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IL-1 R-related protein ST2 suppressed the initial stage of bleomycin-induced lung injury

Naoko Mato [1], Makiko Fujii [2], Yoji Hakamata [3], Eiji Kobayashi [4], Atsuko Sato [5], Morisada Hayakawa [2], Hiromi Ohto-Ozaki [2], Masashi Bando [1], Shoji Ohno [1], Shin-ichi Tominaga [2], and Yukihiko Sugiyama [1]

[1] Division of Pulmonary Medicine, Department of Medicine, Jichi Medical University

[2] Department of Biochemistry, Jichi Medical University

[3] Department of Basic Science, School of Veterinary Nursing and Technology, Nippon Veterinary and Life Science University

[4] Division of Organ Replacement Research, Center for Molecular Medicine, Jichi Medical University

[5] Department of Dermatology, Jichi Medical University

Affiliations: Division of Pulmonary Medicine, Department of Medicine,

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Jichi Medical University, Tochigi, Japan.

Correspondence and requests for reprints: Yukihiko Sugiyama, Division of Pulmonary Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-Shi, Tochigi 329-0498, JAPAN.

TEL: +81-285-58-7349

FAX: +81-285-44-3586

E-mail: sugiyuki@jichi.ac.jp

Short title: ST2 suppressed acute lung injury

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Abstract

Acute lung injury has a range of causes, and occasionally leads to lethal respiratory failure. Despite the advances in treatment, acute lung injury continues to have a high mortality rate, and thus a new therapeutic approach is needed. ST2 is IL-1 receptor-related protein, and its expression is induced by various inflammatory responses. Recently, ST2 has been speculated to exert anti-inflammatory effects; we therefore investigated the role of the ST2 in the murine model of acute lung injury.

To elucidate the function of ST2 *in vivo*, we prepared mice that transiently overexpressed ST2 protein using the hydrodynamic gene transfer method, and then induced lung injury by intratracheal administration of bleomycin.

In bleomycin-treated ST2-overexpressing mice, increase of neutrophils in the bronchoalveolar lavage fluid was markedly suppressed. Additionally, the levels of TNF-α, and IL-6, and as well as the concentration of albumin in BALF were reduced compared with those of controls. Furthermore, the pulmonary architecture in ST2-overexpressing mice remained almost normal, and the survival rate was significantly improved.

From these results, we concluded that ST2 has the potential to suppress the initial stage of acute lung injury, and therefore it may be a useful reagent for the treatment of acute lung injury.

Introduction

Acute lung injury (ALI) can be triggered by various stimuli, including drugs, sepsis, and trauma (1). It is characterized by epithelial and endothelial damage, and is followed by destruction of the alveolar capillary-epithelial barrier. The increased permeability of pulmonary capillary vessels allows the flooding of inflammatory cells and plasma proteins to enter into the lung, and results in a disturbance of gas exchange (1, 2). The mortality rate of ALI patients remains high (40-60%) despite the numerous attempts that have been made to develop new therapies (2). For the treatment of ALI, such factors as mechanical ventilation settings, oxygen concentration maintenance, and the management of fluid balance have been extensively investigated, and new devices and techniques have led to important improvements. However, in spite of these efforts, an effective therapy to attenuate the inflammatory process in ALI has not been established.

Tominaga originally identified the ST2 (IL-1 RL1) gene as an early response gene in mouse fibroblasts (3). The ST2 gene generates at last four different gene products—the soluble secreted form (ST2), the transmembrane form (ST2L), and two variant forms (ST2V and ST2LV)—by alternative splicing (4). Recently, a specific ligand for ST2L was discovered and named IL-33 by Schmitz *et al.* (5). Among ST2 gene products, ST2 has been known to be related to various disorders in humans. An increase of serum ST2 has been reported in such conditions as septic shock (6), severe trauma (6), bronchial asthma (7), and idiopathic pulmonary fibrosis, especially on acute exacerbation (8). Several experimental studies have suggested that the induction of ST2 by various inflammatory stimuli may confer protection against inflammatory damage. For example, it was reported that pre-treatment with ST2 resulted in an attenuation of pro-inflammatory cytokines and an enhanced survival rate in a mouse endotoxin shock model (9). ST2 was also been shown to inhibit IκB degradation and suppressed LPS-induced IL-6 production in THP-1 cells (10). Additionally, in a collagen-induced arthritis model, it was demonstrated that ST2 suppressed the production of inflammatory cytokines and significantly attenuated the disease (11).

Taken together, these findings suggest that it is worthwhile to assess the therapeutic potential of ST2 as an anti-inflammatory agent *in vivo*. In this paper, ST2-overexpressing mice were prepared by the hydrodynamic gene transfer method, and the effects of ST2 on bleomycin-induced lung injury were investigated. Based on the anti-inflammatory effects of ST2, we hypothesized that its *in vivo* overexpression would ameliorate bleomycin-induced lung injury in mice.

Materials and Methods

Mice

Male C57BL/6 mice, 7-8 weeks of age (body weight 18-22g), were purchased from Japan SLC (Hamamatsu, Japan). Mice were maintained under standard conditions and fed rodent chow and water *ad* lithitum. Our proposal were reviewed by the animal ethical committee of Jichi Medical University (permission No. 152), and all experiments were performed in accordance with the Jichi Medical University Guide for Laboratory Animals based on the Helsinki convention for the use and care of animals.

The numbers of mice employed in the different experiments are described in the relevant figure legends.

Plasmids

pCAGGS and pCAGGS-LacZ were kindly provided by Dr. J. Miyazaki (Osaka University, Osaka, Japan) and Dr. T. Murakami (Jichi Medical University, Tochigi, Japan), respectively. pCAGGS was efficient expression vector driven by the chicken β-actin promoter, and pCAGGS-LacZ was constructed by a insertion of the β̃galactosidase reporter gene into pCAGGS. pCAGGS-mST2 was constructed by a insertion of mouse ST2 (mST2) cDNA into the *Xho*1 site of pCAGGS. The EMBL/GeneBank accession number for the nucleotide sequence mST2 is Y07519. mST2 cDNA was originally isolated from cDNA library of BALB / c-3T3 cells after growth stimulation with serum (12). At first, the Hinc 2 fragment of mST2 cDNA containing entire coding region (1582-bp) was inserted into the Xba1 site of pEF-BOS plasmid (pEF-BOS-mST2) using Xba1 linker. Thereafter, the fragment of mST2 was obtained from pEF-BOS-mST2 by digestion with Xba1, and ligated with Xho1 linker. On

the other hand, pCAGGS plasmid was digested with Xho1 and finally mST2 fragment, digested with Xho1, was inserted. Plasmids were purified with a Plasmid Maxi Kit (Qiagen, Valencia, CA).

The pGL3 control vector containing a firefly luciferase reporter gene was purchased from Promega (Promega, Madison, WI).

Cell culture and *in vitro* transfection

To confirm that the constructed plasmid was appropriate for producing soluble recombinant ST2 protein, human embryonic kidney 293T (HEK 293T) cells were cultured and transfected with pCAGGS and pCAGGS-mST2. HEK 293T cells were kindly provided by Dr. T. Kasahara (Kyoritsu University of Pharmacy, Tokyo, Japan), and were cultured in Dulbecco's modified Eagle's medium (Sigma Aldrich, St. Louis, MO) containing 10% (v/v) fetal bovine serum (Thermo Trace, Melbourne, Australia). The amount of plasmid was 10 μg, and transfection was carried out by the calcium phosphate precipitation method.

Western blotting

Twenty hours after the transfection of pCAGGS and pCAGGS-mST2, the culture medium was collected, digested with *N*-Glycosidase-F (Roche, Indianapolis, IN), and subjected to SDS-PAGE and immnunoblotting as described previously (12). In brief, proteins were separated electrophoretically with 10% polyacrylamide gel and transferred to Immobilon-P membrane (Millipore, Bedford, MA) at 100 mA for 1 h. Immunoblotting was performed with rabbit anti-mouse ST2 polyclonal antibody (× 1000 dilution) against the recombinant ST2 protein from *E. coli*, and after incubation for 1 h at room temperature. Then membrane was incubated for 0.5 h with anti-rabbit IgG-HRP (Jackson Immunoresearch, West Grove, PA) (× 5000 dilution) as a secondary antibody, and the protein band was visualized by the ECL system (Amersham Bioscience, Buckinghamshire, UK).

***In vivo* gene transfer**

Plasmid vectors were delivered into mice by hydrodynamic injection, which is an efficient *in vivo* method of gene transfer. Plasmid DNA was diluted in sterile saline, and

the final volume was adjusted to 6.3% of total body weight (13). Under mild anesthesia with diethyl ether, mice were injected with the plasmid solution through the dorsal penile vein in 5 to 8 seconds, using a 26-gauge needle. The amount of plasmid vectors was appropriately chosen in each experiment.

