

Leukotriene (LT)C₄ aggravates bleomycin-induced pulmonary fibrosis in mice

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Summary at a Glance

Bleomycin-induced pulmonary fibrosis is augmented in LTC₄ transgenic mice. LTC₄ overexpression was associated with more fibrosis and higher lavage levels of IL-4, IL-13

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and TGF- β 1.

ABSTRACT

Background and objective: Synthesis of cysteinyl leukotrienes (cys-LTs) is thought to cause inflammatory disorders such as bronchial asthma and allergic rhinitis. Recent reports have suggested that leukotriene (LT) C_4 is an important regulator of pulmonary fibrosis. This study examined the effect of LTC $_4$ in LTC $_4$ synthase-overexpressed transgenic (Tg) mice with bleomycin-induced pulmonary fibrosis. We also investigated the function of lung-derived fibroblasts from Tg mice.

Methods: Bleomycin was administrated to Tg mice and wild-type (WT) mice by intratracheal instillation. Concentrations of interleukin (IL)-4, -13, interferon (IFN)- γ and transforming growth factor (TGF)- β 1 in bronchoalveolar lavage fluid were measured 1, 3, 7, and 14 days after the administration of bleomycin. Lung tissue was examined histopathologically on day 14. In addition, lung-derived fibroblasts from Tg and WT mice were cultured for 7 days. Expression of TGF- β 1 mRNA was measured by real-time PCR.

Results: Both the pathological scores for pulmonary fibrosis (3.8 ± 0.4 vs 2.0 ± 0.1 , $p < 0.05$) and the levels of IL-4 (12.1 ± 2.3 vs < 7.8 pg/ml, $p < 0.05$), IL-13 (26.5 ± 5.2 vs < 7.8 pg/ml, $p < 0.01$), and TGF- β 1 (211.1 ± 30.2 vs 21.3 ± 1.2 pg/ml, $p < 0.01$) on day 14 were significantly greater in Tg than in WT mice. Furthermore, the reduction of LTC $_4$ by pranlukast hydrate, a cys-LT1 receptor antagonist, in fibroblasts from Tg significantly ($p < 0.05$) decreased the expression of TGF- β 1 mRNA (by ~50%), compared with those from WT mice.

Conclusions: Overexpression of LTC $_4$, amplifies bleomycin-induced pulmonary fibrosis

in mice. Our findings suggest a role for LTC₄ in lung fibrosis.

Key words: LTC₄ synthase-overexpressed transgenic mice, bleomycin, pulmonary fibrosis, lung-derived fibroblasts, TGF-β1

Short title: LTC₄ aggravates pulmonary fibrosis

Abbreviations

5-LO: 5-lipoxygenase

LTC₄: leukotriene C₄

IPF: idiopathic pulmonary fibrosis

LTC₄S-Tg: leukotriene C₄ synthase transgenic mice

Cys-LTs: cysteinyl leukotrienes

BAL: bronchoalveolar lavage

CMC: sodium carboxymethyl cellulose

ELISA: enzyme linked immune assay

EIA: enzyme immune assay

FCS: fetal calf serum

TGF-β: transforming growth factor-β

CTGF: connective tissue growth factor

IGF: insulin-like growth factor

FGF-2: fibroblast growth factor

PDGF: platelet-derived growth factor

INTRODUCTION

Pulmonary fibrosis comprises a diverse group of pathologies characterized by inflammatory infiltrates, disruption of alveolar structure, and excessive synthesis and deposition of extracellular matrix.¹⁻³ Furthermore, it is frequently associated with connective tissue diseases such as systemic sclerosis, dermatomyositis, and polymyositis.⁴⁻⁷ Active pulmonary fibrosis is accompanied by an increased number of leucocytes in bronchoalveolar lavage (BAL) fluid.⁸ Although the pathogenesis of pulmonary fibrosis remains unclear, evidence suggests that leukocyte infiltration regulates subsequent fibrosis in the lung.⁹ Progressive fibrosis is thought to be a consequence of aberrant wound healing resulting in progressive scarring.¹⁰ Fibroblast expansion and activation, leading to collagen fibril deposition, is considered a final common pathway. Therefore, much attention has been directed to the mechanisms leading to proliferation and synthesis of extracellular matrix proteins by resident fibroblasts, and to the generation of myofibroblasts. These originate in lung epithelial cells, through epithelial-mesenchymal transition, or in circulating mesenchymal precursors known as fibrocytes.^{11,12}

5-lipoxygenase (5-LO) is expressed in eosinophils, basophils, mast cells, macrophages, platelets, and endothelial cells. The sequential catalytic action of 5-LO on arachidonic acid (AA) generates LTA₄, which is either hydroxylated to LTB₄ or metabolized into cysteinyl leukotrienes (cys-LTs) and leukotriene C₄ (LTC₄) via the LTC₄ synthase (LTC₄S).¹³ LTC₄ is then converted to LTD₄, in the extracellular space, where it is subsequently cleaved to form LTE₄.¹⁴ LTC₄ is thought to cause various inflammatory disorders such as bronchial asthma and allergic rhinitis, and it is secreted by alveolar cells

in patients with idiopathic pulmonary fibrosis (IPF).¹⁵⁻¹⁷ In fact, LTC₄ levels are elevated in BAL fluid and lung tissue in patients with IPF.^{17,18} In addition, 5-LO knockout mice, disruption of LTC₄ synthase, and cys-LT receptor antagonists have all been shown to attenuate pulmonary fibrosis.¹⁹⁻²¹ LTs promote fibroblast chemotaxis, fibroblast proliferation, and collagen synthesis.²²

This study examined the effects of LTC₄ in LTC₄ synthase-overexpressed transgenic (LTC₄S-Tg) mice with bleomycin-induced pulmonary fibrosis. We also investigated the function of lung-derived fibroblasts in LTC₄S-Tg mice.

METHODS

Mice

BALB/c mice were purchased from Japan SLC. LTC₄S-Tg mice with a BALB/c background have been described previously.²³ The transgenic overexpression of *Ltc₄s* is directed by a CMV enhancer/chicken β -actin promoter in the transgenic vector pCAGGs, which has been shown to facilitate widespread transgene expression in mice. The study was approved by the Ethics Committee of the Dokkyo University School of Medicine, and mice were maintained in the animal center under specific pathogen-free conditions.

Bleomycin administration in mice

Study protocols are shown in Figure 1. Protocol 1 was used to induce bleomycin-induced pulmonary fibrosis. Saline alone (bleomycin (-)) as a control or bleomycin sulfate (bleomycin (+); 5 mg/kg body weight; Nippon Kayaku, Tokyo, Japan) was administrated intratracheally to wild-type (WT) (Group 1 (saline) or Group 3 (bleomycin(+)) and

LTC₄S-Tg (Group 2 (saline) or Group 4 (bleomycin)) mice which were both anaesthetized by inhalation of diethyl ether on day (D) 0. Using aseptic techniques, a single incision was made in the neck, and the muscle covering the trachea was snipped to expose the tracheal rings. BAL fluid was obtained before, and on D1, D3, D7, and D14 after the administration of bleomycin or saline, and the lungs were examined histologically on D14.

Bronchoalveolar lavage

The method of BAL and the cells count, and the storage of the fluid sample, have been described previously.²⁴ The remaining cells in the BAL fluid were harvested and analyzed in a subsequent procedure.

Fibroblast isolation from whole lung

Murine lung-derived fibroblasts were generated according to the method reported by Zhang and coworkers.²⁵ The fibroblasts were grown for 14 days to exclude the effects of LTC₄ secreted from the cells and used for 2 to 3 passages.

Lung-derived fibroblast culture

To investigate the functional role of LTC₄ in lung-derived fibroblasts, cells (1×10^5 /ml) from LTC₄S-Tg or WT mice were cultured in the complete medium with 10% FCS in the presence of pranlukast hydrate (pranlukast (+); 10 µg/ml) or 0.5% CMC (pranlukast (-); 10 µg/ml) as a control for 7 days.

Additionally, fibroblasts in LTC₄S-Tg mice were harvested, and the expression of mRNA was analyzed as described below.

Lung collagen measurements

The left lung was exposed, filled with polyethylene glycol, and homogenized. Collagen content was determined using the Sircol Collagen Assay kit (Biocolor Ltd., Belfast, UK),²⁶ and collagen concentration was measured using an ELISA microplate reader (Molecular Devices, Menlo Park, CA) at 540 nm.

Expression of mRNA in lung-derived fibroblasts

Total RNA was extracted from lung-derived fibroblasts using TRIzol, a monophasic solution of phenol and guanidine isothiocyanate (Invitrogen, Carlsbad, CA). Total RNA (5 µg) was immediately reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo(dT) primers (Amersham Pharmacia Biotech, Piscataway, NJ), as per manufacturer instructions. The resultant cDNA products were diluted in Tris-EDTA buffer and used for real-time quantitative PCR.

Real-time quantitative PCR

Quantification of TGF-β1, connective tissue growth factor (CTGF), insulin-like growth factor (IGF)-I, fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF)α, and PDGFβ mRNA expression was performed by real-time RT-PCR on an ABI/PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA), and results were analyzed using ABI/PRISM 7000 SDS v1.0 software (Applied Biosystems). β-actin was used as an internal control (for more detail see Supporting Information online).

Histopathology

The method for perfusion and fixation of the trachea and the right lung have been described previously.²⁴ Sections were cut on a microtome to 4 μ m, mounted on slides, and stained with HE and elastica-Masson to assess fibrosis. Five sections of the entire lung stained with HE were randomly chosen from each mouse. The severity of lung inflammation was assessed using a semiquantitative scoring system, as described previously.^{26, 27} A fluorescence microscope (EX41, Olympus America, Melville, NY) equipped with $\times 20$ and $\times 4$ lenses and an Olympus Magna Fire camera was used to obtain photomicrographs.

Measurement of interleukin (IL)-4, IL-13, interferon (IFN)- γ , TGF- β 1, LTC₄, LTD₄, and LTE₄

Concentrations of IL-4, IL-13, IFN- γ , TGF- β 1, and LTs (LTC₄, LTE₄, or LTD₄) in BAL fluid supernatants were measured using ELISA kits (R & D, MN), and enzyme-immunoassay (EIA) kits (Cayman, Ann Arbor, MI; and Cusabio, Wuhan, Hubei Province China), respectively, as per manufacturer instructions.

Statistical analysis

All data are expressed as mean $\pm$ standard deviation (SD). To determine differences among the 4 groups, we conducted a repeated measures ANOVA. The Student's *t*-test was used to compare expression of mRNA between pranlukast (-) and pranlukast (+), and significance level was set at $p < 0.05$ for all statistical comparisons.

RESULTS

Cell populations in BAL from bleomycin-treated WT and LTC₄S-Tg mice

To examine the effect of LTC₄ on pulmonary inflammation in bleomycin-treated mice, we analyzed cell populations in BAL fluid from WT and LTC₄S-Tg mice on D14 after bleomycin administration. The number of total cells ($p<0.01$), macrophages ($p<0.05$), lymphocytes ($p<0.01$) and neutrophils ($p<0.05$) in LTC₄S-Tg mice significantly increased by between 2 to 5 fold, compared with WT mice (Fig. 2A).

