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In vivo IL-10 gene delivery attenuates bleomycin-induced pulmonary fibrosis by inhibiting the production and activation of TGF-β in the lung

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Abbreviations: IPF, idiopathic pulmonary fibrosis; TGF, transforming growth factor; IL, interleukin; BALF, bronchoalveolar lavage fluid; SA, physiologic saline; hpf, high power fields; LPS, lipopolysaccharide.

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ABSTRACT

Backgroud: Idiopathic pulmonary fibrosis is a devastating disorder for which no effective therapy is established. Among various factors to provoke fibrosis, transforming growth factor (TGF)-β plays a critical role. Interleukin (IL)-10 is a potent immunosuppressive cytokine. However, its effect on the fibrosing process remains unclear.

Methods: We examined whether IL-10 could affect the production and activation of TGF-β, thus attenuate the fibrosis. Mice received an intratracheal injection of bleomycin. On day 1 or day 14, IL-10 gene was delivered in mice with hydrodynamic-based system by rapid intravenous injection of Ringer's solution containing plasmid. Two weeks after the plasmid injection, the mice were examined for fibrosis. The effect of IL-10 on TGF-β production by alveolar macrophages was also assessed.

Results: IL-10 gene treatment, even delivered during the fibrosing phase, significantly suppressed pathologic findings, hydroxyproline content, and TGF-β_1 production of both active and total forms in the lung. Immunohistochemical analyses revealed that alveolar macrophages were one of the major sources of TGF-β_1, and IL-10 diminished the intensity of the staining. IL-10 also suppressed the expression of $\alpha_V\beta_6$ integrin, a molecule that plays an important role in TGF-β activation, on lung epithelial cells. Further, alveolar macrophages from bleomycin-injected mice produced TGF-β_1 spontaneously *ex vivo*, which was significantly suppressed by treatment of the mice *in vivo* or by treatment of the explanted macrophages *ex vivo* with IL-10.

Conclusion: These results demonstrate that IL-10 suppresses the production and activation of TGF-β in the lung, thus attenuates pulmonary fibrosis, even delivered in the chronic phase.

Idiopathic pulmonary fibrosis (IPF) is a devastating disorder for which no effective therapy has been established.[1] Recently, the role of inflammation in fibrosis has been questioned, because inflammation is neither a major histopathologic feature nor a good marker of activity or prognosis for IPF.[1][2] In addition, anti-inflammatory therapies have not proven to be effective.[3] Recent advances in understanding the pathophysiology of IPF suggest that persistence and/or recurrence of injury may alter the microenvironment of the lung, especially the alveolar space, resulting in a dysregulated repair process, which eventually leads to tissue fibrosis.[1][4][5][6] The extent of fibroblastic foci, the most distinctive feature of ongoing pulmonary fibrosis, is predictive of survival in IPF.[2] Therefore, a therapeutic strategy directed at suppressing the fibrosis itself would be appropriate. However, so far, the efficacy of anti-fibrotic agents such as IFN-γ1b or pirfenidone against IPF remains uncertain.[6][7][8]

Bleomycin-induced pulmonary fibrosis has been widely used as an experimental model.[9][10][11][12] However, it does not resemble human IPF in many points.[13][14] For example, bleomycin-induced pulmonary fibrosis is characterized by a uniform and bronchiolocentric distribution, whereas IPF is characterized by temporal heterogeneity and predominance of subpleural lesion. It has a potential for recovery, unlike the human condition. Despite these limitations, bleomycin model has contributed much to elucidate molecular mechanisms of pulmonary fibrosis.[9][10][11][12]

Many factors play an important role in the development of fibrosis. They include profibrotic cytokines, chemokines, eicosanoids, fibrinolytic/fibrinogenic factors, matrix metalloproteinases and their inhibitors, and oxidative stress.[4][5][6] Among them, transforming growth factor (TGF)-β is one of the central mediators. TGF-β is known as an immunosuppressive cytokine. It suppresses proliferation of $CD4^+$ T cells by inhibiting the interleukin (IL)-2 production and maturation of dendritic cells by down-regulating the MHC class II expression.[15] On the other hand, TGF-β is a potent profibrotic cytokine. It enhances fibroblast chemotaxis and proliferation, and induces fibroblast to synthesize collagen.[16] So, TGF-β plays a decisive role in many fibrotic disorders. In IPF patients, TGF-β is mainly produced by alveolar macrophages, and is expressed in the fibroblastic foci.[17] Recent microarray analyses demonstrated that TGF-β_1-related genes are up-regulated in the bleomycin-treated mouse lung.[18] Giri and co-workers blocked the function of TGF-β using a neutralizing Ab and reported that bleomycin-induced fibrosis was suppressed.[9] A chimeric TGF-β_1 soluble receptor reduced the intensity of bleomycin-induced fibrosis in hamsters.[10] Gene transfer of Smad7, an intracellular antagonist of TGF-β signaling, also suppresses pulmonary fibrosis.[11] These studies strongly indicate that TGF-β could be directly involved in the fibrosing process.

Recently, a mechanism of TGF-β activation in the lung has been highlighted. TGF-β is

synthesized as a precursor protein and is released in an inactive (latent) form.[16] The activation occurs extracellularly. Latent TGF-β can be activated by binding to $\alpha_V\beta_6$ integrin[19] or to thrombospondin-1,[20] or by proteases such as plasmin.[21] Among them, $\alpha_V\beta_6$ integrin plays an impotant role in animal models of lung fibrotic disease such as bleomycin-induced pulmonary fibrosis[19] and airway remodeling in allergic inflammation.[22]

IL-10 is an immunosuppressive cytokine.[23] It inhibits the production of many proinflammatory cytokines such as IL-1α, IL-6, IL-8, TNF-α, and GM-CSF from various cells.[23] These *in vitro* studies raise the possibility that IL-10 could be beneficial for the treatment of various immune diseases *in vivo*.[24][25] However, the relationship between IL-10 and pulmonary fibrosis is not fully clarified. In patients with IPF, production of IL-10 protein by alveolar macrophages did not increase, although expression of IL-10 mRNA in alveolar macrophages increased.[26] Concentrations of IL-10 protein in bronchoalveolar lavage fluid (BALF) of patients with IPF were lower than those of normal controls.[27] These findings suggest that IL-10 might be lacking in patients with IPF. In the bleomycin-induced mouse model, the role of IL-10 in inflammation and fibrosis remains controversial,[28][29][30] and especially, the effect of this cytokine on the production of TGF-β in the lung has not been studied. Kradin and co-workers suggested that IL-10 inhibits inflammation but does not affect fibrosis in the pulmonary response to bleomycin.[28] In their report, although IL-10-deficient mice exhibited exaggerated bleomycin-induced inflammation, the intensity of fibrosis was not different from that of wild-type mice.[28] However, they examined only the role of endogenous IL-10 on inflammation and fibrosis and did not examine the effect of exogenous administration of IL-10.

In the present report, with hydrodynamic-based gene delivery system, we elucidated the effect of IL-10 on experimental pulmonary fibrosis. We also studied whether IL-10 played some role in the production of TGF-β in the lung. We found that IL-10 treatment, even delivered in the chronic phase, significantly inhibited the progression of fibrosis by directly suppressing the production and activation of TGF-β in the lung.

METHODS

Mice

Male C57BL/6 mice and C57BL/6-IL-10$^{-/-}$ mice were obtained from Charles River Japan, Inc. (Kanagawa, Japan) and the Jackson Laboratory (Bar Harbor, ME), respectively. All animal experiments were approved by the Animal Research Ethics Board of the Department of Allergy and Rheumatology, University of Tokyo.