To evaluate the liver damage in this method, mice were sacrificed and blood was collected at 24 and 48h after the hydrodynamic gene transfer. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were assayed by Japan Society of Clinical Chemistry (JSCC) transferase method using AST kit and ALT kit (Wako, Osaka, Japan) by Clinical Analyzer Model 7180 (Hitachi High-Technologies Co., Tokyo, Japan)

***In vivo* bioluminescent imaging and luciferase asssay**

In order to examine the expression and distribution of the transgene after hydrodynamic injection, we performed *in vivo* bioimaging. Mice were injected with 20 μg of pGL3 control vector or saline as a control, and at 24 h after the injection, mice were anesthetized with a mixture of ketamine and xylazine, and then D-luciferin (potassium salt; Biosynth,

Staad, Switzerland) was injected into the peritoneal cavity of mice at 2 mg/kg. Five minutes later, the luciferase activity was detected from the ventral surface by using a noninvasive bioimaging system, IVIS (Xenogen, Alameda, CA) (13, 14). Furthermore, to precisely quantify the luciferase activity in individual organ, the liver (quadrate lobe), lung (right lung), heart, kidney, and spleen were excised from mice at 24 h after the hydrodynamic gene transfer. One milliliter of Passive Lysis Buffer (Promega) was added to the organs and homogenized with Polytron homogenizer (Kinematica, Littau, Switzerland). Homogenates were frozen (- 80˚C) and thawed twice and then centrifuged for 10 min at 13000g at 4˚C. The total protein contents of the supernatants were determined by the Bradford method with protein assay dye reagent (Bio rad, Hercules, CA) with calibration using bovine serum albumin (Sigma-Aldrich, St Louis, MO). For the luciferase assay, 10μl of supernatants of homogenates was mixed with 50μl of Luciferase assay reagent II (Promega), and luciferase activity was measured by a luminometor (Lumat LB9507; Berthold Technologies, Bad Wildbad, Germany). The data were demonstrated as the relative light units (RLU)/total protein contents.

Detection of Lac Z protein

For visual confirmation of the gene expression in each organ, 20 μg of pCAGGS-Lac Z and the same dose of pCAGGS, as a control, were introduced. Forty-eight hours after the hydrodynamic injection, mice were sacrificed and each organ (liver, lung) was excised and fixed with a mixture of 0.2% (v/v) glutaraldehyde and 1% (v/v) formaldehyde for 1 h at 4˚C, and the samples were then stained with 1 mg/ml of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (Sigma Aldrich).

ST2-overexpressing mice

In order to prepare ST2-overexpressing mice, pCAGGS-mST2 was introduced by hydrodynamic injection, and pCAGGS was used as a control. Starting at 12 h after the gene transfer, we collected blood from the tail vein and confirmed the concentration of plasma ST2 protein. Concurrently, bronchoalveolar lavage fluid (BALF) and lung homogenate were prepared for examining ST2 protein in the lung. Lavage was performed under deep anesthesia with a lethal dose of pentobarbital, and the trachea was cannulated with an 18-gauge catheter. The lungs were inflated with 1 ml of cold sterile saline, and

then the solution was slowly recovered and centrifuged at 1,500 rpm for 10 min at 4˚C, and the resultant supernatant was collected and analyzed. About 0.8 ml of BALF was consistently recovered, and therefore this amount was used to determine the content of ST2 protein in BALF. To obtain the lung homogenate, the remaining mice were anesthetized as described above and perfusion of pulmonary vessels with 5 ml of saline was carried out via the right ventricle. The lungs were then excised and homogenized in 2 ml of cold saline using a Polytron homogenizer (Kinematica, Littau, Switzerland) on ice. The resultant solution was then centrifuged at 10,000 rpm for 15 min at 4˚C, and the supernatant was subjected to the analysis. The concentrations of ST2 protein in plasma, BALF and lung homogenate were measured by Enzyme-Linked Immunosorbent Assay (ELISA).

mST2 ELISA

The mST2 ELISA system was originally constructed in our laboratory as previously described (7). Briefly, we coated the bottom of 96 well ELISA plate (SUMITOMO BAKELITE Co., Tokyo, JAPAN) with rat monoclonal antibody against mouse T1/ST2

protein (MD Biosciences, Zurich, Switzerland) and samples were added and incubated for 1h at room temperature. Next, rabbit anti-mST2 polyclonal antibody (12) and horseradish peroxidase-labeled antibody against rabbit IgG (GE healthcare UK Ltd., Buckinghamshire, England) were consecutively added. Finally, the solution of O-phenylenediamine was used as substrate and optical density was measured at 450nm with a microplate reader (Inter Medical, Tokyo, Japan).

Induction of bleomycin-induced lung injury

Mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and then bleomycin (Nippon Kayaku Co., Tokyo, Japan) in 50 μl of sterile saline was administered intratracheally as previously reported (15). To determine the appropriate dosage of bleomycin, we preliminarily administered bleomycin at different dose, 2.5mg/kg and 1mg/kg, to mice. In the 2.5mg/kg-injected group, body weight decreased rapidly, and the animals almost died between days 7-10. On the other hand, in the 1mg/kg-injected group, around half of the mice remained alive. Therefore we established that the optimal bleomycin dose for our purpose was 1mg/kg.

In plasmid-introduced mice, at 24 h after the gene transfer (pCAGGS and pCAGGS-mST2) to mice, blood was sampled from the tail vein and the level of ST2 protein was determined. After an additional 24 h, As controls, the same volume of saline or the same dose of bleomycin was administered to mice after the hydrodynamic injection with the same volume of saline (6.3% of the total body weight) without plasmid vectors.

Reverse Transcriptional PCR (RT-PCR) analysis

Bleomycin-induced mRNA expression of several cytokines and mST2 were examined by RT-PCR analysis. Mice that had not undergone the gene transfer were administered bleomycin intratracheally, and after 1, 3, or 7 days, the whole lungs were removed and immediately immersed in 1 ml of TRI reagent (Sigma-Aldrich). After homogenization with a Polytron homogenizer, total RNA was extracted and purified according to the manufacturer's protocol. Contaminated genomic DNA was destroyed by DNase I (Takara, Shiga, Japan), and cDNAs were synthesized using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The nucleotide sequence of primers used were as follows: mST2: (forward) 5'- GCGGAGAATGGAACCAACTA-3', (reverse)

5'-CAATGTGTGAGGGACACTCC-3', β-actin: (forward) 5'-TGTCCCTGTATGCCTCTGGTA-3', (reverse) 5'-ACTGTGTTGGCATAGAGGTC-3', TNF-α: (forward) 5'- CTGGGCAGGGGCCACCACGCTC-3', (reverse) 5'-CTCAGCGCTGAGTTGGTCCCCCTTCTC-3', IL-1β: (forward) 5'-GCTGCTTCCAAACCTTTGAC-3', (reverse) 5'-AGGCCACAGGTATTTTGTCG-3'. IL-33: (forward) 5'-ATGAGACCTAGAATGAAGTATTCCA-3', (reverse) 5'-TTAGATTTTCGAGAGCTTAAACATA-3'. Ten microliters of PCR products were developed by electrophoresis on 1% agarose gels, and the gels were stained with ethidium bromide.

Measurement of cytokines and albumin in BALF

After the treatment with bleomycin, the lungs were lavaged four times with 0.7 ml of cold PBS. After lavage, the total number of cells was immediately counted and the cell type was identified. For the determination of cell types, cells obtained through centrifugation in a cytospin at 800 rpm for 10min were stained with Diff-Quick (Sysmax, Kobe, Japan). The remaining BALF was centrifuged at 1,500 rpm for 10 min at 4˚C, and

the supernatant was analyzed. The concentrations of cytokines (TNF-α, IL-1βIL-6) in BALF were measured using ELISA kits (Biosource, Camarillo, CA), and albumin concentration was measured by a turbidimetric immunoassay using R-ALB-UR (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan). Bronchoalveolar lavage was performed four times of 0.7 ml PBS, and approximately 2.5 ml was continually recovered in each case, the concentrations of each cytokine and albumin were described as pg/ml or μg/ml.

Histology

Mice were sacrificed and pulmonary vessels were perfused with 5 ml of saline via the right ventricle. Then, after the intratracheal injection of 1 ml of 10% (v/v) neutral formalin buffered with 0.2 M cacodylate solution (pH 7.4), the lungs were excised and postfixed with the same fixative for 10 h and were embedded in paraffin. Sections were cut into 6-μm thick slices, which were stained by hematoxylin-eosin (H. E.) solution and Mallory-Azan.

To quantify the histological findings, we calculated the tissue volume density (TVD) using Scion Image software (Scion Corporation, Frederick, MD), and obtained the proportion of airspace consolidation and atelectasis to the total areas.

Analysis of body weight-loss and survival after bleomycin treatment

Twenty-four hours after hydrodynamic gene transfer (pCAGGS and pCAGGS-mST2), bleomycin was administered intratracheally (1 mg/kg). Over 14 days following gene transfer, the mortalities and body weights in each group of mice were monitored.

Statistical analysis

The data are presented as the means±standard deviation. Statistical analyses were performed by Student's *t* test (comparing two groups) or by ANOVA followed by the Turkey's test (comparing multiple groups), and survival analysis was performed by Kaplan-Meier method. SPSS 11.0J (SPSS Inc. Chicago, IL) were used for each statistical analysis. *P* values of < 0.05 were considered statistically significant.

Results

Hydrodynamic gene transfer

Hydrodynamic injection, a strategy used to transfer naked plasmid DNA into animals, was applied to obtain ST2-overexpressing mice. First, in order to confirm the overexpression of the transgene by hydrodynamic injection, we injected the pGL3 control vector into the mice, and observed the luciferase expression in vivo (Figure 1A). Luciferase expression was most prominent around the upper abdomen, and was significantly elevated at day 1. No specific signals were detected in the saline-injected mice.