Concentration of LTC₄, LTD₄, LTE₄, IL-4, IL-13, IFN- γ , and TGF- β 1 in BAL fluid in bleomycin-treated WT and LTC₄S-Tg mice

As shown in Figure 2B and Table S1(supporting information online), we measured the concentration of LTC₄, LTD₄, LTE₄, IL-4, IL-13, IFN- γ , and TGF- β 1 in BAL fluid before treatment administration, and on D1, D3, D7, and D14 in WT and LTC₄S-Tg mice bleomycin-treated and controls. There was no difference for the controls (group 1 and 2). For the bleomycin (+) treated mice (group 3 and 4), the concentration of LTC₄ on D1 and D3 was significantly higher in LTC₄S-Tg mice than in WT mice. On D7 and D14, the concentration of LTC₄ decreased in LTC₄S-Tg mice whereas in WT mice it increased and was significantly higher than in the LTC₄S-Tg mice. As metabolic products of LTC₄, the concentration of LTD₄ in LTC₄S-Tg mice on D1 to D14 was similar to that in WT mice, whereas, the concentration of LTE₄ on D1, D3, D7, and D14 was significantly higher in LTC₄S-Tg mice than in WT mice (Table S1). These results show that LTC₄ metabolism was greater in the Tg mice than in the WT mice.

The concentration of IL-4 at each time point was significantly higher in LTC₄S-Tg mice than in WT mice. The concentration of IL-13 on D3, D7, and D14 was significantly higher in LTC₄S-Tg mice than in WT mice (Table S1). In contrast, the concentration of IFN- γ on D1 was significantly higher in LTC₄S-Tg mice (103.2 $\pm$ 43.5 pg/ml) than in WT mice (49.8 $\pm$ 12.7 pg/ml) (p <0.05), and the concentration of IFN- γ in WT mice on D3 and D7 tended to increase compared with LTC₄S-Tg mice. The concentration of TGF- β 1 on D7 and D14 was significantly higher in LTC₄S-Tg mice (223.7 $\pm$ 21.1 and 211.1 $\pm$ 30.2 pg/ml, respectively) than in WT mice (18.2 $\pm$ 2.7 and 21.3 $\pm$ 1.2 pg/ml, respectively) (p <0.01).

These results indicate that LTC₄ contributes to the increased production of IL-4, IL-13, and TGF- β during bleomycin-induced lung inflammation; although a transient increase in IFN- γ was observed in the Tg mice.

Histopathological analysis in bleomycin-treated WT and LTC₄S-Tg mice

Histopathological analysis of the lung was performed in bleomycin (+)-treated WT (Group 3) and LTC₄S-Tg mice (Group 4) and in bleomycin (-) controls (Group 1 and Group 2) (Fig. 3). Lung sections were stained with HE (Fig. 3A, B, and Figure S1A, B) or elastic-Masson (Fig. 3C, D). No obvious inflammation or fibrosis was observed in lung sections from saline WT (Fig. 3A) or saline LTC₄S-Tg mice (Fig. 3B). Infiltration of cells and fibrosis in interstitial lung tissue on D14 after bleomycin administration increased in LTC₄S-Tg mice (Fig. 3D and Fig S1B) compared to WT mice (Fig. 3C and Fig S1A). As shown in Figure 3G, the pathological scores for lung fibrosis were significantly greater in LTC₄S-Tg mice (3.8 $\pm$ 0.4) than in WT mice (2.0 $\pm$ 0.1) (p <0.05). In addition, the concentration of lung collagen was also significantly higher in bleomycin (+)-LTC₄S-Tg

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mice (70.5 ± 7.1 $\mu\text{g/ml}$) than in bleomycin (+)-WT mice (15.3 ± 8.3 $\mu\text{g/ml}$) ($p < 0.01$) (Fig. 3H). These results indicate that LTC_4 aggravates bleomycin-induced pulmonary fibrosis as well as inflammation.

Effects of LTC_4 on expression of TGF- β 1, CTGF, IGF-I, FGF-2, PDGF α , and PDGF β mRNA in lung-derived fibroblasts of $\text{LTC}_4\text{S-Tg}$ mice

Finally, we analyzed the effect of LTC_4 on the expression of TGF- β 1, CTGF, IGF-I, FGF-2, PDGF α , PDGF β mRNA (Fig. S2), and fibroblast growth factors in lung-derived fibroblasts from $\text{LTC}_4\text{S-Tg}$ mice. The mRNA expression of the growth factors analyzed by real-time PCR is shown as a ratio relative to the expression in fibroblasts of pranlukast (-) WT mice. The relative expression of TGF- β 1 mRNA in pranlukast (+) fibroblasts was significantly decreased by approximately 50% compared with pranlukast (-) fibroblasts ($p < 0.05$). On the other hand, no significant differences between pranlukast (+) and pranlukast (-) fibroblasts in $\text{LTC}_4\text{S-Tg}$ mice for the relative expression of CTGF, IGF-I, FGF-2, PDGF α , and PDGF β mRNA was observed. Similarly, no significant difference between pranlukast (+) and pranlukast (-) fibroblasts in WT mice was noted regarding the relative expression of these growth factors (data not shown). These results indicate that autocrine activation by LTC_4 from lung-derived fibroblast induce the expression of TGF- β 1.

DISCUSSION

Bleomycin is commonly used to induce experimental pulmonary fibrosis in rodents,²⁷ because it causes injury to the lung epithelium. This injury is presumably secondary to DNA damage resulting from the action of reactive oxygen intermediates which are produced by the metal-ion-catalyzed reaction of bleomycin with oxygen.²⁸ These events are followed by an intense inflammatory response. Two weeks after bleomycin administration, the inflammatory and fibroproliferative responses wane, and collagen deposition is detectable.²⁹ Several growth factors have been implicated in the fibroproliferative response that characterizes interstitial pneumonitis.³⁰⁻³⁴ TGF- β and IGF-I have been shown to stimulate collagen matrix synthesis³⁵⁻³⁸ and prevent the induction of apoptosis in fibroblasts.³⁹⁻⁴² FGF-2, PDGF, and IGF-I have been reported to be involved in fibroblast proliferation.^{30,32,43} The mechanism responsible for expression of these growth factors is poorly understood, but recent studies have suggested that cys-LTs may be a contributing factor.^{12,17-22}

In this study, we analyzed how LTC₄ might influence the pathogenesis of pulmonary fibrosis in mice. LTC₄S-Tg mice were used to accurately estimate its role in bleomycin-induced pulmonary fibrosis. Histological and cytological analysis was performed in experimental groups of bleomycin hydrate, or saline-treated WT and LTC₄S-Tg mice. We showed that the number of leukocytes, especially lymphocytes, macrophages, and neutrophils, in BAL fluid of LTC₄S-Tg mice was increased compared to WT mice. LTC₄ secretion is present in BAL fluid in bleomycin (+)-treated mice, in the early phase for LTC₄S-Tg and in the late phase for the WT mice. In LTC₄S-Tg mice LTC₄ is rapidly produced then, it is also quickly metabolized into LTD₄ and LTE₄, which are

also bioactive. LTE_4 is a major cys-LT in tissue in inflammatory models.⁴⁴ The concentration profiles of LTD_4 in WT and $\text{LTC}_4\text{S-Tg}$ mice were the same whereas LTE_4 concentration raised earlier in Tg mice than in WT and had a different profile. This could be due to a faster metabolism or to different sources of this metabolite in Tg mice compared to WT. Further studies are needed to clarify this result.

Concentrations of IL-4 and IL-13 in BAL fluid in the early to late phase were always higher in bleomycin (+)-treated $\text{LTC}_4\text{S-Tg}$ mice than in bleomycin (+)-treated WT mice. The concentration of $\text{TGF-}\beta 1$ in $\text{LTC}_4\text{S-Tg}$ mice, but not in WT mice, was highest in the late phase. Since the LTC_4 receptor, is expressed on macrophages, mast cells, fibroblasts, epithelial cells, and smooth muscle cells which are known to contribute to the pathogenesis of pulmonary fibrosis,^{45,46} excess amounts of cys-LTs might induce the pathogenesis of bleomycin-induced pulmonary fibrosis by orchestrating the functions of the various cells in $\text{LTC}_4\text{S-Tg}$ mice. Here, we observed a Th2-type airway inflammation, which was characterized by eosinophilia and profound Th2 cytokine production. It is known that LTC_4 accelerates the development of the Th2 response, in which dendritic cell/T cell interaction might be modulated bifunctionally by cys-LTs, including LTC_4 .⁴⁷⁻⁵¹ In addition, the intracrine cys-LTs receptor plays a role in the Th2 response by mediating the signaling of eosinophil vesicular transport-mediated IL-4 secretion.⁵² Although the cell source of Th2 cytokines remains unclear in the experimental model of bleomycin-induced pulmonary fibrosis, the high levels of LTC_4 in the Tg mice might serve to accelerate Th2 cytokine production which in turn might augment $\text{TGF-}\beta 1$ production.

Previous reports showed that the reduction in the function of LTC_4 decreased not only pulmonary fibrosis and collagen content, but also production of Th2 cytokines and

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TGF- β 1.^{19,22,53} HUAUX *et al.* showed that IL-4 plays an important role in collagen deposition during the fibroproliferative phase of bleomycin-induced pulmonary fibrosis.⁵⁴ JAKUBZICK *et al.* showed that IL-4 and IL-13 may act on macrophages to promote or sustain the fibroproliferative phase in bleomycin-treated mice.⁵⁵ Furthermore, recent studies have reported that IL-4 and IL-13 induced the expression of TGF- β 1 in mice with bleomycin-induced pulmonary fibrosis.⁵⁶ TGF- β 1 is believed to be a critical mediator of pathological, fibrotic, and remodeling responses in the lung and other organs, and such responses are believed to be mediated by the ability of TGF- β 1 to activate fibrogenic and apoptotic injury pathways.⁵⁷⁻⁵⁹ Therefore, expression of TGF- β 1 is associated with a variety of pulmonary inflammatory diseases, including interstitial lung disease and asthma.^{57,58} On the other hand, we have already reported that the Th2 cytokines milieu might contribute to initiation and acceleration of bleomycin-induced pulmonary fibrosis, but not to its maintenance and prognosis.²⁷ Therefore, Th2-dominant inflammation in the lung might not be essential for the development of bleomycin-induced pulmonary fibrosis, despite the higher amounts of Th2 cytokine in LTC₄S-Tg mice. In this study, bleomycin (+)-treated LTC₄S-Tg mice, compared with bleomycin (+)-treated WT controls, showed an exacerbated pulmonary fibrosis and increased collagen content. Further studies are needed to clarify the mechanism by which this takes place and the role of LTC₄. In contrast, although prostaglandin (PG)E₂, which is produced by the cyclooxygenase pathway, also protects murine lungs from bleomycin-induced pulmonary fibrosis and lung dysfunction,⁶⁰ the levels of PGD₂ and PGE₂ in BAL fluid between LTC₄S-Tg mice and WT mice were similar in our report.²³

Mast cells, eosinophils, and macrophages are known as cellular sources of LTC₄.²³ Recent studies have reported that lung-derived fibrocytes and fibroblasts also secrete