Delivery of IL-10 gene into mice

The plasmid pCAGGS-IL-10[31] was amplified in *Escherichia coli*, and purified with a Qiagen Endo Free plasmid Giga kit (Qiagen GmbH, Hilden, Germany). The plasmid pCAGGS was constructed by introducing the *CAG* (cytomegalovirus immediate-early enhancer-chicken β-actin hybrid) promoter, rabbit β-globin gene sequences including a polyadenylation signal, and an SV40 *ori* into pUC13.[32] An empty pCAGGS plasmid was used as a control. Hydrodynamic-based gene delivery by intravenous injection of the plasmid DNA was performed as described previously.[31][33] Briefly, plasmid DNA in lactated Ringer's solution (0.1ml/g body weight) was injected into the tail vein. The DNA injection was completed within 5 s. With this delivery system, the plasmid was trapped in the liver and produced cytokine there, and then the produced cytokine moved into the bloodstream and perfused the organs.[31][33] Mice received an intravenous injection of plasmid DNA (100 μg; pCAGGS-IL-10 or control pCAGGS) on day 0. For studying the kinetics of IL-10 gene delivery, mice were sacrificed on days 1, 4, 7, 10, 13, and 20. Concentrations of IL-10 in serum and BALF were measured using an ELISA kit (Pharmingen, SanDiego, CA) according to the manufacturer's instructions.

Bleomycin administration and plasmid injection

Seven-week-old animals received an intratracheal injection of 5 mg/kg bleomycin hydrochloride (kindly provided by Nippon Kayaku, Tokyo, Japan) dissolved in 50 μl of physiologic saline (SA) on day 0. Control mice received an intratracheal injection of SA on day 0. Some mice received plasmid DNA (100 μg; pCAGGS-IL-10 or control pCAGGS) or lactate alone on day 1 intravenously. To examine a dose-dependent effect of IL-10-producing-plasmid on the fibrosis, some mice received pCAGGS-IL-10 (3 μg, 10 μg, 30 μg, or 100 μg, respectively) on day 1. On day 7, mice were sacrificed to assess inflammation. On day 14, mice were sacrificed to assess fibrosis of the lung. In some experiments, mice received plasmid DNA or lactate alone on days 14, 19 and 24. On day 28, mice were sacrificed.

BALF analyses

BALF analyses were performed as described previously.[33][34] Briefly, the lungs were lavaged four times with SA (0.5 ml each). The cell suspension was centrifuged at 300 X *g* for 10 min at 4° C. The supernatants were collected and stored at -70° C for measurement of the concentration of TGF-β_1. The cells were re-suspended in 1 ml of SA with 1% BSA (Wako, Osaka, Japan), and the total cell number was counted with a haemocytometer. Cytospin samples were prepared by centrifuging the suspensions at 300 rpm for 10 min. On the basis of the findings made with Diff-Quick staining (Kokusai-Shiyaku, Kobe, Japan), cell differentials were counted with at least 300 leukocytes in each sample. TGF-β_1 concentrations in BALF were measured directly (before acdification) for the detection of active form of TGF-β_1 and after complete activation by acidification for the detection of total TGF-β_1 using an ELISA kit (R & D systems, Minneapolis, MN). In this ELISA kit, TGF-β_1 Ab is not mouse specific and is reactive to human and rat TGF-β_1. The values were determined by comparison with a standard curve prepared from known concentrations of TGF-β_1. We could detect more than 7.8 pg/ml of TGF-β_1 protein using this kit (data not shown). We measured TGF-β_1 concentrations two or three times using the ELISA kit and confirmed their reliability.

Histologic examinations

Histologic examinations were performed as described previously.[33][34] After perfusion with SA, lung tissue was fixed by instilling 10% neutralized buffered formalin (Wako) through the trachea, and embedded in paraffin. Five-micrometer thick sections were stained with H&E or with Azan to evaluate collagen deposition. The extent of inflammation or fibrosis was evaluated using previously reported scoring system with a slight modification.[35][36] In sections, viewed at 400x, the lesions were defined as follows: inflammation score, absence of inflammation = 0, mild inflammatory changes (no obvious damage to the lung architecture) = 1, moderate inflammatory injury (thickening of the alveolar septae) = 2, severe inflammatory injury (pneumonitis that distorted the normal architecture) = 3. We also defined fibrosis score as follows: absence of lesion = 0, occasional small localized subpleural foci = 1, thickening of interalveolar septa and subpleural foci = 2, continuous thickening of subpleural fibrous gangue and interalveolar septa = 3. Each section was evaluated by two researchers independently in a blind fashion. The mean value was adopted.

Hydroxyproline assay

The hydroxyproline assay was performed as described previously.[12] Briefly, the left lung was cut out and hydrolyzed in 2 ml of 6 N HCl (Wako) at 110° C for 12 h. The pH of the samples was then adjusted to between 6 and 7. A 2-ml aliquot from the total sample volume of

20 ml was added to 1 ml of 1.4% chloramine T (Wako) and incubated at room temperature for 20 min, then 1 ml of 3.15 M perchloric acid (Wako) was added and incubated for 5 min, followed by the addition of 1 ml of Erlich's solution (1 M *p*-dimethylaminobenzaldehyde (Wako) in ethylene glycol monomethyl ether (Wako)). After incubation at 60° C for 20 min, absorbance was analyzed at 570 nm with a Microplate Reader (Bio-Rad Laboratories, Hercules, CA). The amount of hydroxyproline was determined by comparison with a standard curve prepared from known concentrations of reagent hydroxyproline (Sigma, St. Louis, MO).

Immunohistochemistry for TGF-β_1 and $\alpha_V\beta_6$ integrin

Immunohistochemistry was performed using Vectastain ABC kits (Vector Laboratories, Burlingame, CA) as described previously.[12] Briefly, the tissue was deparaffinized and rehydrated with decreasing concentrations of ethyl alcohol. The slides were boiled in 0.05 M citric acid for 7 min. After cooling down to room temperature, the slides were treated with blocking solution containing 5% normal goat serum, 2% casein, and 3% BSA for 45 min. Anti TGF-β_1 Ab (3 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) or anti $\alpha_V\beta_6$ integrin Ab (5 μg/ml; Chemicon International, Temecula, CA) [22] was applied to the tissue and incubated at 37º C for 30 min. This TGF-β_1 Ab is not mouse specific and is reactive to human and rat TGF-β_1. After a wash with PBS, biotinylated goat anti-rabbit IgG Ab (for anti TGF-β_1 Ab) or biotinylated goat anti-mouse IgG Ab (for anti $\alpha_V\beta_6$ integrin Ab) was applied and the slides were incubated at 37 ºC for 30 min. After further washing, avidin-biotin alkaline phosphatase complex was applied and the slides were incubated at 37 ºC for 30 min before the addition of substrate solution. Color development was stopped by rinsing the slides in distilled water. The slides were counterstained with neutral red. Positively immunostained cells were enumerated directly in 10 random high power fields (hpf; 400x).