Next, to confirm the transgene expression in each organ, several organs (liver, lung, heart, kidney, spleen) were excised and the luminescence activity was measured after hydrodynamic gene transfer of pGL3 control vector. Figure 1B and C demonstrated that the liver showed outstanding luminescence activity, and thus the liver the only target organ of this gene transfer method. The other organs of pGL3-injected mice showed lower luminescence activity than the liver, however, higher activity than that of control mice. Furthermore, to detect the transgene expression visually, pCAGGS-LacZ was

administered in the same manner, and the liver and lung were excised and stained with the X-gal solution. As shown in Figure 1D, the liver was clearly stained blue, however, there was almost no detectable β-galactosidase expression in the lung (Figure 1E). This outcome corresponded to the results of the bioluminescent assay.

Transient overexpression of mST2 protein

Before *in vivo* application, to confirm the production of the mature mST2 protein from the constructed plasmid, pCAGGS and pCAGGS-mST2 were transferred into HEK 293T cells. The culture supernatant was collected for Western blotting. As shown in Figure 2A, both the glycosylated mature form of ST2 (60-70 kDa) and the core peptide (37 kDa) obtained by digestion with *N*-Glycosidase F were clearly detected. The results indicated that the mature ST2 protein was appropriately expressed and secreted from the cultured cells using our constructed plasmid.

We then carried out *in vivo* gene transfer into mice using hydrodynamic injection. First, we determined the appropriate amount of plasmid for obtaining the maximum plasma concentration of the ST2 protein. As shown in Figure 2B, various amounts of plasmid

vectors (10-100 μg) were introduced, and the concentration of plasma ST2 protein increased significantly in accordance with the amount of plasmid administered. It reached a maximum level when 50 μg of the plasmid was injected, but no significant increase was observed when the amount of plasmid was increased from 50 to 100 μg. Therefore, the amount of the plasmid was fixed at 50 μg for the following *in vivo* experiments.

We next examined the time-kinetics of the introduced ST2 protein. As shown in Figure 2C, the ST2 protein level in plasma attained a peak at around 12-24 h (18.1-27.6 □g/ml) and gradually decreased until 21 days (4.07 □g/ml). Empty vector-injected mice showed no increase in ST2 level from the beginning to the end of the trial, indicating that the injection of plasmids itself did not exert any influence on the endogenous ST2 level. For examination of the level of local ST2 protein in lung, 1 ml of saline was injected intratracheally to obtain BALF, and almost constant recovery of 0.8 ml was obtained. The level of BALF ST2 protein was 10.81±3.31 □g/ml, as measured by ELISA, and therefore the BALF ST2 protein content was estimated at 8.65±2.65 ng/BALF. As regards the lung homogenate, the lungs were excised and homogenized in 2ml of saline. Since the

concentration of ST2 protein was 23.57±12.72 □g/ml, the content of ST2 protein in lung homogenate was 47.14±25.43 ng (Figure 2D, 2E).

Since the hydrodynamic method has been known to cause liver damage, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were monitored after the injection. In this study, AST/ALT levels were elevated at day 1, however, returned to the normal range by day 2. There were no significant time-course differences in the serum AST/ALT concentration between empty vector–introduced mice and ST2-overexpressing mice (Figure 2F).

mRNA expression of pro-inflammatory cytokines, endogenous ST2, and IL-33 in bleomycin-induced lung injury

TNF-α and IL-1β are pro-inflammatory cytokines and they have been reported to be upregulated immediately after bleomycin exposure, and to play principal roles in the exacerbation of lung injury (16, 17). In the present study, therefore, we examined the lung mRNA expression of the pro-inflammatory cytokines TNF-α and IL-1β after the intratracheal injection of bleomycin. As shown in Figure 3, bleomycin-treatment

remarkably increased the mRNA levels of TNF-α and IL-1β at day 1, demonstrating the acute lung injury occurred, and then the levels of both cytokines were decreased from day 3-7. We also examined the levels of lung endogenous ST2 and IL-33 mRNA, which were weakly and constitutively expressed without bleomycin treatment, however these mRNA gradually increased in a time dependent manner from day 3 to day 7 after bleomycin treatment.

Analysis of BALF cell counts and cell types after bleomycin-administration

To examine the acute extravasation of inflammatory cells into the pulmonary interstitium by bleomycin, bronchoalveolar lavage was performed at day1 and day3 after bleomycin treatment, and we analyzed the total cell counts and cell types. Compared to the levels in control mice treated with saline, the total cell numbers and numbers of neutrophils were significantly elevated at day 1 in bleomycin-treated mice (Figure 4A, B).

We then examined whether or not the ST2 protein affected the initial inflammatory phase in acute lung injury, using bleomycin-treated ST2-overexpressing mice and mice

injected with empty vector as controls. The time course was presented in Figure 5. We firstly confirmed that the hydrodynamic injection itself did not affect the total cell counts in empty vector and pCAGGS-ST2-injected mice (Figure 4A). And in mice treated with empty vector plus bleomycin, the total number of cells immediately increased on day 1, as well as mice treated with bleomycin alone. However, in ST2-overexpressing mice, the total cell number was significantly reduced compared with the other bleomycin treated mice. Furthermore, in mice with empty vector plus bleomycin, numbers of neutrophils were prominently elevated at day 1. In contrast, a significant decrease in the number of neutrophils and lymphocytes was evident on day 1 in ST2-overexpressing mice. (Figure 4B).

On the other hand, on day 3, the total cell counts in ST2-overexpressing mice was estimated to be almost equivalent to that in mice treated with bleomycin alone, as well as empty vector injected plus bleomycin treated mice. However, even though there were no significant statistical differences, the counts of neutrophils were relatively lower and the counts of lymphocytes were higher in ST2-overexpressing mice, compared to the other bleomycin-treated groups.

Pro-inflammatory cytokines and albumin levels in BALF

In the present study, total cell counts and neutrophils were particularly increased in the initial phase of bleomycin-induced lung injury, we next assayed the concentrations of pro-inflammatory cytokines in BALF at day1 after the bleomycin-treatment, and were compared between groups. In bleomycin treated mice, significant increases of TNF-□IL-1□□□□□□□□□□□□□ and IL-6 were demonstrated, and levels were almost equivalent or slightly lower than those in the mice treated empty vector plus bleomycin. In ST2-overexpressing mice, the concentration of TNF-α was significantly reduced compared with that of mice injected with empty vector plus bleomycin or that of mice treated with bleomycin alone (Figure 6A). In addition, the level of IL-6 was also significantly decreased in ST2-overexpressing mice (Figure 6B). The level of IL-1β was slightly lower in ST2 overexpressing mice, but there were no statistical differences (data not shown).

The albumin concentration in BALF is frequently used as an indicator of pulmonary vascular permeability in a case of acute lung injury. And in fact, bleomycin-treatment

induced significant increase of albumin levels in bleomycin-treated mice in the present study. However, in ST2-overexpressing mice, the albumin concentration was significantly lower than in other bleomycin-treated mice (Figure 6C).

Histological examination of the effect of ST2 protein on the bleomycin-induced lung injury

Next, we histologically investigated the effect of ST2 protein on acute phase of bleomycin-induced lung injury (Figure 7A, B). Mice treated only with saline exhibited a small-scale interstitial edema at the early phase (day 1), but no inflammatory cells were observed in the perivascular area at that time (Figure 7A, saline). We also examined the impact of hydrodynamic injection histologically, plasmid-injection itself did not affect the pulmonary architecture (Figure 7A, EV, ST2). On the other hand, both the mice treated with bleomycin alone and those treated with empty vector plus bleomycin-treated showed an accumulation of inflammatory cells around the broncho-vascular bundle, and in higher magnification, many neutrophils with segmented nuclei and some round mononuclear cells were recognized (Figure 7B, bleomycin, EV plus bleomycin). In

contrast, rare inflammatory cells were observed in the peribronchial area in ST2-overexpressing mice, but accumulations of neutrophils were not observed (Figure 7A, B, ST2+bleomycin).

In general, following the inflammatory phase in bleomycin-induced lung injury, connective tissues and extracellular matrix are increased (17). We performed Mallory-Azan and H.E. staining of the lung tissue sections on day 7 after administration of bleomycin (Figure 8, A; entire images at lower magnification, B; central regions at higher magnification). Mice treated saline alone and mice injected plasmid vector alone resulted in no inflammatory change; no alteration of the pulmonary architecture was observed (Figure 8A saline, EV, ST2). However, a large number of inflammatory cells was observed in the mice treated with bleomycin and that those treated with empty-vector plus bleomycin; the infiltrating cells accumulated in the sub-pleural and peribronchial areas, and the airspaces showed wide-ranging collapse (Figure 8A, bleomycin, EV+bleomycin). Furthermore, there was a large amount of blue-stained connective tissue throughout the alveolar interstitium, especially in the severely damaged area (Figure 8B, bleomycin, EV+bleomycin). On the other hand, in ST2-overexpressing mice, some

inflammatory cells were observed, but the areas of inflammation were restricted. Most of the alveolar structure looked nearly normal, and it was clear that there was less connective tissue showed than the other bleomycin-treated mice (Figure 8A,B, ST2+bleomycin).

Next, we utilized Scion Image software and quantified tissue volume density (TVD) to visualize the histological results at day 7 after bleomycin-treatment. TVD was presented as the ratio of the collapsed high density area per total area, and it increased in proportion to the extent of lung injury. Mice treated with bleomycin alone and treated with empty vector plus bleomycin showed significantly higher TVD than saline-treated mice. On the other hand, in ST2-overexpressing mice treated with bleomycin, TVD was significantly lower than in other bleomycin treated mice (Figure 8C).