LTC₄.¹² PERNG *et al.* reported that LTC₄ directly induces TGF- β 1 production in the airway epithelium via the p38 kinase pathway.⁵³ Furthermore, fibroblasts differentiate into myofibroblasts in TGF- β 1-exposed human lung fibroblasts and are simultaneously expressed by the immunolocalization of α -SMA.⁶¹ These findings indicate that lung-derived fibroblasts may need sufficient levels of continuous LTC₄ to self-proliferate, and consequently might sustain production of TGF- β 1 during a fibroproliferative phase. In our study, LTC₄ secretion from fibroblasts was higher in LTC₄S-Tg mice than in WT mice. In addition, antagonizing cys-LTs with pranlukast in fibroblasts from LTC₄S-Tg mice, but not in those from WT mice, lead to decreased cell viability and expression of α -SMA and TGF- β 1 mRNA (data not shown). These results suggest that lung-derived fibroblasts proliferate by an autocrine mechanism via self-secretion of LTC₄ and/or through secondary production of TGF- β 1. The excess LTC₄ in a living body might have contributed to hyper-proliferation of fibroblasts derived from lungs of the Tg mice compared with those of WT mice. On the other hand, we showed that pranlukast had no effect on the expression of CTGF, FGF-2, IGF-I, or PDGF mRNA in the fibroblasts from LTC₄S-Tg mice even though, mRNA expression of these growth factors in the fibroblasts was higher in LTC₄S-Tg mice than in WT mice (bleomycin +). KEVIN *et al.* reported that murine and human fibroblasts express both cys-LT1 and cys-LT2 receptor.¹² Although cys-LT2 receptor is considered to have a low affinity to cys-LTs compared with the cys-LT1 receptor,⁶² excess cys-LTs might exert their effects on bleomycin-induced fibrosis through not only the cys-LT1 receptor but also the cys-LT2 receptor. Several investigators have reported that the cys-LT2 receptor also plays an important role in the pathogenesis of pulmonary fibrosis in a study that analyzed cys-LT2 receptor-deficient mice.⁶³ Because pranlukast is mainly a cys-LT1 receptor antagonist, expression of

TGF- β 1 and other growth factors might be induced by excess LTC₄ through the pathway of cys-LT₂ receptor signaling in LTC₄S-Tg mice.

In conclusion, although the nature of LTC₄ in the pathogenesis of lung fibrosis has not been elucidated at a molecular level, the present findings suggest that over-expression of LTC₄ aggravates bleomycin-induced pulmonary fibrosis via augmenting production of some fibrogenic growth factors.

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Figure legends

Figure 1

Diagram of study protocols. WT and LTC₄S-Tg mouse models of bleomycin-induced pulmonary fibrosis (bleomycin (+); Group 3 and Group 4, respectively) and saline (bleomycin (-); Group 1 and Group 2, respectively) as a control. BAL fluid was obtained prior to the administration of bleomycin and on day (D)1, D3, D7, and D14. The lung was histologically examined on D14.

Figure 2

Cell type (A) on D14, and time kinetics of LTC₄, LTD₄, LTE₄, IL-4, IL-13, IFN- γ , and TGF- β 1 concentration (B) prior to the administration of bleomycin on D1, D3, D7, and D14 in BAL fluid. ○ Group 1: saline WT mice; ○ Group 2: saline LTC₄S-Tg mice; ● Group 3: bleomycin-treated WT mice; ● Group 4: bleomycin-treated LTC₄S-Tg mice. Data are expressed as mean $\pm$ SD for each group (n=8 per each time point in group). Significance was set at $p<0.05$ for comparison of bleomycin (+)-treated lung fibrosis in WT mice (Group 3). * $p<0.05$, ** $p<0.01$. M Φ , macrophages; Lym, lymphocytes; Neu, neutrophils.

Figure 3

Histological analysis of bleomycin-induced lung fibrosis [C, D; bleomycin (+)] and saline treatment [A, B: bleomycin (-)], as a control for the administration of bleomycin, in WT or LTC₄S-Tg mice. Lung sections were stained with HE (A, B) and elastica-Masson

(MG) (C, D). Scale bar represents 100 μ m. Original magnification, $\times 100$ for HE and MG.

Quantification of pulmonary fibrosis and collagen production. Score for lung fibrosis (E) and collagen concentration (F) in lung tissue was determined on D14 after administration of bleomycin.  Group 1: saline WT mice;  Group 2: saline LTC₄S-Tg mice; 

Group 3: bleomycin-treated WT mice;  Group 4: bleomycin-treated LTC₄S-Tg mice.

Data are presented as mean $\pm$ SD for each group (n=7 per group). Significance was set at $p < 0.05$ for comparison of bleomycin (+)-treated lung fibrosis in WT (Group 3). * $p < 0.05$,

** $p < 0.01$.

Supporting Information available online

Figure S1

Histological analysis of bleomycin-induced lung fibrosis [A:bleomycin (+)] in WT or LTC₄S-Tg mice. Lung sections were stained with HE (A, B). Scale bar represents 100 μ m. Original magnification, $\times 100$ for HE and MG. Quantification of pulmonary fibrosis and collagen production.  Group 1: saline WT mice;  Group 2: saline LTC₄S-Tg mice;  Group 3: bleomycin-treated WT mice;  Group 4: bleomycin-treated LTC₄S-Tg mice. Data are presented as mean $\pm$ SD for each group (n=7 per group). Significance was set at $p<0.05$ for comparison of bleomycin (+)-treated lung fibrosis in WT (Group 3). * $p<0.05$, ** $p<0.01$.

Figure S2

Expression of TGF- β 1, CTGF, IGF-I, FGF-2, PDGF α , and PDGF β mRNA in LTC₄S-Tg mice. Lung-derived fibroblasts (1×10^5 /ml) in LTC₄S-Tg mice in the complete medium with pranlukast hydrate (pranlukast (+)) or 0.5% CMC (pranlukast (-)), as a control for pranlukast hydrate, were cultured for 7 days and then harvested. White and black bars represent pranlukast (+) and pranlukast (-), respectively, in LTC₄S-Tg mice. Data are expressed as mean $\pm$ SD for each group (n=5 per group). Significance was set at $p<0.05$ for comparison of pranlukast (-) in LTC₄S-Tg mice. mRNA expression of the growth factors analyzed by real-time PCR is shown as a ratio relative to the expression in fibroblasts of pranlukast (-) WT mice. * $p<0.05$.

FIGURE 1

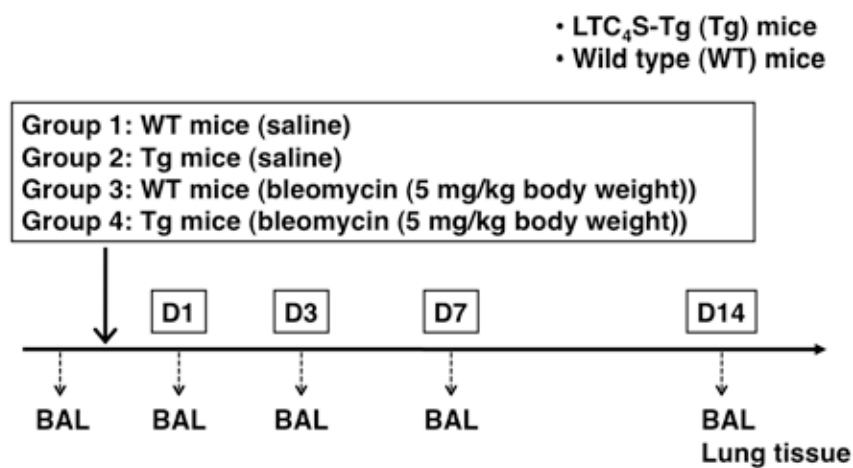


Fig 1

FIGURE 2a

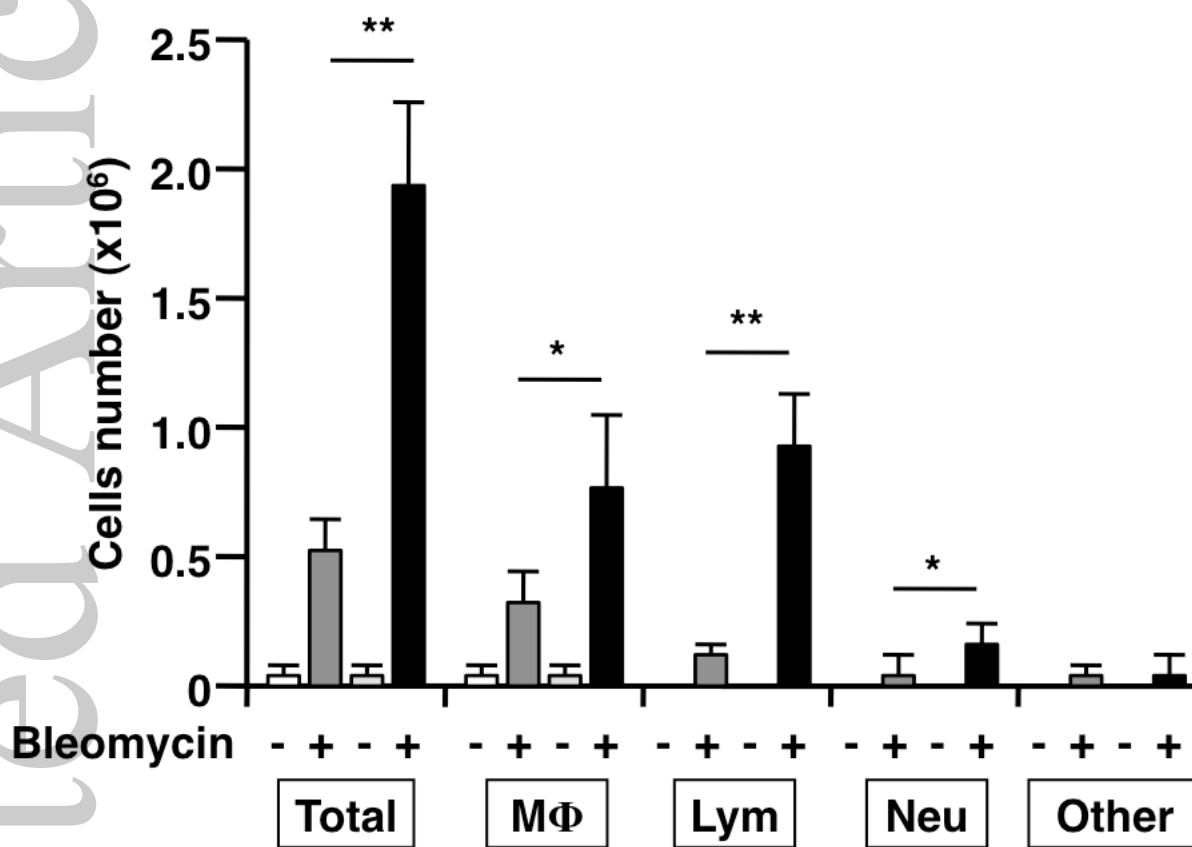


Figure 2a

FIGURE 2b

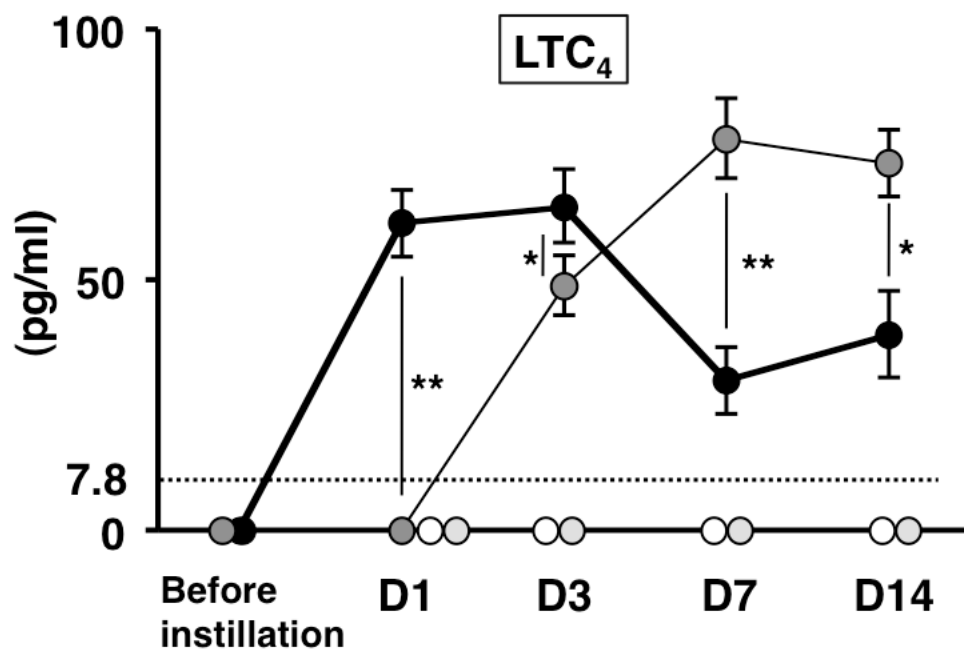


Fig_2b1_(LTC4)