Cytokine production by alveolar macrophages of C57BL/6 mice

Alveolar macrophages were isolated using previously reported methods with a slight modification.[21][37][38][39] On day 7, alveolar macrophages were obtained by lavageing the lung with sterile PBS containing 1 mM EDTA (Wako). They were centrifuged and re-suspended in DMEM (GIBCO BRL, Grand Island, NY) supplemented with 10% FCS, 10 mM HEPES (GIBCO BRL), 0.1 mM nonessential amino acid (GIBCO BRL), 1 mM sodium pyruvate, 2 mM sodium glutamate (Sigma), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), and 5.0 x 10^{-5} M 2-ME (Sigma). The cells were plated at density of 2 x 10^6 cells/ml in tissue culture flasks. After 2 h at 37º C, nonadherent cells were removed by rinsing plates twice with medium. After trypsin/EDTA (GIBCO BRL) treatment, the cells were centrifuged. Then, they were cultured in serum-free medium for 24 h. In another

experiment, cells from bleomycin-injected mice were cultured in serum-free medium with IL-10 (12.5, 25, 50, 100, or 200 ng/ml, respectively; Genzyme Techne, Minneapolis, MN) or dexamethasone (0.1 or 1 M; Sigma) for 24 h. Cell numbers were counted with a haemocytometer. Over 90% of the adherent cells were determined as macrophages based on morphologic criteria with Diff-Quick staining. We also confirmed the purity of macrophages by flow cytometry with anti-CD11b mAb (M1/70; Pharmingen) (data not shown). TGF-β_1 concentrations in the supernatant were measured before and after activation by acidification using an ELISA kit and data were expressed as picograms of TGF-β_1 per 10^5 macrophages.

Cytokine production in the macrophage cell line J774.1

The macrophage-like cell line J774.1 was obtained from Riken Bioresource Center (Ibaraki, Japan). Cells were stimulated with lipopolysaccharide (LPS; 1 μg/ml; Sigma) in serum-free medium in the presence of IL-10 for 24 hours. Then, TGF-β_1 concentrations in the supernatants were measured.

Statistics

The results are presented as mean (SEM). Statistical analysis was performed by one-way ANOVA followed, when differences were significant, by appropriate post hoc tests using Turkey test. For analysis of the score about inflammation and fibrosis, we used non-parametric methods (Kruskal-Wallis test) because they were discrete value. For analysis of the differences between two groups, we used Student's *t* test. A p value of <0.05 was considered statistically significant. For the primary findings, we showed 95% confidence intervals (CI) for the mean differences.

RESULTS

IL-10 expression in serum and in BALF after hydrodynamic-based gene therapy by intravenous injection of plasmid DNA in C57BL/6 mice

First, we examined the kinetics of IL-10 gene delivery after the intravenous injection of plasmid DNA in C57BL/6 mice. Samples were collected at specific points in time after the injection of plasmid (100 μg; pCAGGS-IL-10 or control pCAGGS). The temporal pattern of IL-10 protein expression in serum and in BALF was confirmed (fig 1). The level of IL-10 peaked 1 day after the injection, and gradually decreased thereafter. On day 13, the IL-10 concentration in the BALF in the pCAGGS-IL-10 treated mice was 25.2 (8.6) pg/ml, and that in the control pCAGGS treated mice was 2.9 (1.1) pg/ml, respectively. There was still a significant difference between the two groups ($p<0.05$). On day 20, IL-10 delivery did not differ between mice that received pCAGGS-IL-10 and mice that received control pCAGGS (data not shown).

Effect of IL-10 gene delivery on bleomycin-induced acute lung inflammation

Then, we examined the effect of IL-10 gene delivery on bleomycin-induced lung inflammation. Mice received an intratracheal injection of bleomycin or SA on day 0. An injection of plasmid (100 μg; pCAGGS-IL-10 or control pCAGGS) or lactate alone was performed after the bleomycin administration (on day 1). On day 7, mice were evaluated. Bleomycin injection significantly increased cell numbers in BALF (total cells, macrophages, neutrophils, and lymphocytes) as compared with those of SA-injected mice. IL-10 delivery slightly reduced cell numbers in BALF as compared with those of lactate-treated mice (LAC) or control plasmid-treated mice (CONT). However, they were not significantly different (fig 2A). Histologic examination showed that IL-10 gene delivery did not suppress the infiltration of inflammatory cells into the alveoli and interstitium in the lung (fig 2B and 2C).

IL-10 gene delivery suppresses bleomycin-induced pulmonary fibrosis

Next, we elucidated the effect of IL-10 gene delivery on bleomycin-induced pulmonary fibrosis. Mice received an intratracheal injection of bleomycin on day 0 and an intravenous injection of plasmid on day 1. Then mice were sacrificed on day 14. In mice that received bleomycin, focal fibrotic lesions with thickened intraalveolar septa were induced by the bleomycin administration (fig 3). First, we decided the optimal dose of IL-10 plasmid. *In vivo* IL-10 gene delivery suppressed the hydroxyproline level in a plasmid-dose dependent manner and 100 μg of pCAGGS-IL-10 per mouse was the optimal dose (fig 3A). Based on this datum, we used 100 μg of IL-10 plasmid for the following experiment. IL-10 treatment strongly suppressed fibrotic lesions (fig 3B). We confirmed by Azan staining that IL-10 treatment suppressed collagen deposition in the lung (fig 3C). A quantitative evaluation of the histologic

findings made by scoring fibrotic change showed that IL-10 treatment significantly suppressed the intensity of pulmonary fibrosis (fig 3D). *In vivo* IL-10 delivery suppressed the increase in hydroxyproline level in the lung as compared with that of control plasmid-treated mice (fig 3E; SA; 769 (32) μg/g tissue, BLEO; 1,184 (102) μg/g tissue, LAC; 1,357 (120) μg/g tissue, CONT; 1,064 (66) μg/g tissue, IL-10; 725 (76) μg/g tissue; CONT *v* IL-10, $p<0.05$, 95% CI 1.22 to 676.69). Moreover, IL-10 gene delivery suppressed concentrations of both total form of TGF-β_1 (fig 3F; SA; 14.9 (3.2) pg/ml, BLEO; 86.6 (7.9) pg/ml, LAC; 97.7 (6.6) pg/ml, CONT; 82.1 (5.7) pg/ml, IL-10; 58.7 (4.6) pg/ml; CONT *v* IL-10, $p<0.05$, 95% CI 0.15 to 46.64) and its active form (fig 3G; SA; 1.4 (1.0) pg/ml, BLEO; 18.7 (1.4) pg/ml, LAC; 18.6 (1.7) pg/ml, CONT; 19.0 (1.7) pg/ml, IL-10; 11.1 (2.3) pg/ml; CONT *v* IL-10, $p<0.05$, 95% CI 1.22 to 14.57) in the BALF, which is the most prominent isoform of TGF-βs detected during bleomycin-induced injury.[37] These results suggested that *in vivo* IL-10 gene delivery suppressed the development of pulmonary fibrosis induced by bleomycin. In a preliminary study, we delivered the IL-10 gene 3 days before injecting bleomycin and examined the effect on inflammation and fibrosis. The bleomycin-induced inflammation and fibrosis were both significantly suppressed (data not shown), which was consistent with a previous report.[30]

IL-10 gene delivery suppresses the expression of TGF-β_1 and $\alpha_V\beta_6$ integrin in the lung

Next, we examined the expression of TGF-β_1 in the lung using immunohistochemistry. In bleomycin-injected mice, TGF-β_1 was detected mainly in alveolar macrophages, epithelial cells, and the interstitium (fig 4A). This finding was consistent with a previous study on human IPF.[17] In bleomycin-injected, IL-10-treated mice, TGF-β_1 expression in the lung, especially in macrophages, was decreased (SA; 1.7 (0.3) /hpf, BLEO; 16.6 (1.4) /hpf, LAC; 16.7 (1.5) /hpf, CONT; 13.6 (0.8) /hpf, IL-10; 8.7 (0.8) /hpf). Next, we examined the expression of $\alpha_V\beta_6$ integrin on epithelial cells. In lactate-treated mice or control pCAGGS-treated mice, the expression of $\alpha_V\beta_6$ integrin was up-regulated in lung epithelial cells by bleomycin injection as compared with that of saline-injected mice. IL-10 gene delivery suppressed this expression (fig 4B; SA; 0.5 (0.2) /hpf, BLEO; 3.9 (0.5) /hpf, LAC; 4.6 (0.6) /hpf, CONT; 3.5 (0.5) /hpf, IL-10; 2.2 (0.5) /hpf).