Body weight loss and survival rate in bleomycin-induced lung injury

We monitored the body weight loss during the 14 days after bleomycin administration in ST2-overexpressing mice and empty vector-injected mice (Figure 9A). During the first one week, most of the experimental mice remained alive, but their body weights decreased to varying degrees. In the second week, although the difference between the

two groups was not statistically significant, the empty vector-injected mice continuously lost body weight, while the body weight of the ST2-overexpressing mice tended to remain constant.

Finally, we compared the survival rate for 14 days after bleomycin administration between ST2-overexpressing mice and the other group of mice (saline, bleomycin, and empty vector plus bleomycin) (Figure 9B). The survival rate of ST2-overexpressing mice was 84.0% at day 14 and mean survival time was 13.44 days (95% C.I. 12.92-13.96). The survival rate of mice treated with an empty vector plus bleomycin was 43.5% and mean survival time was 11.7 days (95% C.I. 10.58-12.81), and that of mice receiving bleomycin treatment alone was 63.6% and 12.2 days (95% C.I. 10.76-13.60). ST2-overexpressing mice showed significant high survival rate than the other control groups. No mice died from hydrodynamic injection only (data not shown).

Discussion

Some recent reports have suggested that ST2 protein is induced by various inflammatory stimuli and may play an anti-inflammatory role *in vivo* (6-10). Tajima *et al.* reported the

increase of serum ST2 protein in acute exacerbation of IPF (8), and he also reported that ST2 gene expression was induced in the murine lung by intratracheal administration of bleomycin (18). Therefore, we speculated that ST2 was closely related with lung inflammation, and we induced acute lung injury by bleomycin in the present study, as was also reported in Tajima *et al.*, and then assessed the effect of ST2 protein *in vivo*.

In order to examine the effect of ST2 protein in mice, at first we planed to purify and to administer recombinant ST2 protein to mice. However, unlike with *in vitro* study, in order to attain the high ST2 level and to maintain it *in vivo*, enormous amount of ST2 protein was need. Then, we employed hydrodynamic injection method, which is an efficient method for transient gene transfer to adult animals (13, 14, 19), and high levels of target gene expression can be achieved by the rapid injection of naked plasmid in a large volume. As well as the previous report (19), we confirmed the main transgene-expressing organ was liver, but ST2 protein was released into the systemic circulation and attained the high level from 12 h after the gene transfer. The transgene expression level depends on the injection speed, injection volume, target organ, and the choice of promoter of the plasmid vectors. We used the pCAGGS vector, which was

driven by the chicken β-actin promoter and which enabled us to maintain a high level of expression in mammalian cells. However, the appropriate plasma ST2 level to achieve an anti-inflammatory effect for mice treated with bleomycin has not been established *in vivo*. According to a previous report, 100 μg of ST2 protein is required to prevent the endotoxin shock in mice (9). Therefore, in this study, we used a high dose of plasmid (50 μg/mouse) to obtain the maximal plasma concentration of ST2 (18.1-27.6 μg/ml).

In the present report, we demonstrated that ST2 protein hindered the invasion of neutrophils and the subsequent capillary leakage in acute lung injury for the first time. In addition, TNF-α, IL-1β and IL-6 are known to play pivotal roles in the induction of lung inflammation, leukocyte migration, and production of other cytokines and chemokines (20-21), and these cytokines were elevated in BALF in bleomycin-induced acute lung injury. However, we found that the levels of TNFα, and IL-6 in BALF were significantly decreased in ST2-overexpressing mice at day 1 after bleomycin-treatment. In the previous *in vitro* experiments, it was demonstrated that ST2 inhibited the production of pro-inflammatory cytokines in macrophages derived from bone marrow (9, 11), as well as in a monocytic leukemia cell line (10). Therefore, in the present *in vivo* models, we

speculate that overexpressed plasma ST2 infiltrated into the alveolar spaces, where it affected alveolar macrophages to suppress the production of cytokines, and subsequently suppressed the migration of neutrophils and increase of vascular permeability.

In contrast, we did not detect any significant differences in BALF findings from day3 after the bleomycin treatment. We speculated that the following etiological explanation might account for the BALF findings from day 3; (1) ST2 would play an anti-inflammatory role only during the initial phase of the acute lung injury, and thus could not stop the infiltration of inflammatory cells after 3 days, (2) ST2 overexpression in this system peaked at 12-24 h after gene transfer, and then ST2 rapidly declined; thus the effects of ST2 would diminish along with attenuation of the plasma concentration of ST2, (3) ST2 would play the adverse effects from day 3 after bleomycin- treatment. In association with (2), hydrodynamic gene transfer is certainly an invasive approach and thus repetitive transfer was difficult in our pilot study, because the mice were often dead by repeated injection. Therefore, a method for maintaining high ST2 levels even after 48 h of gene transfer must be developed to assess the effects of ST2 accurately in this model. And in association with (3), it was reported that serum ST2 was elevated in some

autoimmune disease (22), and ST2L have been reported to play a important role in differentiation of helper T cells and they induces the proliferation and activation of Th2 response (22-23). Furthermore, the recent paper reported that recombinant ST2 boosted hepatic Th2 response *in vivo* and increased the Th2 cytokines and advanced liver fibrosis (24). It suggests the ambivalent dual effects of ST2 protein; ST2 suppress the pro-inflammatory cytokines from macrophage in fact, but it would exert the response of Th2 lymphocytes. Considering these reports, we speculate overexpresssed-ST2 protein may affect the lymphocyte-proliferation in this model, and which would play the adverse effect in the increase of total cells from day 3.

We found that endogenous ST2 mRNA was induced by bleomycin-treatment, and newly we detected that IL-33 was also concurrently increased at day 3 after bleomycin-treatment, whereas pro-inflammatory cytokines (TNF-α, IL-1β) were increased immediately after bleomycin-treatment. The effects of ST2 and IL-33 have been well investigated in allergic disease; IL-33 is known to induce the production of Th2 cytokines and IgE very strongly and the proliferation of eosinophils (6, 25). Tajima *et al.* reported that endogenous ST2 and Th2 cytokines (IL-4, IL-5) were slowly induced after

bleomycin-treatment, similar to our study. From these results, endogenous ST2, Th2 cytokines and IL-33 would affect the progress of lung injury following the initial phase of acute lung injury. It is thought to be important whether overexpressed-ST2 affected the IL-33 production or not, however, we could not approach that problem because of the difficulty of keeping the level of ST2 at day 3 after bleomycin-treatment.

From day 7 after bleomycin treatment, the pulmonary remodeling was progressed, and we found the subpleural collapse and increase of collagen fibers rather than the peribronchial accumulation of inflammatory cells. And around half of the bleomycin-treated mice and half of the mice treated empty vector plus bleomycin-treated died during day 7-14, suggesting that the pulmonary remodeling phase (day 7-14) were most responsible for the mortality. In ST2-overexpressing mice, the histological findings and survival rate after bleomycin-treatment were improved compared to other bleomycin-tread mice. Pulmonary remodeling was known to be mostly caused by the degradation of extra-celllar matrix by neutrophils (26) and initial increase of neutrophils play a critical role in progress of lung inflammation and fibrosis (27). Considering the previous reports and our present data, we speculated that inhibiting the infiltration of

neutrophils by ST2 at an early stage of lung injury helped to protect against proteolysis, and minimized histological change, and improved the survival, even though the peribronchial inflammation, as indicated by the BALF finding, could not be completely suppressed.

In order to conclusively determine the roles of ST2, it will be necessary to conduct similar experiments using ST2 knockout (KO) mice. And although several studies using ST2 KO mice have been reported, unfortunately both specific soluble ST2 and transmembrane ST2L were absent in these mice, because the disrupted DNA of KO mice corresponds to a common portion of ST2 and ST2L (28). Therefore, utilizing available KO mice for the studies of the involvement of ST2 in the lung inflammation would complicate the outcome. However, we speculate that previous results and the findings of the current study suggest that bleomycin would cause serious damage to the ST2 specific KO mice.

In conclusion, our present study confirmed that ST2 possessed the ability to suppress lung inflammation via inhibiting the elevation of pro-inflammatory cytokine levels and the accumulation of neutrophils, and via reducing the vascular permeability. Further

investigation into the etiology of the effects of ST2 is required before the clinical applications, but ST2 might be a new candidate for the treatment of lethal acute lung injury.

Legends to figures

Figure 1. Detection of the gene expression transferred by the hydrodynamic injection

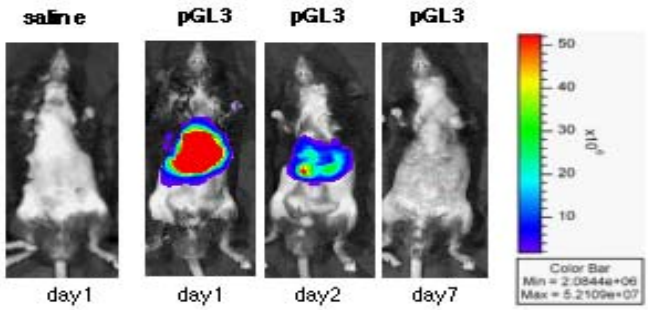
(A) *In vivo* imaging of the mice after the hydrodynamic injection of saline and pGL3 control vector. The luciferase expressions were detected from ventral surface of the body by IVIS up to 7 days. The right panel indicates the photon intensity correlating with the gene expression. The data are representative of four separate experiments showing similar results.