FIGURE 2b

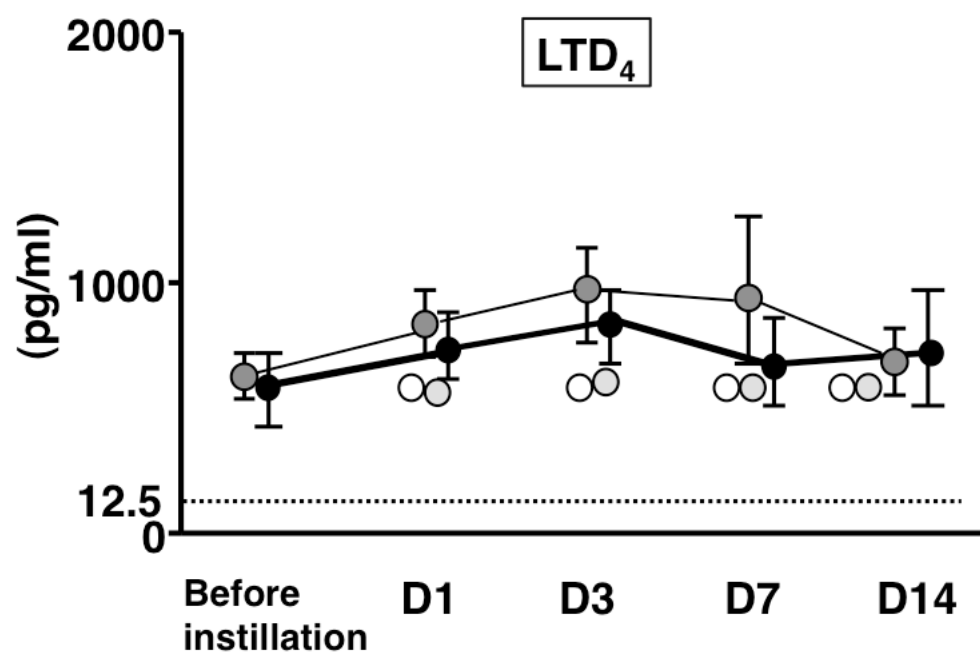


Fig_2b2_(LTD4)

FIGURE 2b

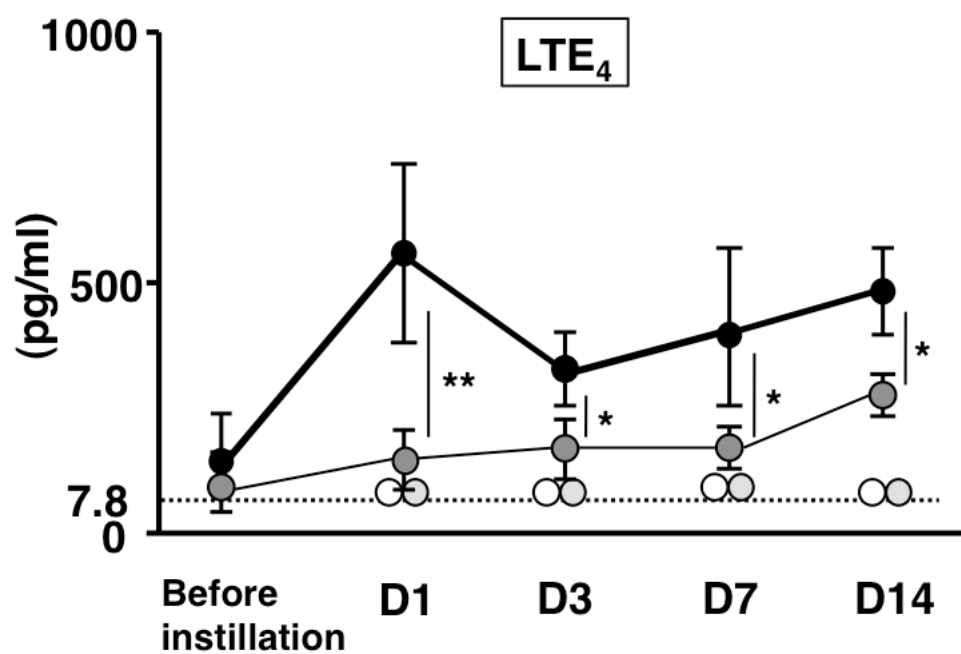


Fig_2b3_(LTE4)

FIGURE 2b

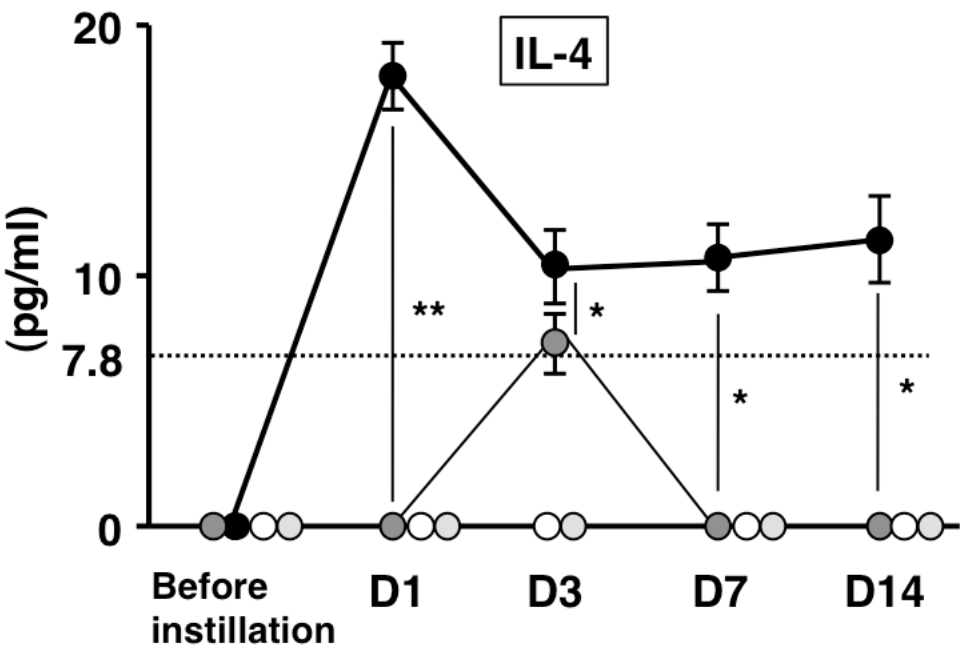


Fig_2b4_(IL-4)

FIGURE 2b

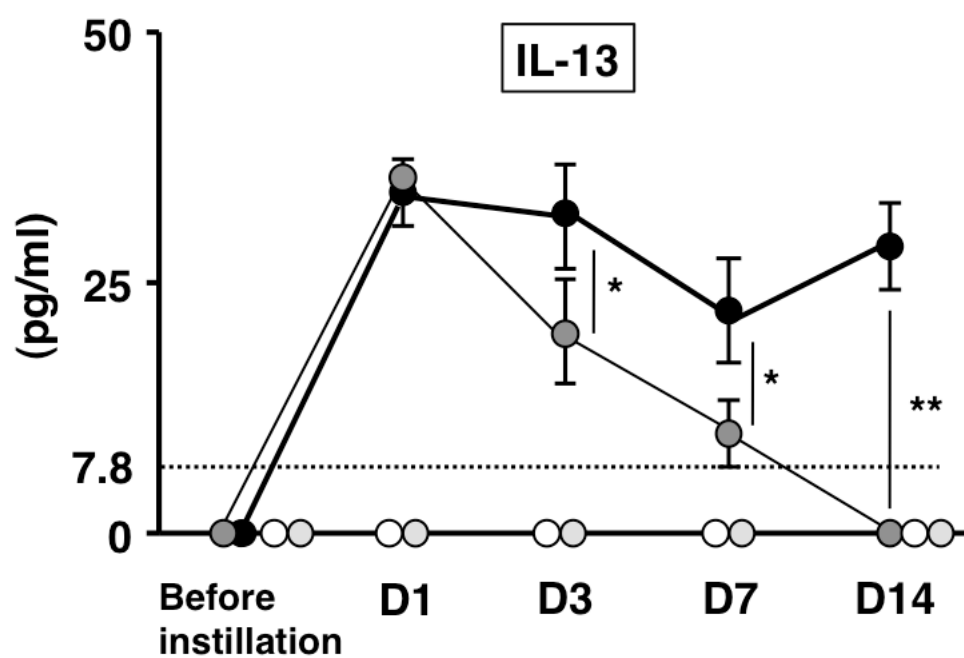


Fig_2b5_(IL-13)

FIGURE 2b

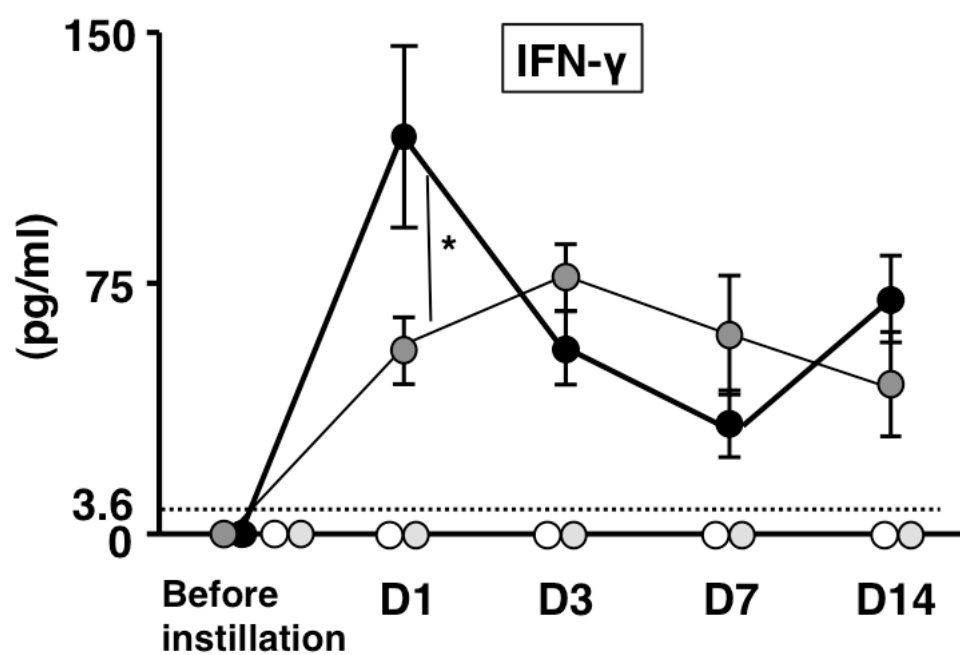


Fig_2b6_(IFN- γ)