***In vivo* IL-10 gene delivery suppresses TGF-β_1 production by alveolar macrophages**

We then confirmed the effect of IL-10 using explanted alveolar macrophages *ex vivo* (fig 5). Alveolar macrophages explanted from the bleomycin-injected mice spontaneously produced both total and active forms of TGF-β_1, and IL-10 gene delivery suppressed their production (fig 5A; total form; SA; 8.6 (1.3) pg/ml, BLEO; 51.8 (6.5) pg/ml, LAC; 48.6 (6.7) pg/ml, CONT; 54.8 (5.9) pg/ml, IL-10; 33.4 (2.3) pg/ml; CONT *v* IL-10, $p<0.05$, 95% CI 0.75 to 41.89; fig

5B; active form; SA; 2.1 (0.8) pg/ml, BLEO; 15.5 (0.9) pg/ml, LAC; 15.0 (1.9) pg/ml, CONT; 13.6 (1.1) pg/ml, IL-10; 8.9 (0.4) pg/ml; CONT *v* IL-10, $p<0.05$, 95% CI 0.13 to 9.17). Moreover, production of both total and active forms of TGF-β_1 by explanted alveolar macrophages obtained from IL-10-deficient mice with bleomycin injection was up-regulated as compared with wild-type mice (fig 5C; total form; Wild; 48.0 (4.8) pg/ml, IL-$10^{-/-}$; 63.9 (4.8) pg/ml, $p<0.05$, 95% CI 1.29 to 30.44; fig 5D; active form; Wild; 17.3 (1.5) pg/ml, IL-$10^{-/-}$; 25.0 (2.2) pg/ml, $p<0.05$, 95% CI 1.98 to 13.42). These results indicated that IL-10 suppressed the production of TGF-β_1 by alveolar macrophages. In another experiment, we examined the production of endogenous IL-10 by alveolar macrophage. IL-10 production by explanted alveolar macrophages slightly increased by bleomycin treatment as compared with that of SA-injected mice (data not shown). IL-10 gene delivery induced a slight additional increase in the endogenous IL-10 production, although it was not significantly different from that of control plasmid-treated mice (data not shown).

IL-10 suppresses TGF-β_1 production by activated alveolar macrophages

Then we examined the direct effect of IL-10 on TGF-β_1 production in alveolar macrophages activated by the instillation of bleomycin. Alveolar macrophages explanted from the bleomycin-injected mice were cultured in the presence or absence of IL-10, and TGF-β_1 production was examined. As shown in fig 6A and 6B, alveolar macrophages from the bleomycin-injected mice produced more total and active TGF-β_1 than did the macrophages from the SA-injected mice. Although the production of total amounts of TGF-β_1 seemed to increase at 25 ng/ml of IL-10, it was not significantly different from that without IL-10. IL-10 significantly suppressed their TGF-β_1 production at a high concentration (total form; BLEO; 56.8 (2.2) pg/ml, IL-10 (200 ng/ml); 36.1 (1.9) pg/ml; BLEO *v* IL-10 (200 ng/ml), $p<0.05$, 95% CI 0.85 to 40.53; active form; BLEO; 18.8 (1.7) pg/ml, IL-10 (100 ng/ml); 9.1 (1.2) pg/ml, IL-10 (200 ng/ml); 9.6 (0.6) pg/ml; BLEO *v* IL-10 (100 ng/ml), $p<0.05$, 95% CI 0.92 to 18.55; BLEO *v* IL-10 (200 ng/ml), $p<0.05$, 95% CI 0.40 to 18.03). On the other hand, dexamethasone did not suppress the production, consistent with a previous report.[37] These results suggested that IL-10 could, at least in part, directly suppress the production of TGF-β_1 by activated alveolar macrophages. In another experiment, we stimulated the macrophage cell line J774.1 with bleomycin, however, the production was not affected (data not shown). Instead, we stimulated J774.1 cells with LPS. The production of TGF-β_1 was up-regulated by LPS (fig 6C). With LPS stimulation, IL-10 also suppressed the production at a concentration of as low as 25 ng/ml (LPS; 125.8 (15.5) pg/ml, IL-10 (25 ng/ml); 86.9 (4.4), IL-10 (50 ng/ml); 85.6 (6.2) pg/ml, IL-10 (100 ng/ml); 88.2 (7.3) pg/ml; LPS *v* IL-10 (25 ng/ml), $p<0.05$, 95% CI 3.94 to 73.74; LPS *v* IL-10 (50 ng/ml), $p<0.05$, 95% CI 5.27 to 75.07; LPS *v* IL-10 (100 ng/ml),

$p<0.05$, 95% CI 2.69 to 72.48). We also confirmed that stimulation with LPS up-regulated the expression of TGF-β_1 mRNA of J774.1 cells and IL-10 suppressed their expression (data not shown).

IL-10 gene delivery is effective in the chronic fibrosing process

Finally, we examined whether IL-10 gene delivery could suppress chronic fibrosing process even if delivered when pulmonary fibrosis has developed. As lung fibrosis had developed on day 14 (fig 3), gene delivery of IL-10 was started at this time point, and was repeated on days 19 and 24 during the further development of fibrosis. On day 28, the mice were evaluated. IL-10 gene delivery suppressed pulmonary fibrosis, which was assessed by histologic findings (fig 7A). Result from Azan staining demonstrated that collagen deposition in the lung was attenuated by IL-10 treatment (fig 7B). The effect of IL-10 was also confirmed by histologic scoring (fig 7C) and by hydroxyproline assay (fig 7D; SA; 962 (75) μg/g tissue, BLEO; 1,740 (80) μg/g tissue, LAC; 1,834 (131) μg/g tissue, CONT; 1,791 (128) μg/g tissue, IL-10; 1,365 (81) μg/g tissue; CONT v IL-10, $p<0.05$, 95% CI 19.27 to 832.29). These results showed that IL-10 could suppress fibrosing process itself even delivered in the fibrosing phase.

DISCUSSION

The results of the present study clearly demonstrated that *in vivo* IL-10 gene delivery, even delivered during the chronic phase, suppressed the development of pulmonary fibrosis. They also indicated that alveolar macrophages are an important source of TGF-β_1, and that IL-10 strongly suppressed the production of both total and active forms of TGF-β_1 from alveolar macrophages, thus attenuating the intensity of the fibrosis.

In a previous study, Arai and co-workers reported that IL-10 gene delivery before bleomycin-administration suppresses pulmonary fibrosis, probably by inhibiting the initial inflammatory process which is induced by bleomycin-injection.[30] However, the effect of gene delivery after the insult has not been clarified in their report, which hinders the realization of gene therapy for human IPF patients. In contrast, in the current study, we clearly demonstrated that our gene delivery system was effective even after fibrosis has already developed (fig 7). A reason for the discrepancy in the results would be partly due to a difference in the gene delivery system. Arai and co-workers employed a HVJ-liposome system,[30] while we used a hydrodynamic-based gene delivery system. The latter system could achieve a higher concentration of IL-10 protein in the lung (fig 1) than the former system. It is considered that in our case, certain amount of IL-10 delivered into the lung suppressed the fibrosis even if it was delivered after the insult. In another study, we applied the same system and clarified that IL-10 gene delivery was also effective for regulating allergic airway inflammation.[33] Although the hydrodynamic-based gene delivery system cannot be directly applied to humans, these findings strongly indicate that if IL-10 could be delivered selectively and effectively in the lung, it could be a very effective option for the treatment of human lung diseases in future. Therefore, for future clinical applications, progress in the site-specific delivery of IL-10 must be further achieved.