(B*)*(C) *Ex-vivo* luciferase assay of the individual organs. The luciferase activities of liver, lung, heart, kidney, and spleen at 24 h after the hydrodynamic injection of saline (control) and pGL3 control vector were demonstrated as the relative light units (RLU) per total protein contents. The luciferase activity of the liver was demonstrated independently using another scale in Figure C. Data are means ± SD, n=4, and are representative of two independent experiments. *$P < 0.05$, **$P < 0.01$ versus relevant organ of saline injected group, #$P < 0.01$ liver versus other organs of pGL3 injected mice.

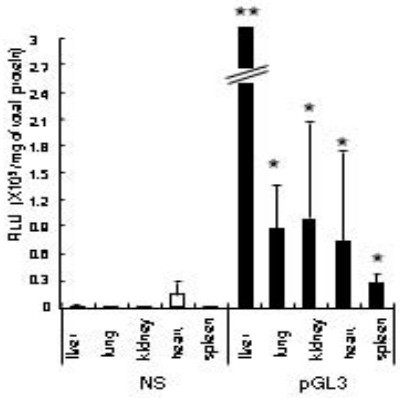
(D)(E) General view of the left lobe of the liver (D), and right lobe of the lung (E), at 48 hours after the hydrodynamic injection of pCAGGS or pCAGGS-LacZ. Each organ was excised and stained with X-gal solution. Blue staining indicates the expression of β-galactosidase.

Figure 1

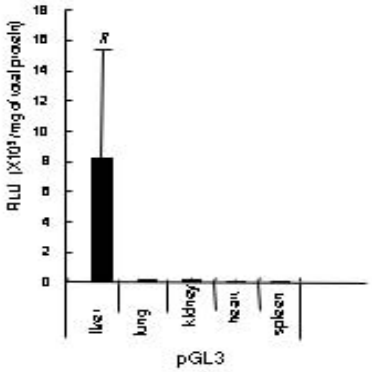
A


saline
pGL3
pGL3
pGL3
day1
day1
day2
day7
50
40
30
20
10
Color Bar

B


liver
lung
kidney
heart
spleen
NS
pGL3

C


liver
lung
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spleen
pGL3

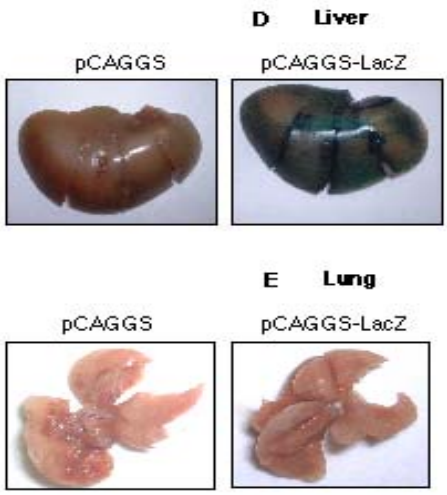


Figure 2. Assessment of ST2 gene transfer *in vitro* and *in vivo*

(A) *In vitro* detection of mouse ST2 protein in the supernatants of HEK 293T cells after the gene transfer of pCAGGS and pCAGGS-mST2. These plasmid vectors were transferred by the calcium-phosphate method, and 24 hours later, supernatants were collected and subjected to Western blotting. The presence of mature glycosylated ST2

protein (black arrowhead, 60-70 kDa) and deglycosylated core peptide (white arrowhead, 37 kDa) was confirmed.

(B) Concentration of plasma ST2 protein in mice injected with various amounts of plasmids. Mice were sacrificed at 12 h after the hydrodynamic injection of plasmid (pCAGGS-mST2) or same volume of saline a control, and the plasma was collected to measure the ST2 concentration. The data are presented as the means ± SD, n=4, and are representative of three independent experiments. *$P < 0.05$ compared to saline injected mice, #$P < 0.05$ compared to other plasmid dose.

(C) Time kinetics of plasma ST2 protein after the hydrodynamic injection with 50 μg of plasmid. Mice were bled consecutively from the tail vein on each day, and plasma was prepared. The data are presented as the means ± SD, n=4, and are representative of three independent experiments. *$P < 0.05$ pCAGGS-injected group versus pCAGGS-mST2-injected group at the same plasmid dose, and #$P < 0.05$ compared to other timing in pCAGGS-mST2-injected group.

(D) (E) ST2 protein in lung homogenate (D) and BALF (E) at 24 hours after hydrodynamic injection. The data are presented as means ± SD, n=3, *$P < 0.05$ compared to pCAGGS-injected mice.

(F) Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels after the hydrodynamic gene transfer. The data are presented as the means ± SD, n=3, and are representative of two independent experiments. * $P <0.05$ compared to saline injected mice (without plasmid-injection).

Figure 2

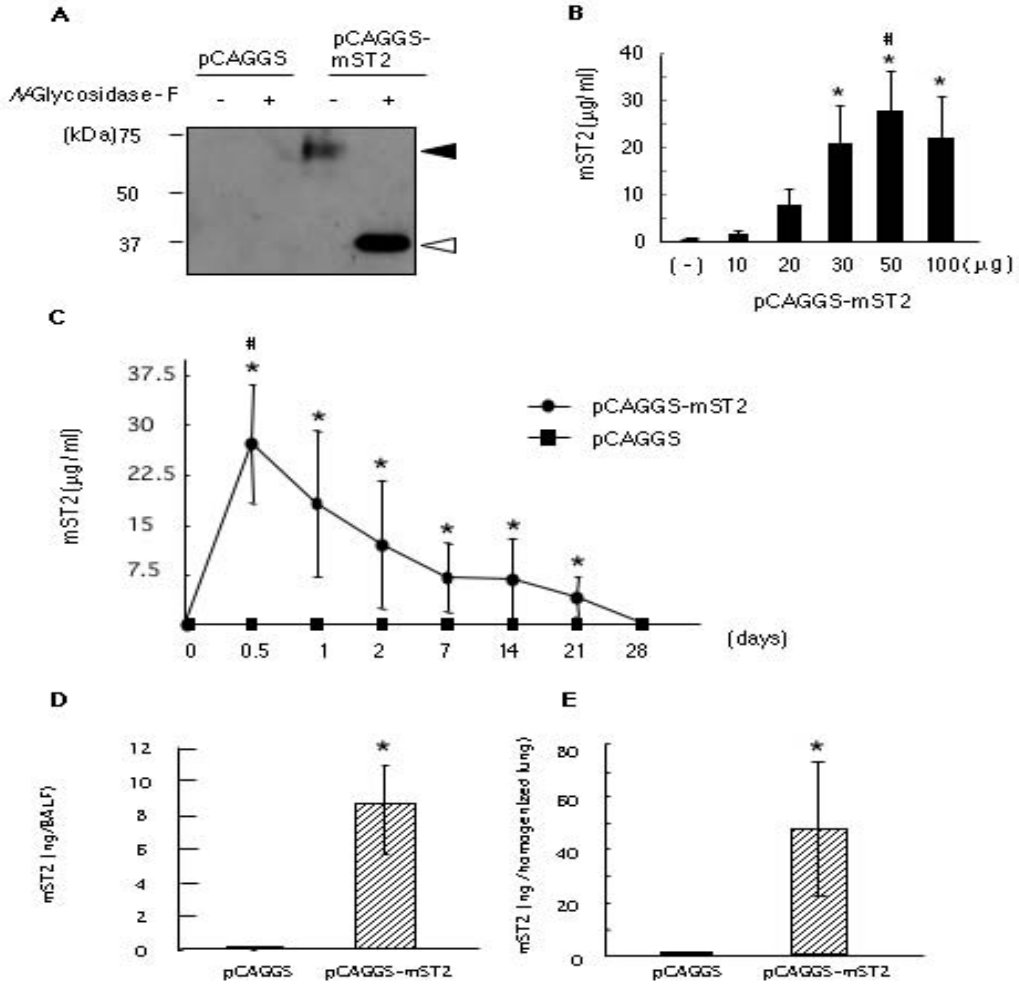

A
pCAGGS
pCAGGS-mST2
N-Glycosidase-F
- + - +
(kDa)75
50
37
B
mST2(μg/ml)
40
30
20
10
0
(-) 10 20 30 50 100(μg)
pCAGGS-mST2
C
mST2(μg/ml)
37.5
30
22.5
15
7.5
pCAGGS-mST2
pCAGGS
0 0.5 1 2 7 14 21 28
(days)
D
mST2 (ng/BALF)
12
10
8
6
4
2
0
pCAGGS
pCAGGS-mST2
E
mST2 (ng/homogenized lung)
80
60
40
20
0
pCAGGS
pCAGGS-mST2

Figure 2

F

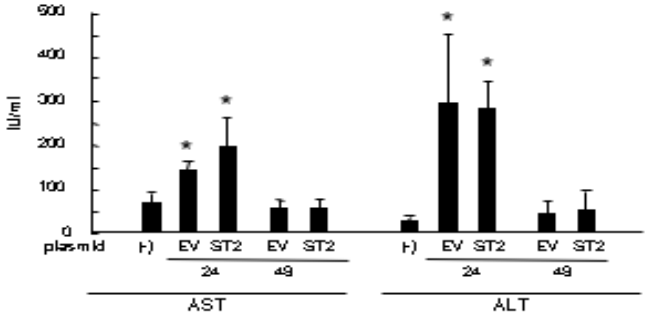


Figure 3. mRNA expression of pro-inflammatory cytokines, ST2, and IL-33 after bleomycin treatment

Bleomycin was administered intratracheally to wild type mice, and at 1, 3, and 7 days after, whole lung was excised and each mRNA expression was assayed. Mice without

bleomycin treatment were controls. β-actin was house keeping gene and endogenous control. Similar results were obtained in three separate experiments.