FIGURE 2b

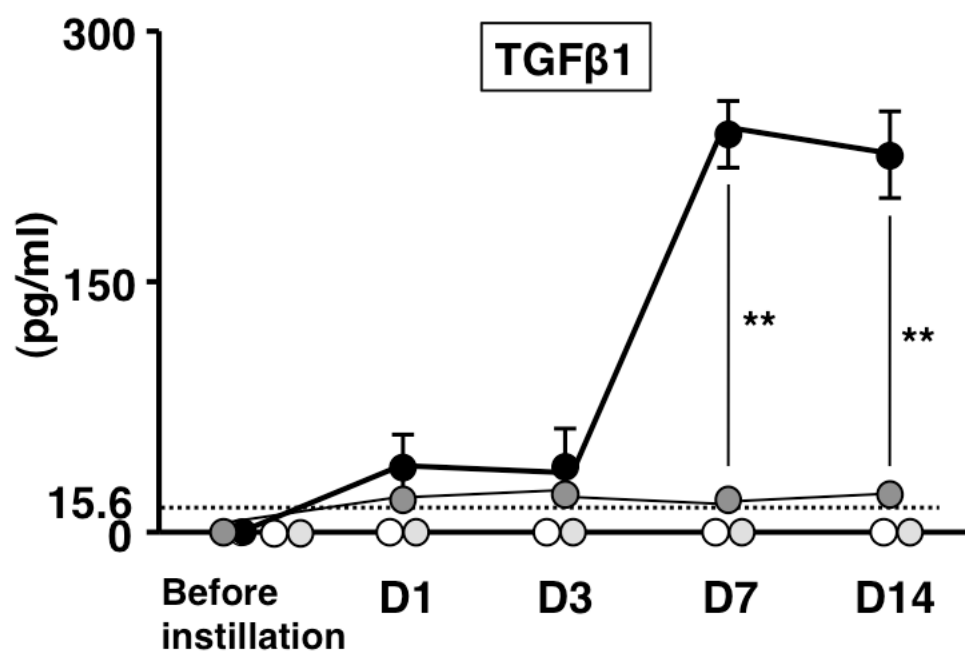
Fig_2b7_(TGF- β 1)