Among many pro-fibrotic cytokines, TGF-β is a key growth factor that directly promotes the synthesis of collagen by many kinds of fibroblasts including those of the lung, and thus plays a key role for developing fibrosis.[16] So far, therapeutic trials to regulate TGF-β function in experimental pulmonary fibrosis have focused on the alteration of TGF-β signal transduction.[9][10][11] On the other hand, the approach to suppress TGF-β production or activation itself has rarely been successful so far. Khalil and co-workers examined the influence of dexamethasone on the production of TGF-β_1 by alveolar macrophages from bleomycin-treated rats, but they did not find any suppressive effect.[37] We obtained the same result with dexamethasone (fig 6).

So far, the relation between IL-10 and TGF-β has been unclear. In the present study, we for the first time demonstrated that IL-10, a cytokine that possess various immunoregulatory effects, suppressed TGF-β_1 production in the lung (figs 3-6). We confirmed that IL-10 suppressed the

production of TGF-β_1 by stimulated alveolar macrophages both *in vitro* and *in vivo* (figs 5 and 6). We also found that IL-10 suppressed the expression of TGF-β_1 mRNA of LPS-stimulated macrophage-like cell line J774.1 (data not shown), which suggested that IL-10 could suppress the mRNA expression of TGF-β_1, and thus suppress the production of TGF-β_1 protein. Further, the decrease in TGF-β_1 production by alveolar macrophages correlated with the suppression of fibrosis (figs 3 and 4). These results strongly suggest that inhibition of TGF-β_1 production from alveolar macrophages by IL-10 could lead to the suppression of fibrosis. However, the effect of IL-10 on other cells than macrophages such as epithelial cells or fibroblasts should be considered, because suppression of TGF-β_1 production from alveolar macrophages by IL-10 was not so strong (figs 5 and 6). For example, Arai and co-workers reported that IL-10 reduced constitutive and TGF-β-induced type I collagen mRNA expression in fibroblasts *in vitro*.[30] This effect on fibroblasts might play some role in the suppression of fibrosis by IL-10

The mechanisms underlying the production, activation, and signal transduction of TGF-β, especially in the lung, are complicated.[5][16] Although our results indicated that IL-10 apparently suppressed TGF-β_1 in the lung, the mechanism of the suppression needs to be further elucidated. For example, results from *in vitro* study suggested that IL-10 directly had some effect on TGF-β_1 production from macrophages at a high concentration (fig 6). Judging from the time course study (fig 1), our gene delivery could produce such a high concentration in serum. However, it could not produce a high concentration of IL-10 in the lung, although the values in the BALF may underestimate the true concentration of IL-10 in alveolar lining fluid. Therefore, indirect mechanisms of IL-10 to down-regulate TGF-β_1 production through other cytokines affecting TGF-β_1 in the lung may also play an important role. We also demonstrated that IL-10 treatment attenuated the expression of $\alpha_V\beta_6$ integrin on epithelial cells (fig 4B). This finding suggests that down-regulation of the $\alpha_V\beta_6$ integrin expression might be one of the mechanisms of IL-10 to suppress TGF-β activation. On the other hand, IL-10 might suppress the $\alpha_V\beta_6$ integrin expression on epithelial cells indirectly by suppressing the production of TGF-β itself that up-regulates the expression of $\alpha_V\beta_6$ integrin.[40] Further, thrombospondin-1[20] and plasmin[21] also play an important role in the activation of TGF-β. Therefore, effects of IL-10 on these molecules that were involved in the proteolytic cleavage and activation of TGF-β also should be further investigated.

Our results showed that not all the IL-10 treated mice had reduced fibrosis score. Generally, the response to bleomycin varies considerably between individuals. This would be the reason why not all the mice that received IL-10 expressing plasmid had reduced fibrosis score. Therefore, we used certain number of mice for each *in vivo* experiment (n = 12) to confirm the reliability of the result and found that IL-10 gene delivery significantly suppressed the

development of pulmonary fibrosis.

In summary, IL-10 suppressed the production and activation of TGF-β in the lung, thus attenuated pulmonary fibrosis. Therefore, IL-10 gene delivery could become a novel strategy for treating pulmonary fibrosis. For future clinical applications, progress in the site-specific delivery of IL-10 as well as an understanding the mechanism of activation of TGF-β must be further achieved.

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FOOTNOTES

Competing interests: no competing interests to disclose.

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FIGURE LEGENDS

Figure 1 IL-10 expression in serum and in BALF after hydrodynamic-based gene therapy by intravenous injection of plasmid DNA in C57BL/6 mice. Mice received an intravenous injection of pCAGGS-IL-10 (100 μg; IL-10) or control pCAGGS (100 μg; CONT) on day 0. Concentrations of IL-10 in serum (A) and in BALF (B) were measured at the indicated times after the injection using ELISA. Values are presented as means ± SEM for 6 mice per group. *p<0.05 and ***p<0.001 compared with the value for CONT.

Figure 2 Effect of IL-10 gene delivery on bleomycin-induced acute lung inflammation. Mice received an intratracheal injection of bleomycin (5 mg/kg) on day 0. Control mice received physiologic saline (SA) on day 0. Some mice received an intravenous injection of pCAGGS-IL-10 (100 μg; IL-10), control pCAGGS (100 μg; CONT), or lactate (LAC) on day 1. On day 7, mice were analyzed (n = 12 per group). (A) BALF cell analysis. BAL was performed. Leukocytes were identified based on morphologic criteria (n = 12). (B) Histologic findings (H&E). Lungs were excised and subjected to H&E staining. Scale bar, 100 μm. (C) Quantitative evaluation of the histologic findings by scoring inflammation (n = 12). In sections, the lesions were defined as described in METHODS. Horizontal bars indicate mean scores for each group. ***p<0.001 compared with the value for SA.

Figure 3 IL-10 gene delivery suppresses bleomycin-induced pulmonary fibrosis. Mice received an intratracheal injection of bleomycin (5 mg/kg) on day 0. Control mice received physiologic saline (SA) on day 0. Some mice received an intravenous injection of pCAGGS-IL-10 (100 μg; IL-10), control pCAGGS (100 μg; CONT), or lactate (LAC) on day 1. On day 14, mice were analyzed (n = 12 per group). (A) A dose-dependent effect of IL-10-producing-plasmid on the development of pulmonary fibrosis. Lungs were excised and a hydroxyproline assay was performed (n = 12). (B) Histologic findings (H&E). Lungs were excised and subjected to H&E staining. Scale bar, 100 μm. (C) Azan staining. Scale bar, 100 μm. (D) Quantitative evaluation of the histologic findings by scoring fibrotic change (n = 12). In sections, the lesions were defined as described in METHODS. Horizontal bars indicate mean scores for each group. (E) Hydroxyproline assay. Lungs were excised and a hydroxyproline assay was performed (n = 12). (F) Concentrations of total TGF-β_1 in BALF. Supernatant of BALF was assayed for TGF-β_1 after activation by acidification using an ELISA kit (n = 12). (G) Concentrations of active form of TGF-β_1 in BALF. Supernatant of BALF was assayed for TGF-β_1 before acidification (n = 12). **p<0.01 and ***p<0.001 compared with the value for SA. #p<0.05 and ##p<0.01 compared with the value for CONT.

Figure 4 IL-10 gene delivery suppresses the expression of TGF-β_1 and $\alpha_V\beta_6$ integrin in the lung. (A) Immunohistochemistry for TGF-β_1. Mice were treated as described in Figure 3. On day 14, lungs were excised and staining for TGF-β_1 was performed (blue). Counter-staining was performed with neutral red. Scale bar, 100 μm. Insertion; staining for TGF-β_1 in alveolar macrophages (blue). (B) Immunohistochemistry for $\alpha_V\beta_6$ integrin. On day 14, lungs were excised and staining for $\alpha_V\beta_6$ integrin was performed (blue). Counter-staining was performed with neutral red. Scale bar, 40 μm.