Figure 3

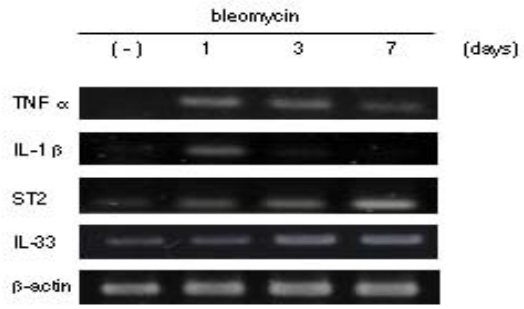


Figure 4. Analysis of total cell numbers and cell types in BALF

BALF analysis was performed in mice with or without hydrodynamic gene transfer, at day 1 and day 3 after the administration of bleomycin. Total cell numbers (A) and cell

types (B) in BALF are shown. The data are presented as the means±SD of four independent experiments. Mϕ means macrophage. (A) saline: n=5, bleomycin: n=6; EV (pCAGGS) or ST2 (pCAGGS-mST2): n=3, EV or ST2 +bleomycin: n=10. (B) saline: n=5, bleomycin: n=6; EV or ST2+bleomycin: n=8. *$P < 0.05$ ST2 plus bleomycin versus bleomycin alone, **$P < 0.01$ ST2 plus bleomycin versus EV plus bleomycin, #$P < 0.01$ compared to saline, ¶ $P < 0.01$ compared to EV and ST2 (without bleomycin), § $P < 0.05$ compared to bleomycin alone.

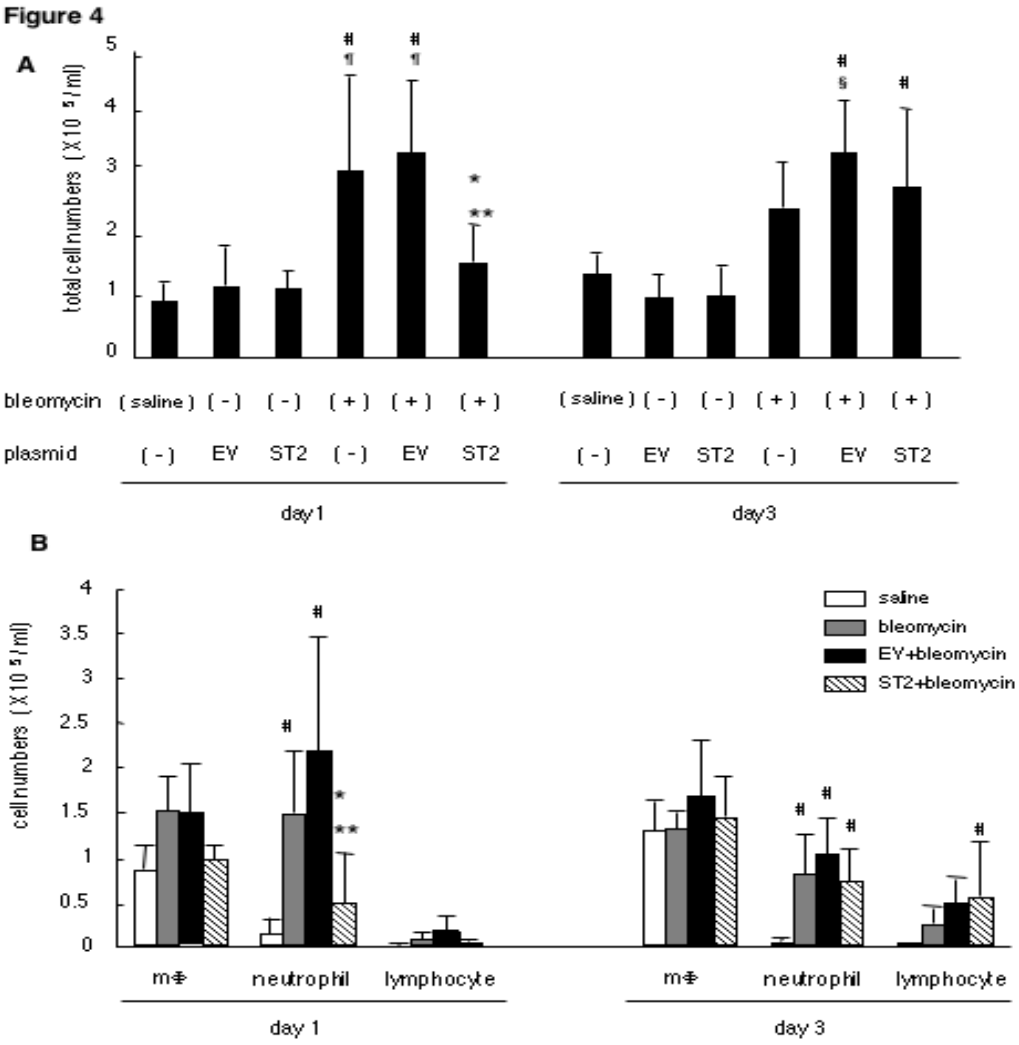


Figure 5. Time course of the experiments

The time course of the experiments to examine the effect of ST2 for the bleomycin-induced acute lung injury was presented schematically. The gene transfer was performed by the hydrodynamic injection of plasmids.

Figure 5

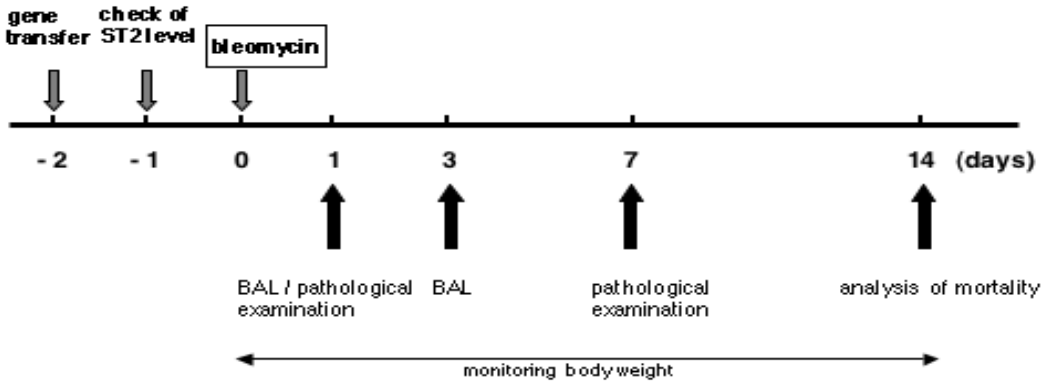


Figure 6. Pro-inflammatory cytokines and albumin concentration in BALF

The concentrations of TNF-α (A), IL-6 (B), and albumin (C) were analyzed at day 1 after the treatment with bleomycin or saline. Cytokines were measured by ELISA. The albumin concentration was measured by turbidimetric immunoassay. Data are representative of two independent experiments. saline: n=5, bleomycin alone: n=6, EV

(pCAGGS) or ST2 (pCAGGS-mST2) plus bleomycin: n=8. *$P < 0.05$ ST2 plus bleomycin versus EV plus bleomycin, #$P < 0.01$ compared to saline, ¶ $P < 0.01$ compared to EV and ST2 (without bleomycin).

