-1$^{-/-}$ mice treated with 7 U/kg of bleomycin. Survival rates of WT mice at 24 dpa were over 80%. In contrast, an approximately 60% survival rate was observed for Del-1$^{-/-}$ mice at just 10 dpa (Fig. 4B). The survival rates of Del-1$^{-/-}$ mice then plummeted to 30% at 10 dpa and to 0% at 16 dpa (Fig. 4B). This outcome illustrated that Del-1 plays an important role in the development of consequences during bleomycin-induced pulmonary fibrosis.

4. Discussion

We show here that Del-1, an endogenous anti-inflammatory molecule, regulates bleomycin-induced pulmonary fibrosis. We demonstrate that Del-1 expression is suppressed in the lung of mice treated with bleomycin compared to lung of control mice, and that Del-1$^{-/-}$ mice with bleomycin-induced pulmonary fibrosis exhibited increased collagen deposition and TGF-β production in the lungs compared to WT counterparts. Finally, we illustrate that Del-1$^{-/-}$ mice with bleomycin-induced pulmonary fibrosis displayed higher weight loss and mortality than did WT counterparts.

Although TGF-β is a well-known profibrotic cytokine, its exact role in pulmonary fibrosis remains undefined. Our findings suggest that Del-1 may inhibit activation of TGF-β. How does Del-1 then suppress TGF-β activation? TGF-β is secreted as a Large Latent Complex (LLC) into the ECM and becomes active only after encountering activators [22,23]. Although the mechanism underlying activation of TGF-β has not been fully defined, integrin-independent and integrin-dependent mechanisms have been proposed. In the integrin-dependent activation, $\alpha_v\beta_6$ and $\alpha_v\beta_3$ integrins bind to an RGD motif present in latency-associated peptide (LAP), a component of LLC, thereby activating TGF-β [22,24]. This notion is supported by a report which showed that administration of neutralizing anti-integrin α_v antibody into mice challenged with CCL18 reduces collagen deposition in the lung [25]. Intriguingly, Del-1 was shown to bind to α_v integrins [17,26]. It is therefore tempting to speculate that Del-1, secreted from endothelial cells and resident/infiltrated macrophages, may compete with LAP for α_v integrins located on epithelial cells, endothelial cells, or macrophages, thus inhibiting activation of macrophage-derived TGF-β in the extracellular matrix, leading to the inhibition of transdifferentiation of fibroblasts to myofibroblasts, a primary source of deposited collagen in the extracellular matrix of the fibrotic lung (Supplementary Figure). *In vitro* assessment of competition for α_v integrins between recombinant Del-1 and latent TGF-β associated with LAP will be required to validate this possibility.

If our model is the case, Del-1 function may be downregulated in human patients with pulmonary fibrosis. How do we explore this possibility? First, DNA sequencing of promoter and coding regions of Del-1 genomic DNA isolated from the patients will reveal, if any, single nucleotide polymorphism, insertion or deletion. Subsequently, effect of this identified mutation in Del-1 genomic DNA will be validated by *in vitro* Del-1 activity assay. Second, assessment of Del-1 RNA or protein levels in the fibrotic lung tissues harvested from the patients by RT-PCR or Western blotting, respectively, will verify an association in human between Del-1 mRNA or protein levels and pulmonary fibrosis.

Elucidation of the role for Del-1 in pulmonary fibrosis will better the understanding of molecular pathogenesis of this disease and thus contribute to the development of effective therapeutics.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.009.

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