Figure 5 Production of TGF-β_1 by explanted alveolar macrophages. (A, B) IL-10 gene delivery suppresses TGF-β_1 production in explanted alveolar macrophages. Mice received an intratracheal injection of bleomycin on day 0 and an intravenous injection of plasmid on day 1. On day 7, BAL was performed. The cells were plated in tissue culture flasks. After 2 h at 37º C, nonadherent cells were removed. After trypsin/EDTA treatment, the cells were re-suspended and cultured in serum-free medium for 24 h (n = 8 per group). (A) Concentrations of total TGF-β_1 in supernatants were measured after activation by acidification using an ELISA kit and data were expressed as picograms of TGF-β_1 per 10^5 macrophages (n = 8). (B) Concentrations of active form of TGF-β_1 in supernatants were measured before acidification (n = 8). ***$p<0.001$ compared with the value for SA. #$p<0.05$ compared with the value for CONT. (C, D) Deletion of the IL-10 gene increases TGF-β_1 production in explanted alveolar macrophages. IL-10-deficient mice (IL-$10^{-/-}$) or wild-type mice (wild) received bleomycin. On day 7, BAL was performed. Alveolar macrophages were treated as described in (A, B) (n = 8 per group). (C) Concentrations of total TGF-β_1. Data were expressed as picograms of TGF-β_1 per 10^5 macrophages (n = 8). (D) Concentrations of active form of TGF-β_1 (n = 8). *$p<0.05$ compared with the value for wild-type mice.

Figure 6 Effect of IL-10 on production of TGF-β_1 by activated alveolar macrophages. Mice received an intratracheal injection of bleomycin or SA on day 0. On day 7, BAL was performed. The cells were plated at a density of 2 x 10^6 cells/ml in tissue culture flasks. After 2 h at 37º C, nonadherent cells were removed. Then, after trypsin/EDTA treatment, macrophages from bleomycin-injected mice were cultured with IL-10 or dexamethasone (DEX) in serum-free medium for 24 h (n = 8 per group). (A) Concentrations of total TGF-β_1 in supernatants were measured and data were expressed as picograms of TGF-β_1 per 10^5 macrophages (n = 8). (B) Concentrations of active form of TGF-β_1 in supernatants were measured (n = 8). ***$p<0.001$ compared with the value for macrophages from SA-injected mice. #$p<0.05$ compared with the value for macrophages from bleomycin-injected mice without IL-10. (C) Effect of IL-10 on TGF-β_1 protein production induced by LPS in a

macrophage cell line. J774.1 cells were incubated with LPS for 24 h in the presence or absence of IL-10. TGF-β_1 concentrations in the supernatants were measured after activation by acidification using an ELISA kit (n = 8). ***p<0.001 compared with the value for macrophages without LPS stimulation. #p<0.05 compared with the value for macrophages that were stimulated with LPS without IL-10.

Figure 7 IL-10 gene delivery suppresses pulmonary fibrosis even after fibrosis has already developed. Mice received an intratracheal injection bleomycin or SA on day 0. Some mice received intravenous injection of pCAGGS-IL-10 (IL-10), control pCAGGS (CONT), or lactate (LAC) on day 14, on day 19, and on day 24. On day 28, mice were sacrificed (n = 12 per group). (A) Histrogical findings (H&E). On day 28, lungs were excised and subjected to H&E staining. Scale bar, 100 μm. (B) Azan staining. Scale bar, 100 μm. (C) Quantitative evaluation of the histological findings by scoring fibrotic change (n = 12). Horizontal bars indicate means of each group. (D) Hydroxyproline assay (n = 12). On day 28, lungs were excised and hydroxyproline assay was performed. ***p<0.001 compared with the value for SA. #p<0.05 compared with the value for CONT.

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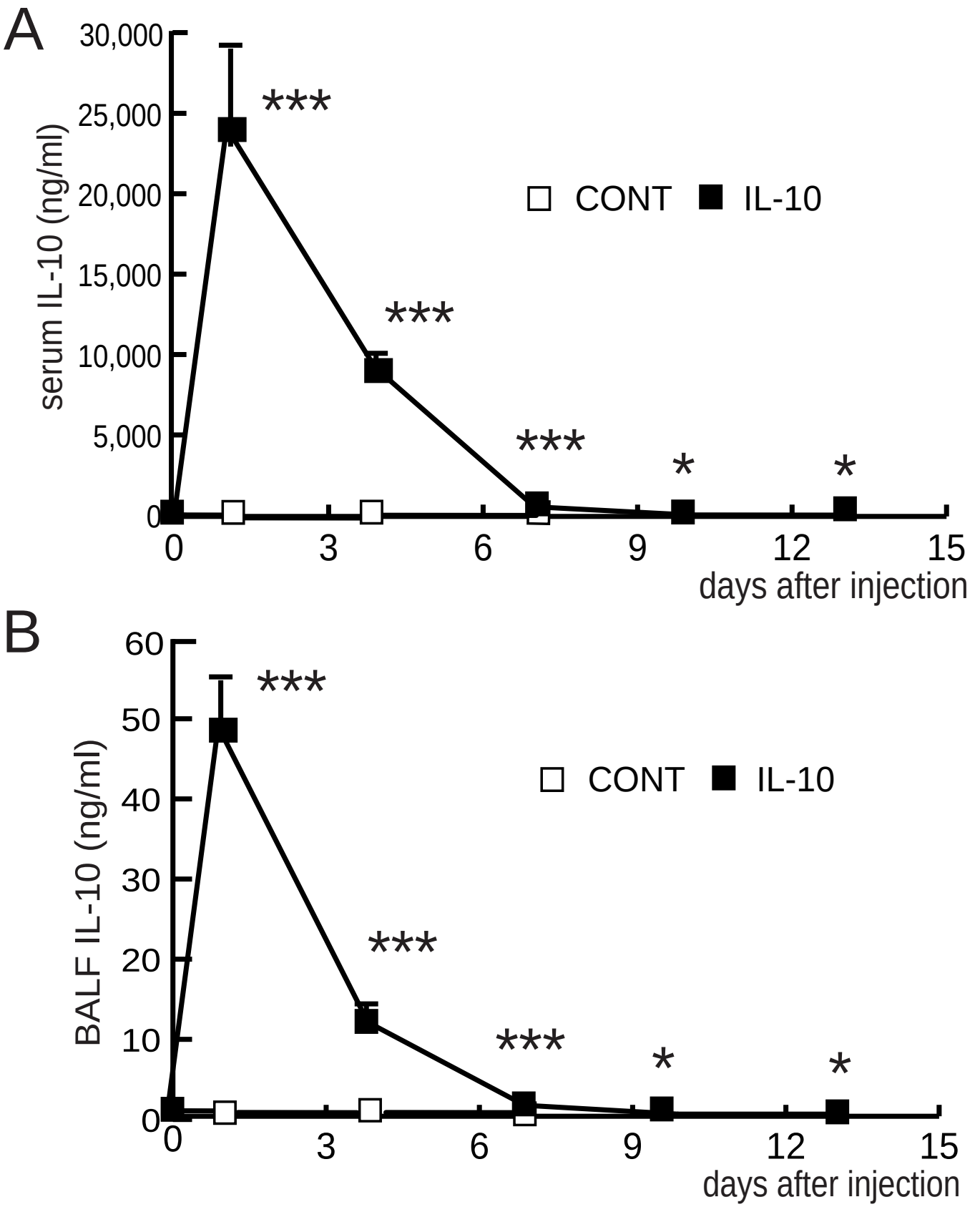
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FIGURE 1