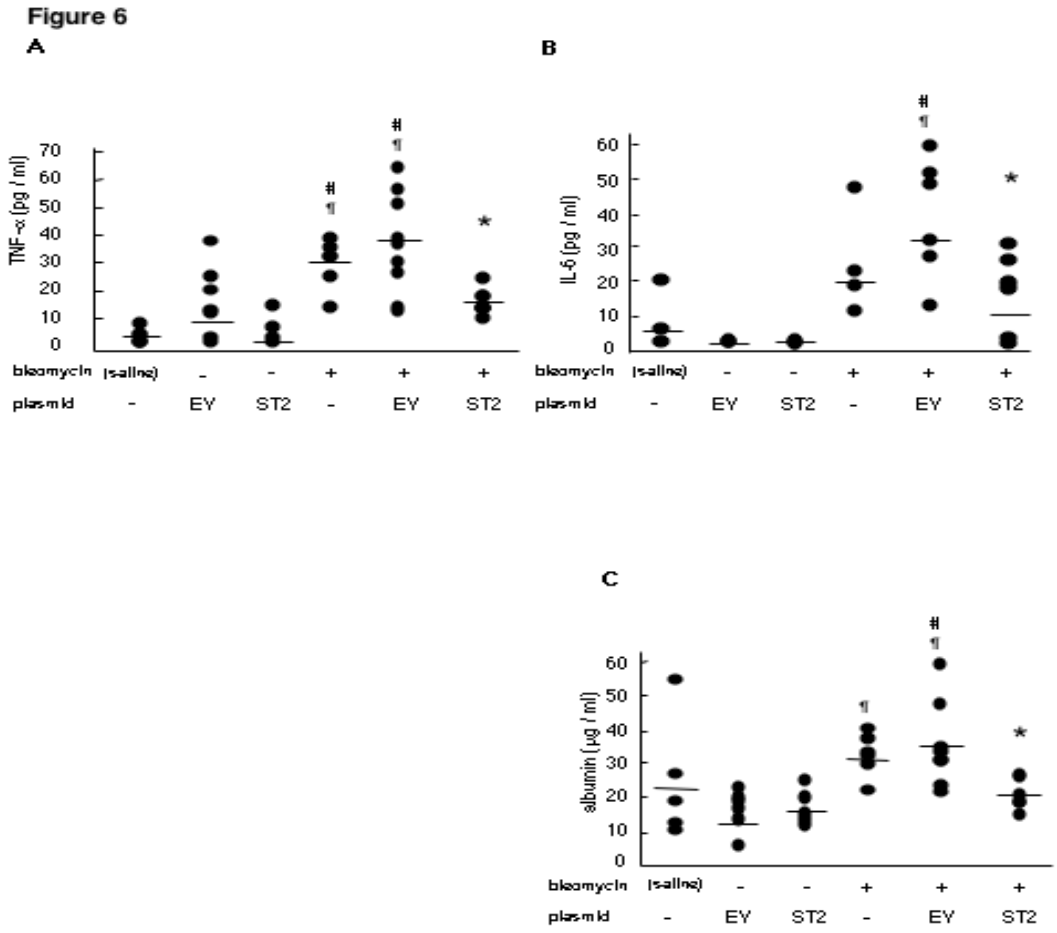


Figure 7. Histological findings at day 1 after the treatment with bleomycin

Photomicrographs of lung sections from mice treated with saline, EV (pCAGGS) alone, ST2 (pCAGGS-mST2) alone, bleomycin alone, EV plus bleomycin, ST2 plus bleomycin, at day 1 after bleomycin-treatment. Photograph of Figure 7A was taken at lower magnification and 7B was especially taken at around broncho-vascular bundles at higher magnification of mice treated with saline, bleomycin alone, EV plus bleomycin and ST2 plus bleomycin. Sections were stained with H&E. The arrow in Figure 7A point to the accumulation of inflammatory cells, and the arrowheads in 7B point to neutrophils. Scale bar = 200 μm (A), 10 μm (B). br: bronchiole; v: vessel.

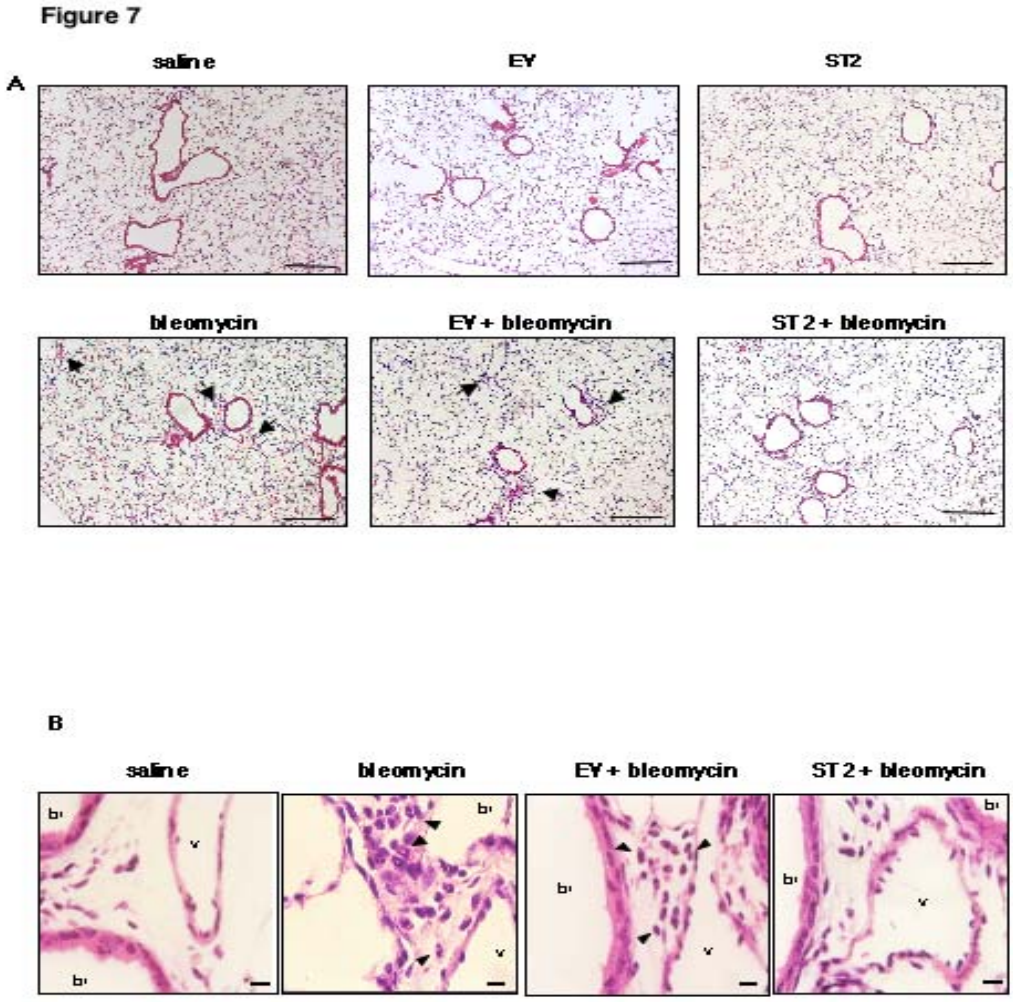


Figure 8. Histological findings at day 7 after the treatment with bleomycin

Photomicrographs of lung sections from mice treated with saline, EV (pCAGGS) alone, ST2 (pCAGGS-mST2) alone, bleomycin alone, EV plus bleomycin and ST2 plus bleomycin at day 7 after the treatment with bleomycin. The sections in Figure 8A was

stained with H.E and that in 8B was stained with the Mallory Azan method. The scale bar in A= 640 μm. The scale bar in B= 50 μm.

(C) Tissue volume density is demonstrated as the proportion of air space consolidation per total volume. n=3 (saline) and n=4 (bleomycin, EV plus bleomycin, ST2 plus bleomycin), and data are representative of three independent experiments. *$P < 0.05$ bleomycin alone versus ST2 plus bleomycin, **$P < 0.01$ EV plus bleomycin versus ST2 plus bleomycin, #$P < 0.01$ compared to saline.