FIGURE 3a

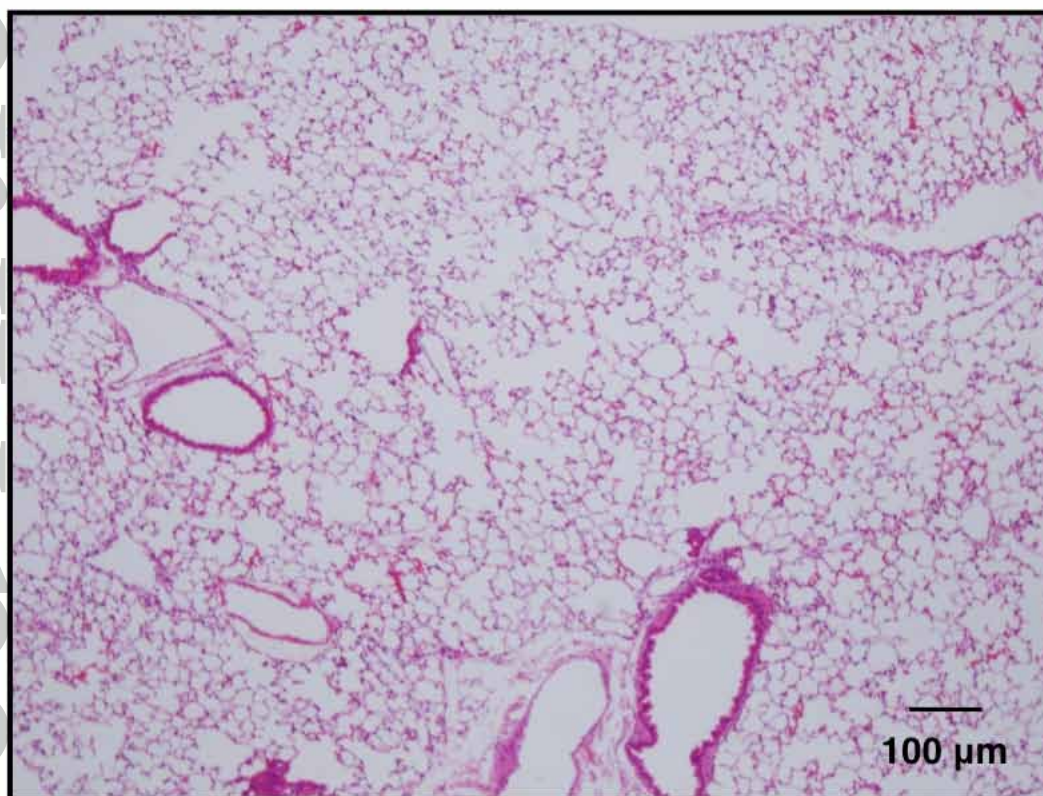


Fig 3a

FIGURE 3b

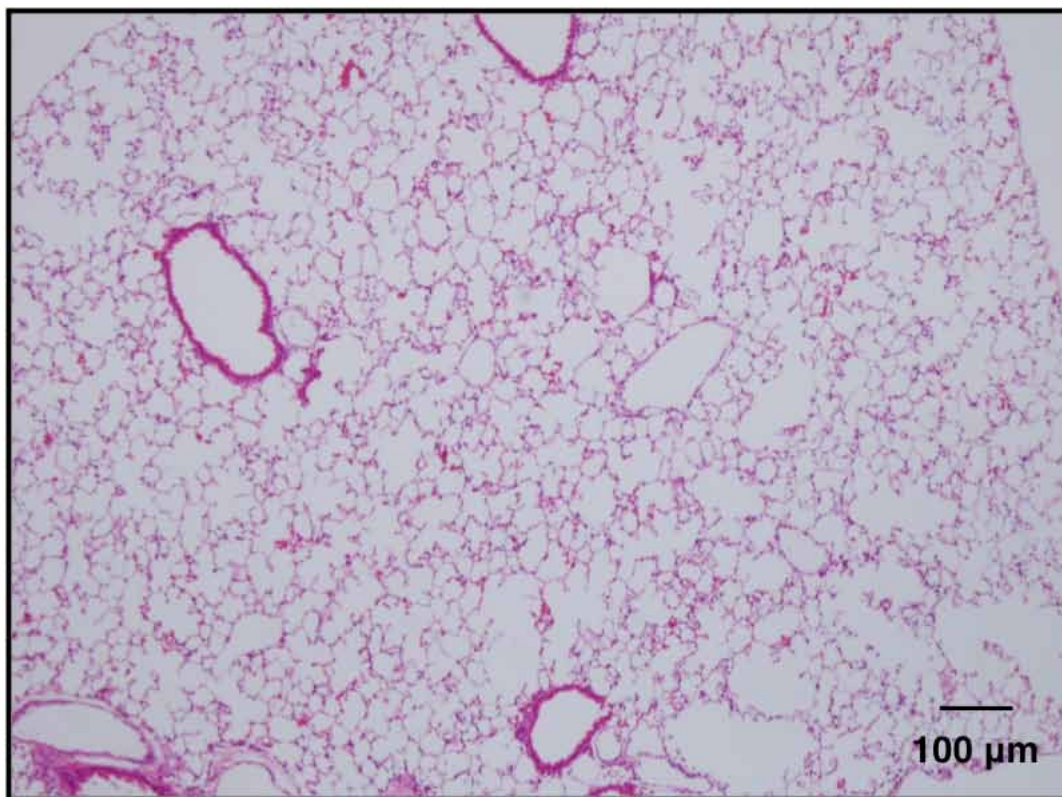


Fig 3b

FIGURE 3d

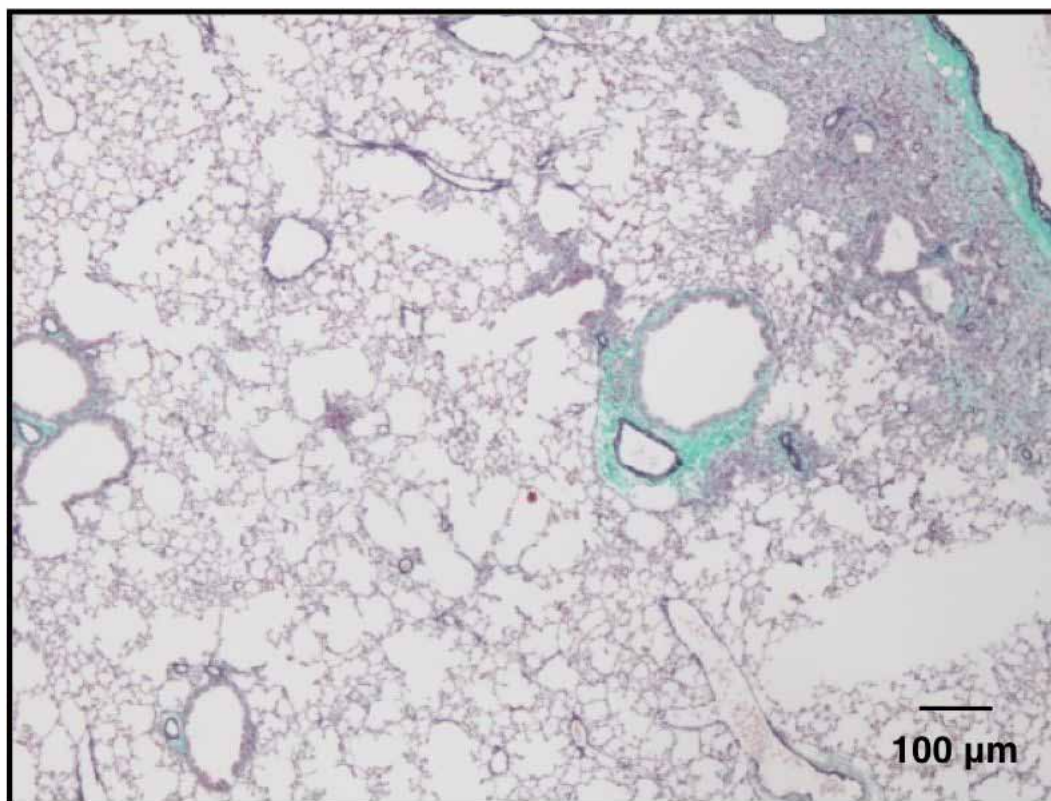


Fig_3C

FIGURE 3f

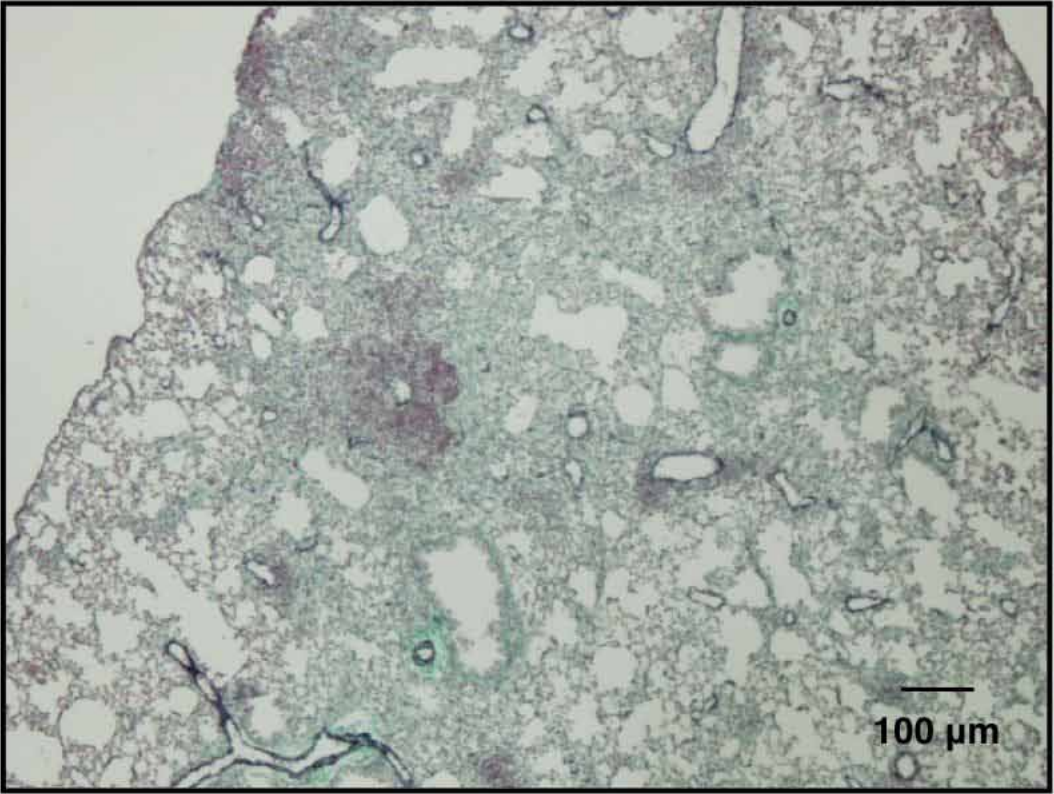


Fig_3D

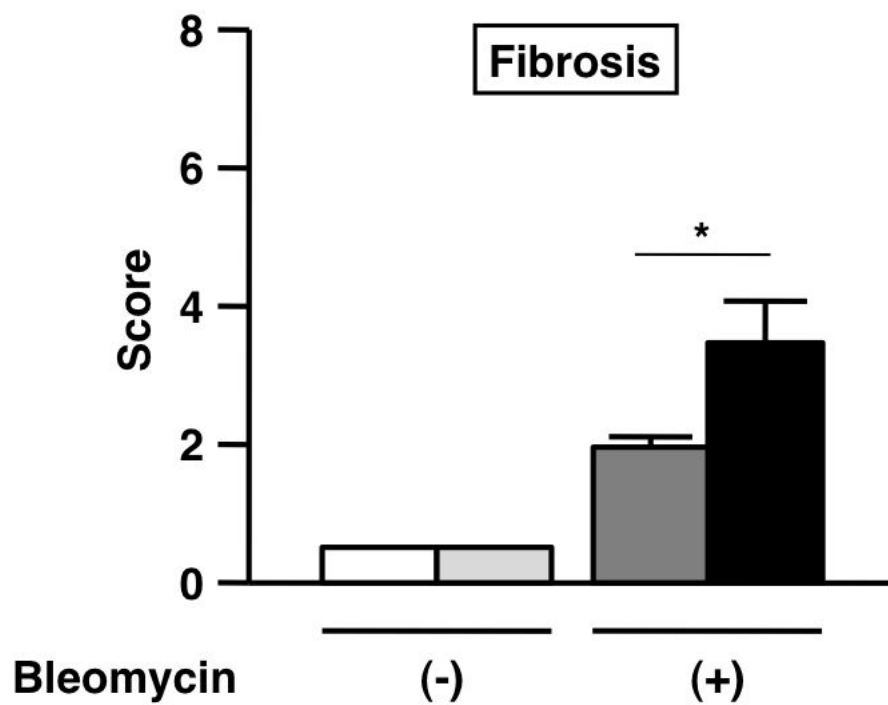
FIGURE 3g

Fig_3E

FIGURE 3h

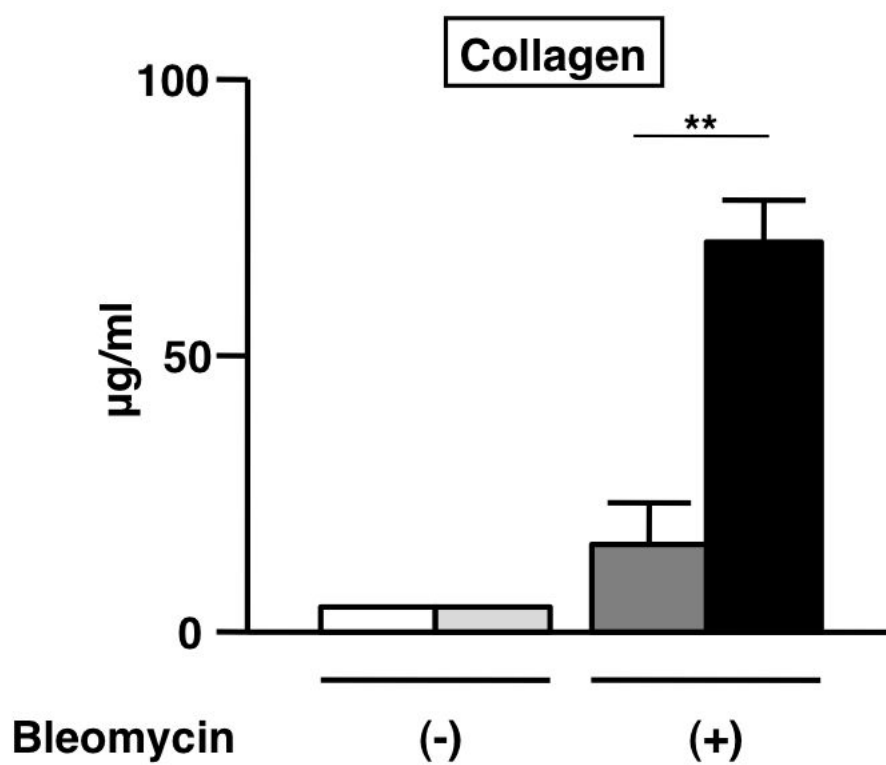


Fig_3F

Table 1

Concentrations of LTC₄, LTE₄, IL-4 and IL-13, in BAL fluid in bleomycin-treated WT and LTC₄S-Tg mice

Concentration in BAL fluid (pg/ml ±SD)	Bleomycin treated mice	Day 1	Day 3	Day 7	Day 14
LTC₄ and LTE₄					
LTC₄	WT	<7.8	46.2±4.9	75.3±8.8	71.3±7.2
	LTC₄S-Tg	60.5±8.9	68.2±10.5	25.1±6.9	32.3±13.2
	p	<i>p</i> <0.01	<i>p</i> <0.05	<i>p</i> <0.01	<i>p</i> <0.05
LTE₄	WT	121.1±23.8	135.6±25.6	141.6±18.8	182.3±19.1
	LTC₄S-Tg	584.1±162.3	382.1±52.3	458.8±80.4	492.5±62.3
	p	<i>p</i> <0.01	<i>p</i> <0.05	<i>p</i> <0.05	<i>p</i> <0.05
Interleukins 4 and 13					
IL4	WT	<7.8	<7.8	<7.8	8.0±0.7
	LTC₄S-Tg	18.1±1.2	10.5±1.5	11.2±1.3	12.1±2.3
	p	<i>p</i> <0.01	<i>p</i> <0.05	<i>p</i> <0.05	<i>p</i> <0.05
IL13	WT	-	19.8±6.3	10.5±2.6	<7.8
	LTC₄S-Tg	-	30.5±7.3	23.5±7.2	26.5±5.2
	p	-	<i>p</i> <0.05	<i>p</i> <0.05	<i>p</i> <0.01