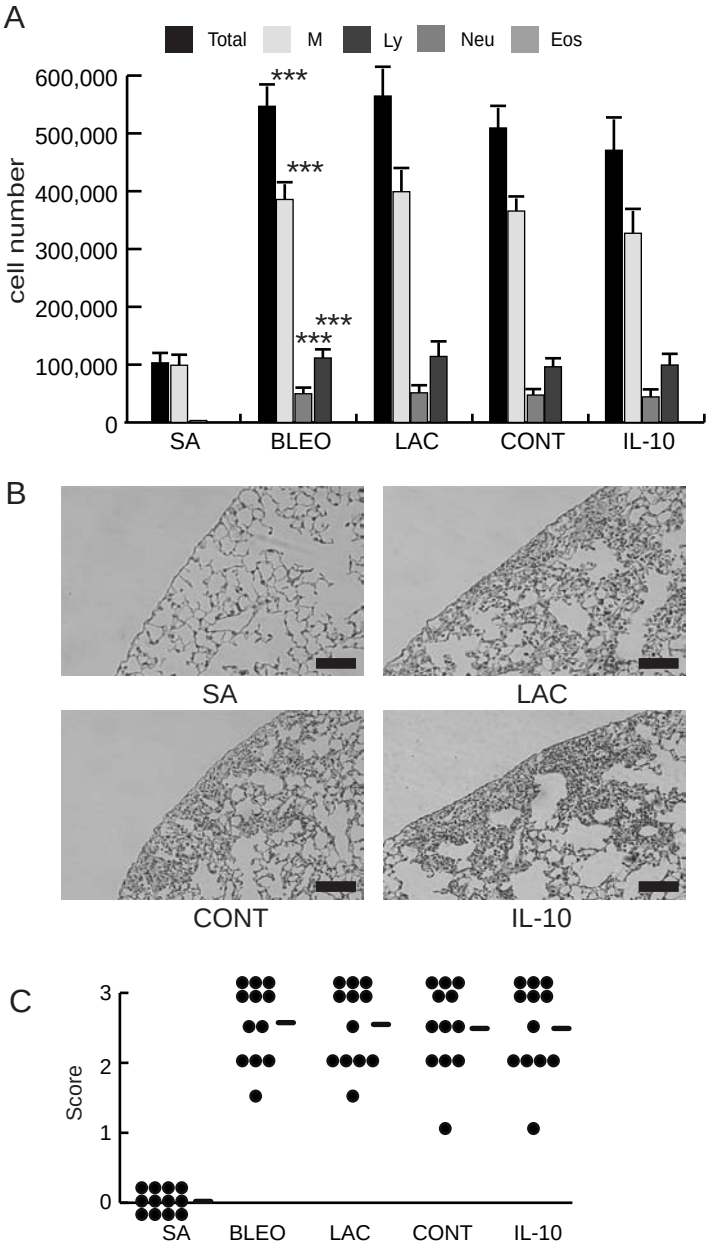
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FIGURE 2

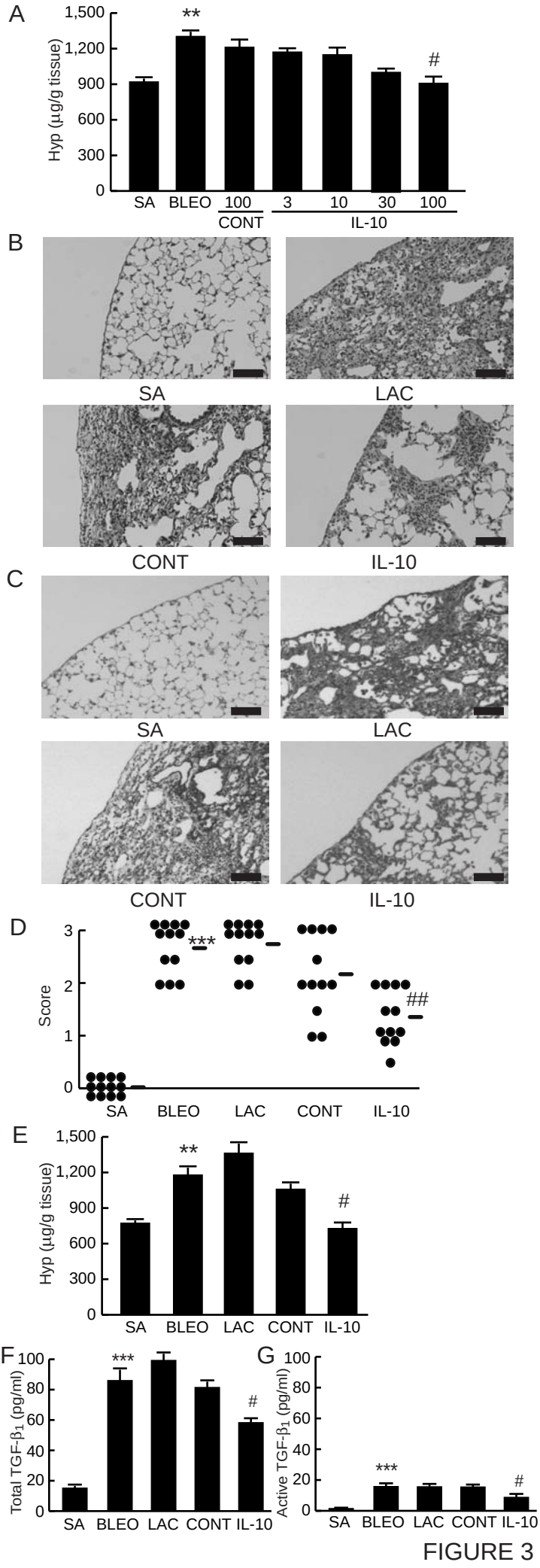
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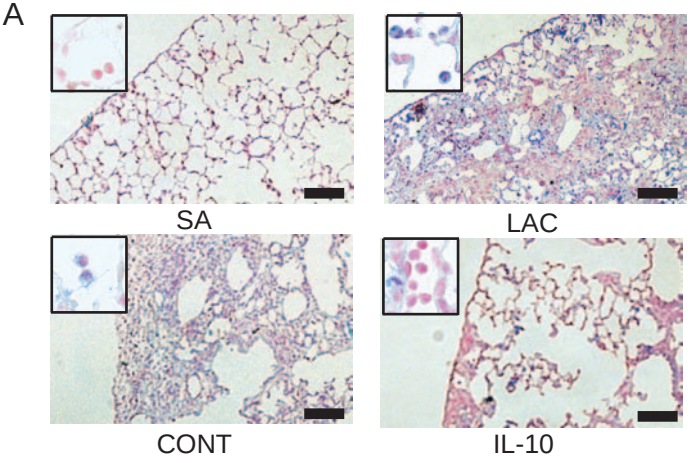
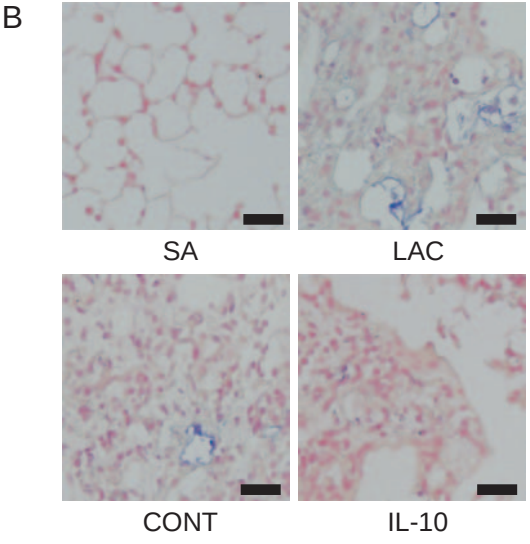
FIGURE 3





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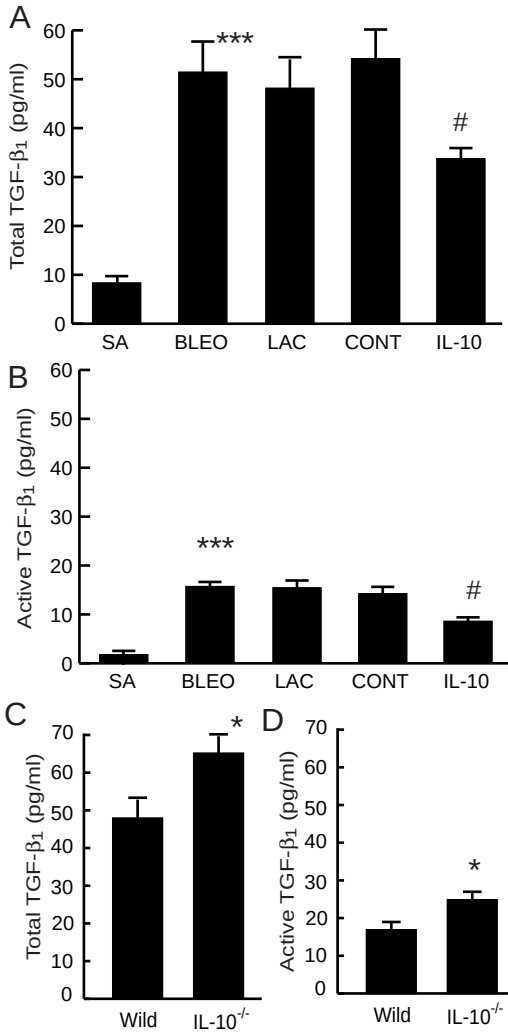
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FIGURE 5

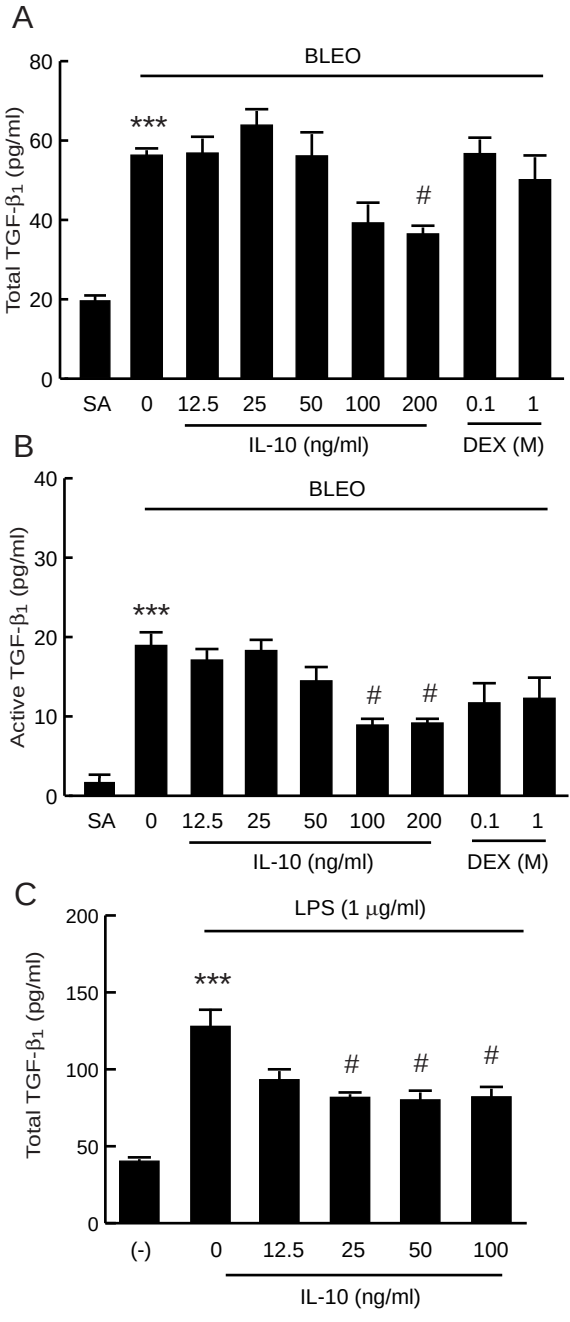
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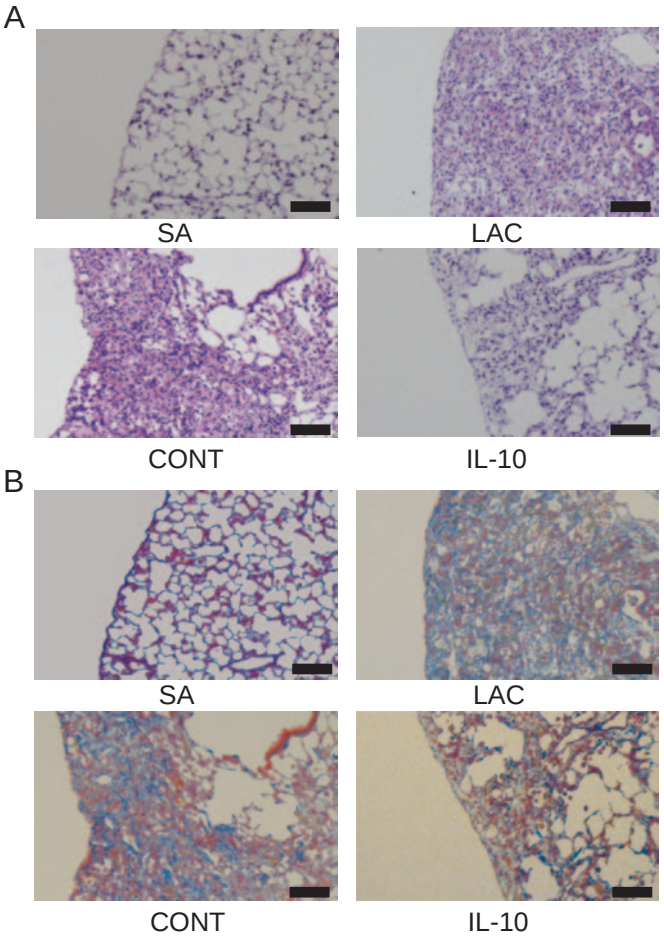
FIGURE 6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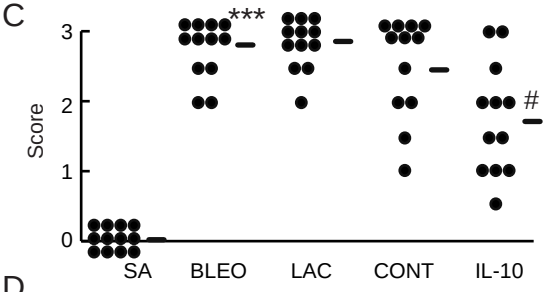
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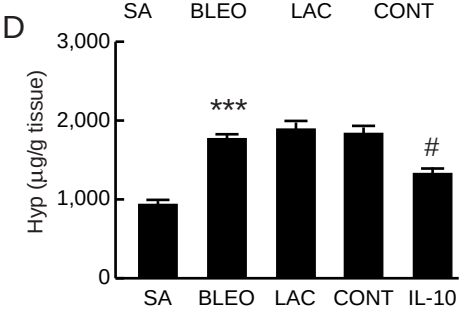
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FIGURE 7