FIGURE 3c

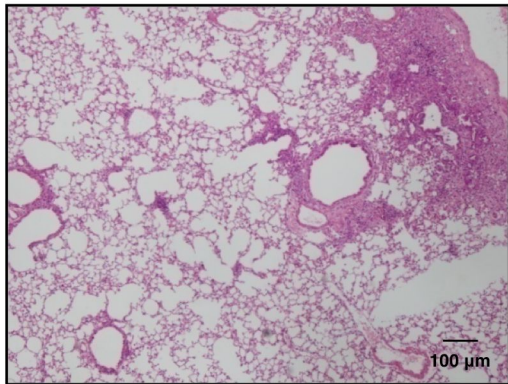


FIGURE 3e

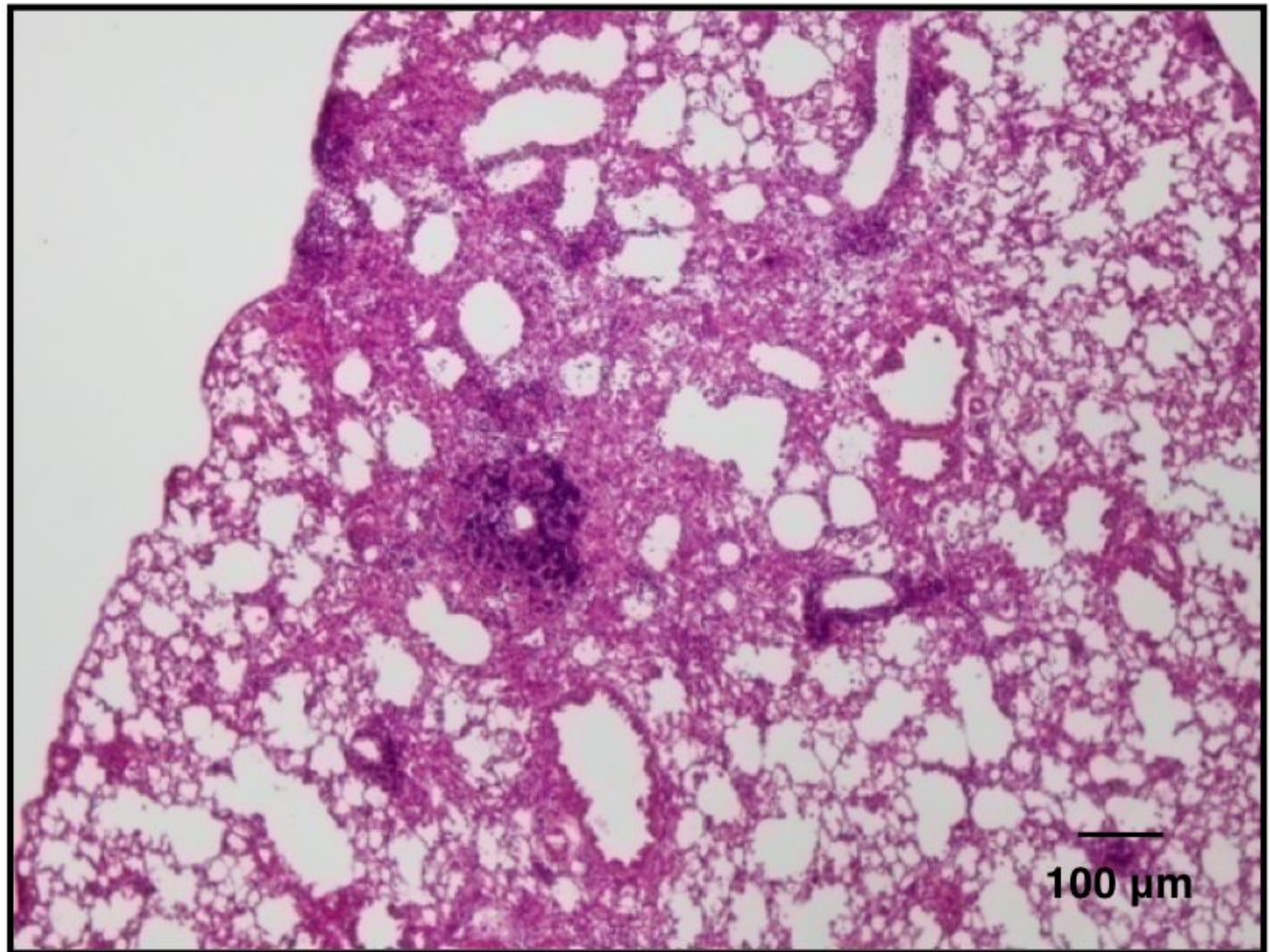


FIGURE 4

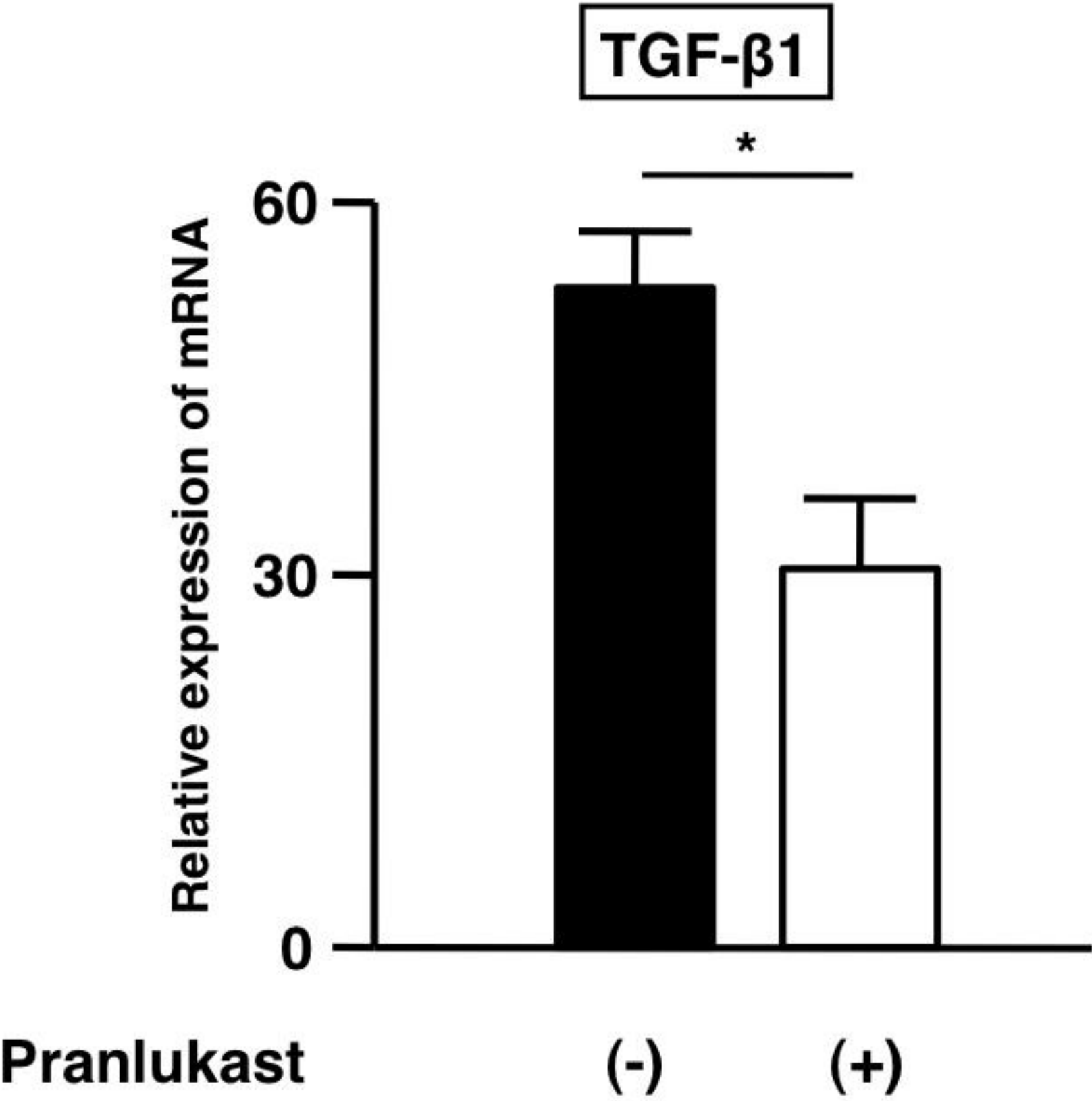


FIGURE 4

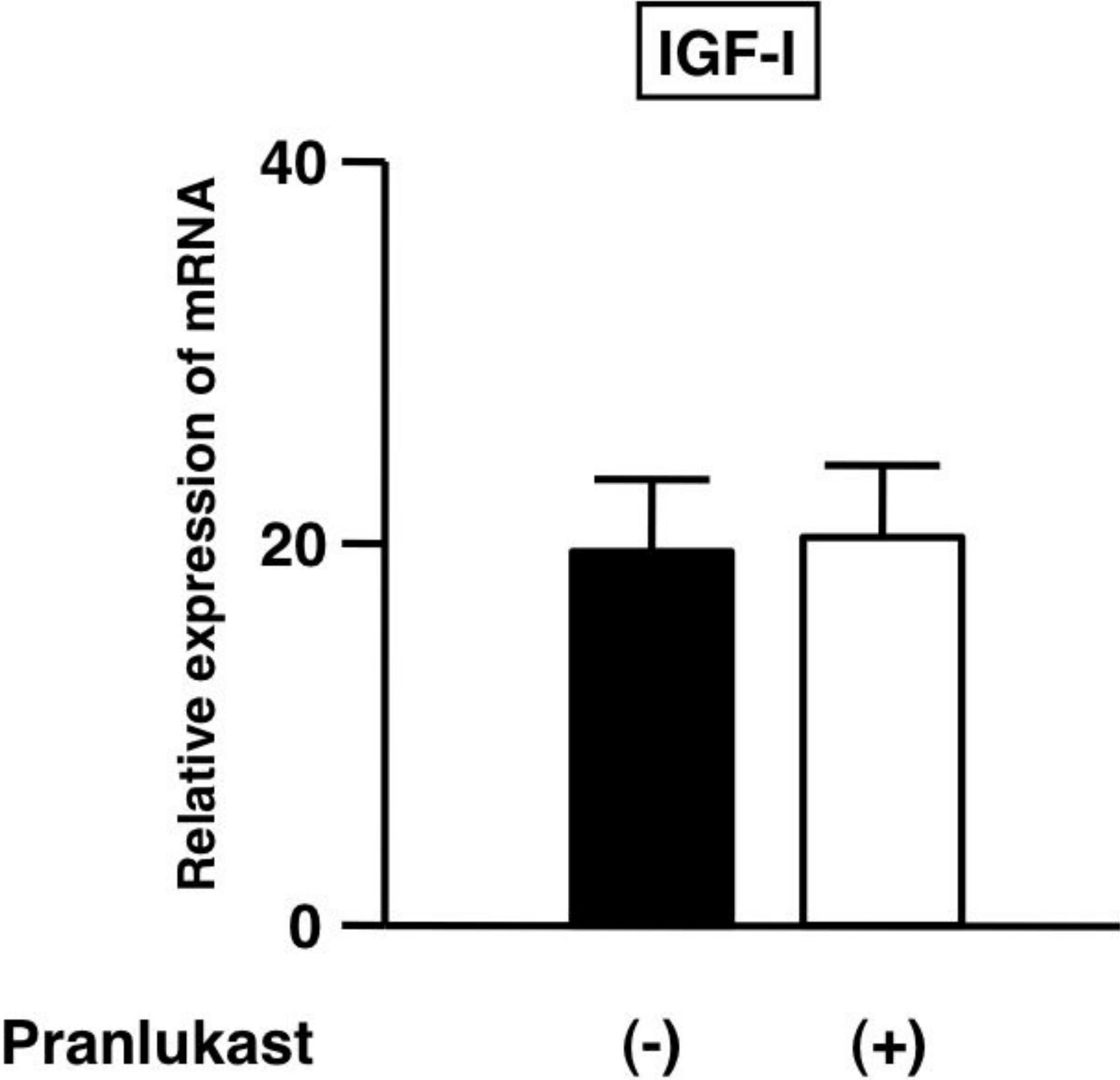


FIGURE 4

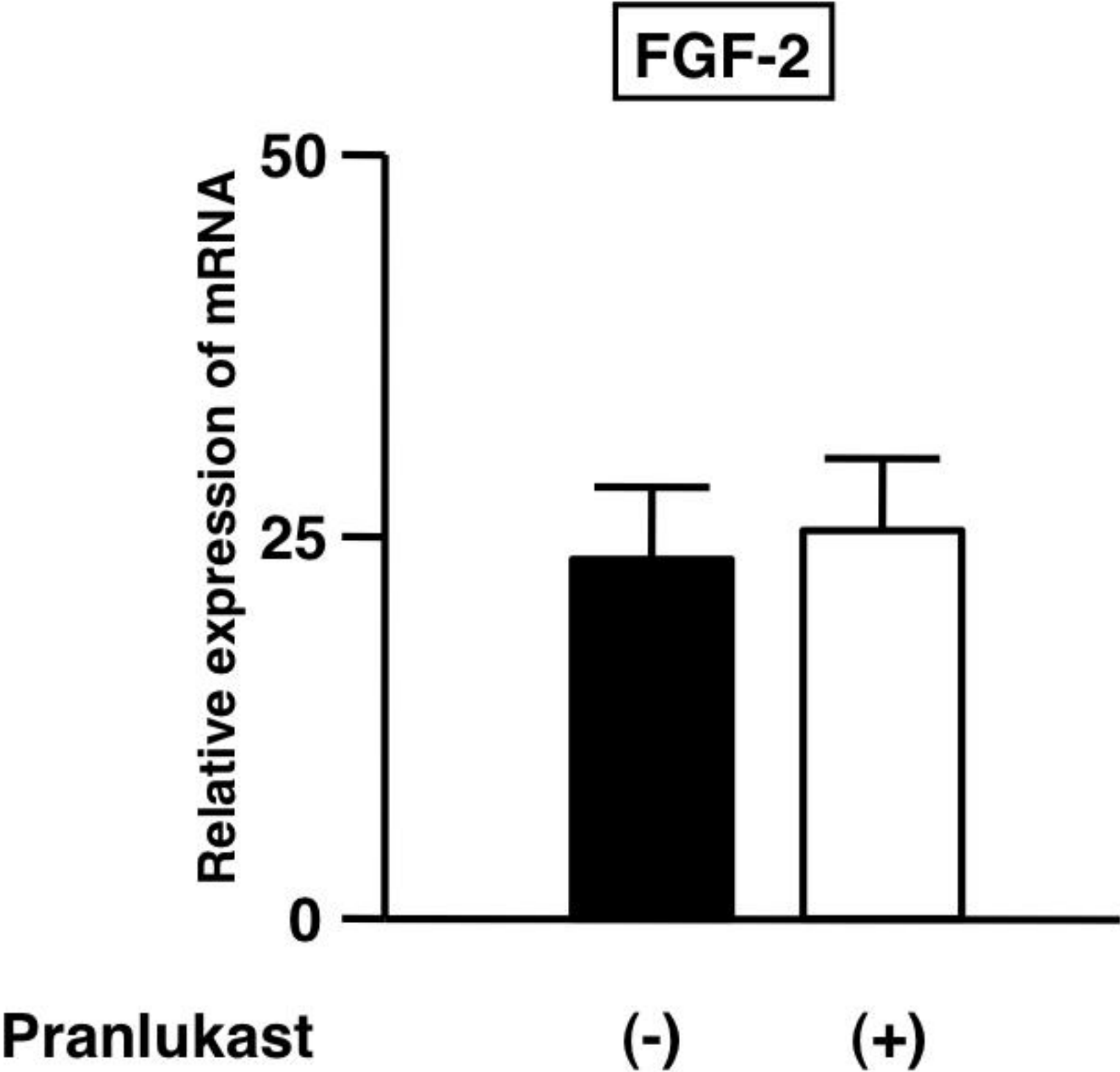


FIGURE 4

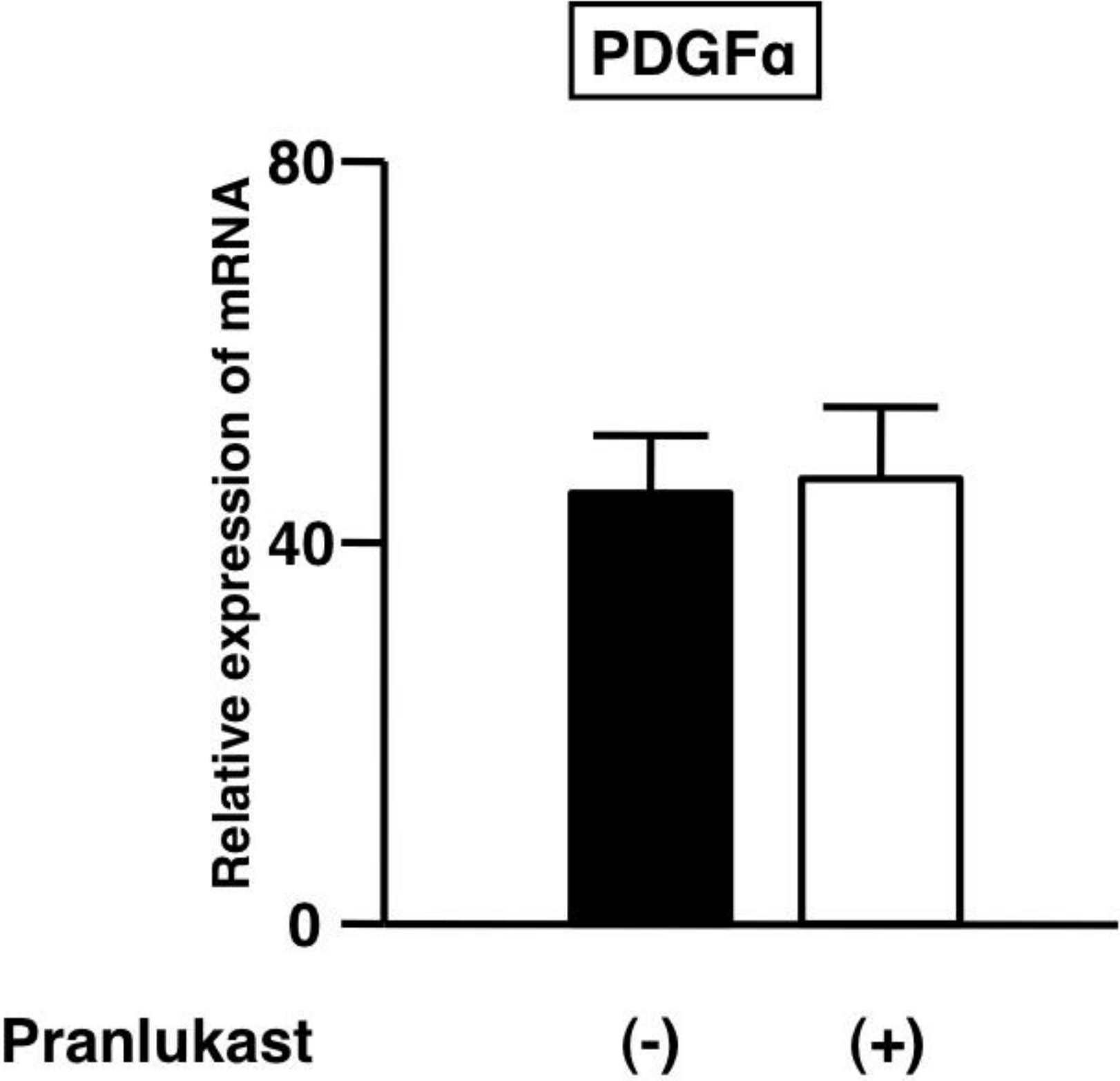
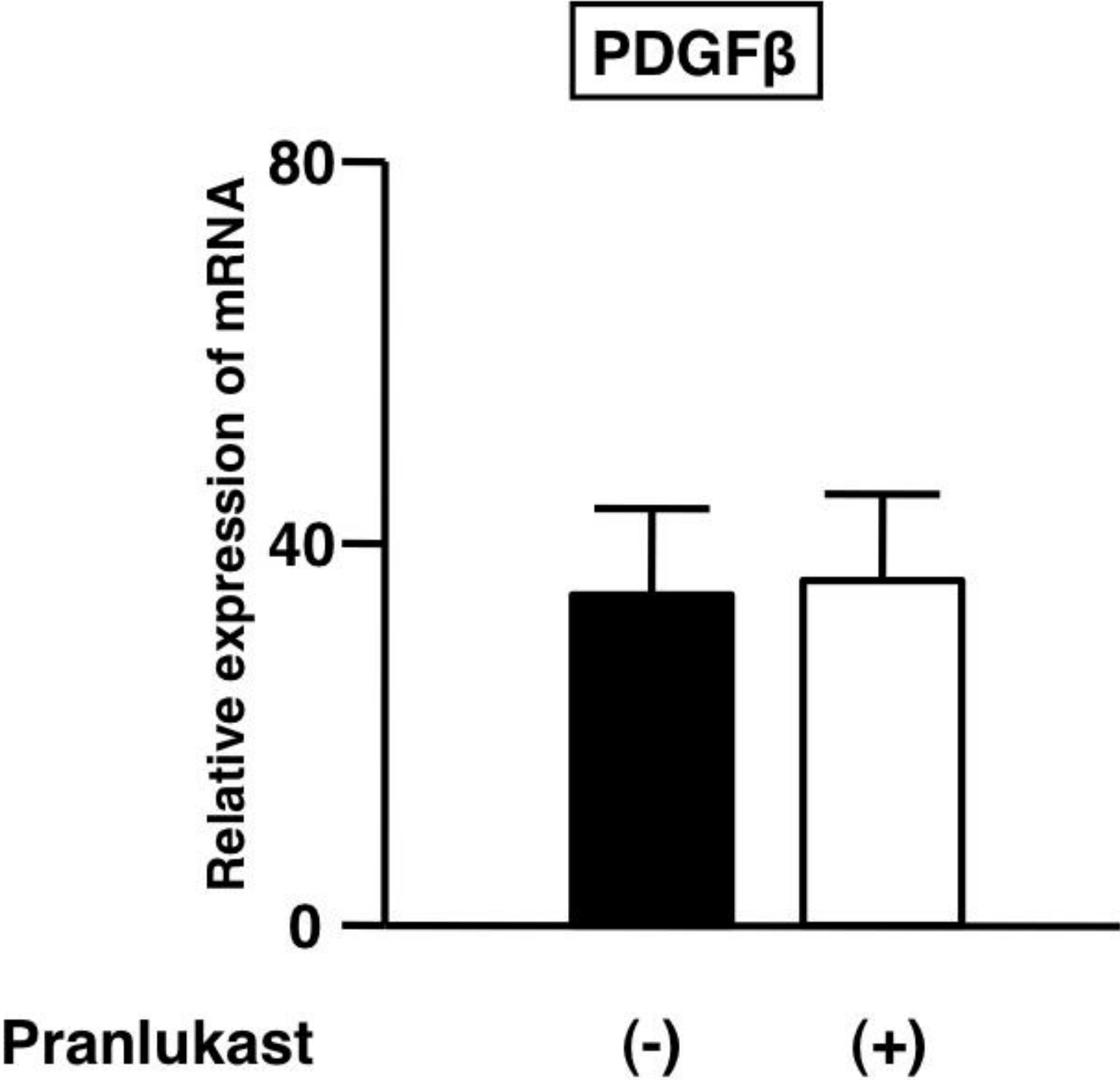


FIGURE 4



Supplementary Information

Leukotriene (LT)₄ aggravates bleomycin-induced pulmonary fibrosis in mice

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METHODS

PCR conditions were as follows: 4 µl of cDNA was mixed with 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems) and 1.5 µl of primer mix (15 pmol) and adjusted to a final volume of 25 µl. Thermal cycler (Applied Biosystems) conditions were according to manufacturer instructions. Amplification of standard cDNA and sample cDNA was carried out in 96-well reaction plates. Each plate contained the same standard cDNA. Cycling conditions included a hot start at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15s, and 60 °C for 1 min. Product accumulation was monitored by SYBR green fluorescence. Data from two runs were averaged, and relative expression levels were determined from a standard curve of serial dilutions of cDNA samples. Quantification of TGF-β1, CTGF, IGF-I, FGF-2, PDGFα, and PDGFβ mRNA levels were expressed as an absolute number of copies after normalization to β-actin mRNA levels. Primers for real-time quantitative RT-PCR were as follows: mouse β-actin, 5'-CCAGCCTTCCTTCTTGGGTAT (forward); and 5'-TGGCATAGAGGTCTTTACGGATGT (reverse). Mouse TGF-β1, CTGF, IGF-I, FGF-2, PDGFα, and PDGFβ primers were obtained from Takara Bio (Madison, WI).