Figure 8

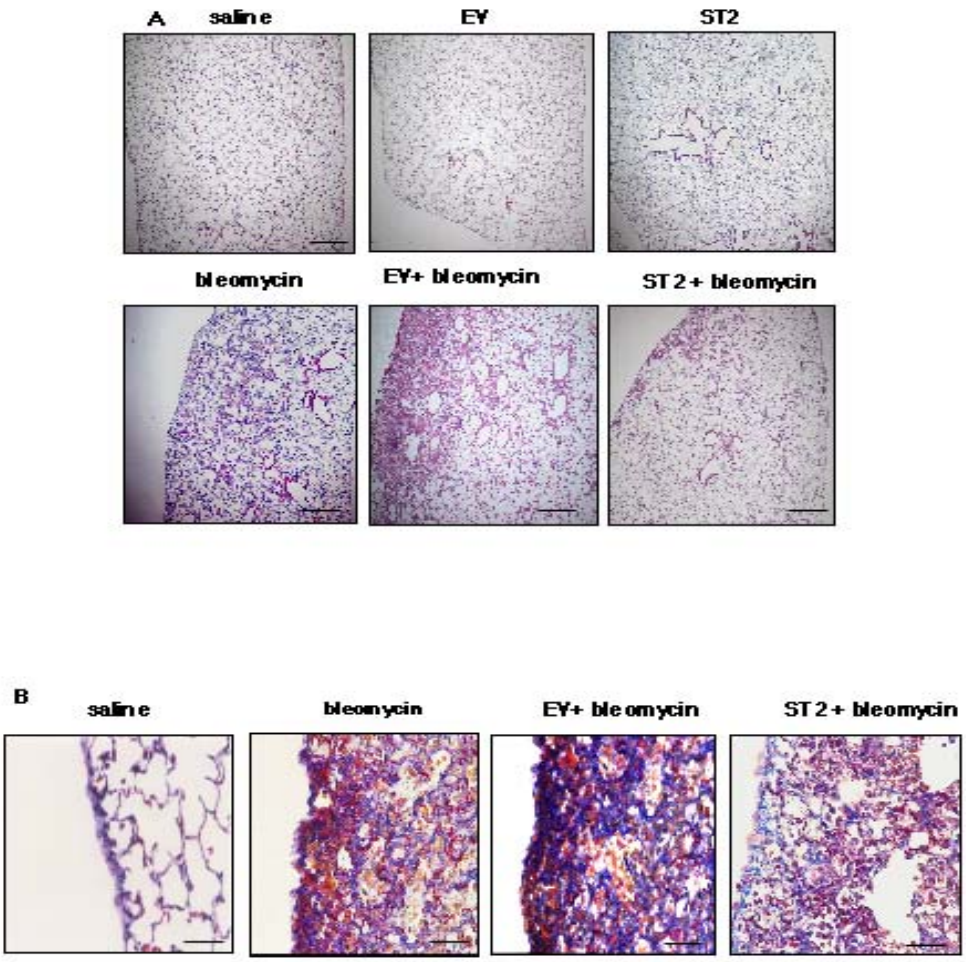
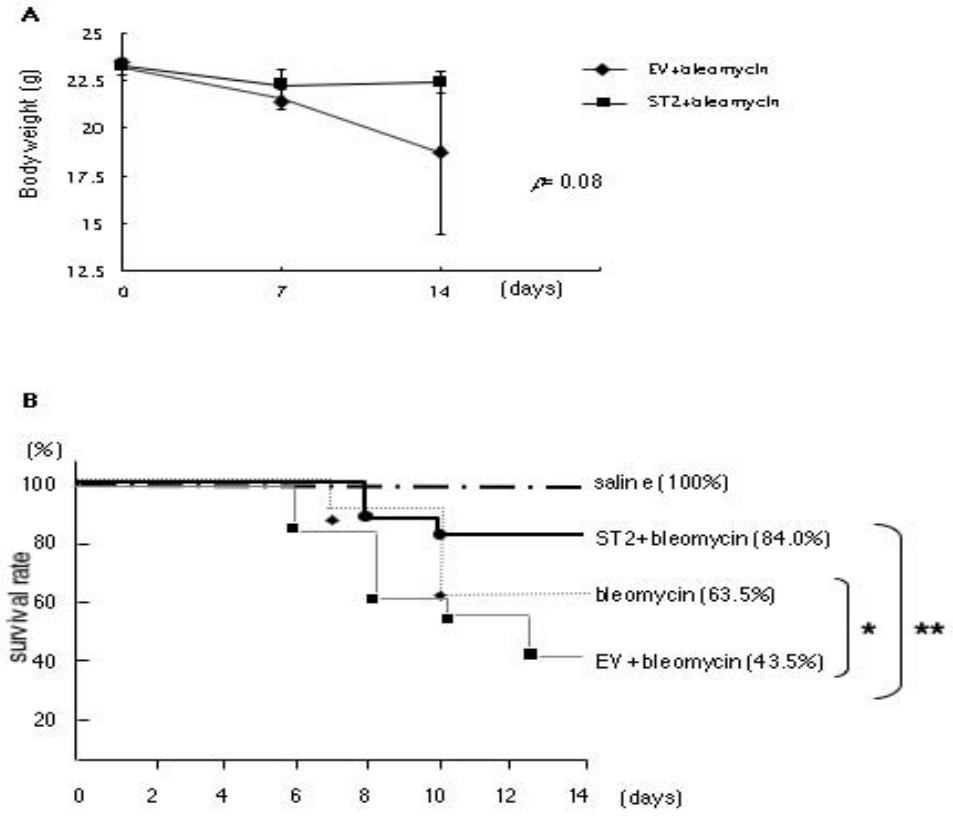


Figure 9. Body weight-loss and survival rate of mice with bleomycin-induced lung injury

(A) Comparison of body weight loss in mice treated wit EV plus bleomycin and ST2 plus bleomycin between day 0-14 (n=8 in both group).

(B) Survival rate of mice were monitored for 14 days and analyzed by Kaplan-Meyer method. We excluded mice that died by injurious tracheotomy or deep anesthesia. saline: n= 7, bleomycin alone: n=14, EV or ST2 plus bleomycin: n=23. *$P < 0.05$ bleomycin alone versus ST2 plus bleomycin, **$P < 0.01$ EV plus bleomycin versus ST2 plus bleomycin.

Figure 9



(supplement data)

Serum ST2 level after the injection of LPS at 7.5mg/kg intravenously. This model mimicked the human septic shock. The concentration of serum ST2 was examined using the mouse ST2 ELISA system. Serum ST2 were markedly elevated 12-24h after LPS injection and rapidly declined at 48 h. *P<0.05, compared to other time points.

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References

1. Bedrossian CW. Iatrogenic and toxic injury. In: Dail DH, Hammar SP, editor. Pulmonary Pathology. Springer-Verlag, New York. 1988. p. 511-517.

2. Ware LB, Matthay MA. The acute respiratory syndrome. *N Engl J Med* 2000; 342: 1334-1349.

3. Tominaga S. A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. *FEBS Lett* 1989; 258: 301-304.

4. Tominaga S, Kuroiwa K, Tago K, Iwahana H, Yanagisawa K, Komatsu N. Presence of expression of novel variant form of ST2 gene product in human leukemic cell line UT-7/GM. *Biochem Biophys Res Commun* 1999; 264: 14-18.

5. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF, Kastelein RA. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005; 23: 479-90.

6. Brunner M, Krenn C, Roth G, Moser B, Dworschak M, Jensen-Jarolim E, Spittler A, Sautner T, Bonaros N, Wolner E, Boltz-Nitulescu G, Ankersmit HJ. Increased levels of

soluble ST2 protein and IgG1 production in patients with sepsis and trauma. *Intensive Care Med* 2004; 30: 1468-1473.

7. Oshikawa K, Yanagisawa K, Tominaga S, Sugiyama Y. ST2 protein induced by inflammatory stimuli can modulate acute lung inflammation. *Biochem Biophys Res Commun* 2002; 299: 18-24.

8. Tajima S, Oshikawa K, Tominaga S, Sugiyama Y. The increase in serum soluble ST2 protein upon acute exacerbation of idiopathic pulmonary fibrosis. *Chest* 2003; 124: 1206-1214.

9. Sweet MJ, Leung BP, Kang D, Sogaard M, Schulz K, Trajkovic V, Campbell CC, Xu D, Liew FY. A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. *J Immunol* 2001; 166: 6633-6639.

10. Takezako N, Hayakawa M, Hayakawa H, Aoki S, Yanagisawa K, Endo H, Tominaga S. ST2 suppresses IL-6 production via the inhibition of IkappaB degradation induced by the LPS signal in THP-1 cells. *Biochem Biophys Res Commun* 2006; 341: 425-432.

11. Leung BP, Xu D, Culshaw S, McInnes IB, Liew FY. A novel therapy of murine collagen-induced arthritis with soluble T1/ST2. *J Immunol* 2004; 173: 145-150.

12. Takagi T, Yanagisawa K, Tsukamoto T, Tetsuka T, Nagata S, Tominaga S. Identification of the product of the murine ST2 gene. *Biochim Biophys Acta* 1993; 1178: 194-200.

13. Inoue S, Hakamata Y, Kaneko M, Kobayashi E. Gene therapy for organ grafts using rapid injection of naked DNA: application to the rat liver. *Transplantation* 2004; 77: 997-1003.

14. Hakamata Y, Murakami T, Kobayashi E. "Firefly rats" as an organ/cellular source for long-term in vivo bioluminescent imaging. *Transplantation* 2006; 81: 1179-1184.

15. Howell DC, Johns RH, Lasky JA, Shan B, Scotton CJ, Laurent GJ, Chambers RC. Absence of proteinase-activated receptor-1 signaling affords protection from bleomycin-induced lung inflammation and fibrosis. *Am J Pathol* 2005; 166: 1353-65.

16. Kelley J, Newman RA, Evans JN. Bleomycin-induced pulmonary fibrosis in the rat. Prevention with an inhibitor of collagen synthesis. *J Lab Clin Med* 1980; 96: 954-964.

17. Lindenschmidt RC, Tryka AF, Godfrey GA, Frome EL, Witschi H. Intratracheal versus intravenous administration of bleomycin in mice: acute effects. *Toxicol Appl Pharmacol* 1986; 85: 69-77.

18. Tajima S, Bando M, Ohno S, Sugiyama Y, Oshikawa K, Tominaga S, Itoh K, Takada T, Suzuki E, Gejyo F. ST2 gene induced by type 2 helper T cell (Th2) and proinflammatory cytokine stimuli may modulate lung injury and fibrosis. *Exp Lung Res* 2007; 33: 81-97.

19. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther* 1999; 10: 1735-1737.

20. Piguet PF, Collart MA, Grau GE, Kapanci Y, Vassalli P. Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. *J Exp Med* 1989; 170: 655-663.

21. Jordana M, Richards C, Irving LB, Gauldie J. Spontaneous in vitro release of alveolar-macrophage cytokines after the intratracheal instillation of bleomycin in rats. Characterization and kinetic studies. *Am Rev Respir Dis* 1988; 137: 1135-1140.

22. Kuroiwa K, Arai T, Okazaki H, Minota S, Tominaga S. Identification of human ST2 protein in the sera of patients with autoimmune diseases. *Biochem Biophys Res Commun* 2001; 284:1104-1108

23. Trajkovic V, Sweet MT, Xu D. T1/ST2-an IL-1 receptor-like modulator of immune responses. *Cytokine and Grouwth Factor Review* 2004; 15: 87-95.

24. Amatucci A, Novobrantseva T, Gilbride K, Brickelmaier M, Hochman P, Ibraghimov A. Recombinant ST2 boosts hepatic Th2 response in vivo. *J Leukoc Biol.* 2007; 82: 124-132.

25. Kurowska-Stolarska M, Kewin P, Murphy G, Russo RC, Stolarski B, Garcia CC, Komai-Koma M, Pitman N, Li Y, McKenzie AN, Teixeira MM, Liew FY, Xu D. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J Immunol.* 2008; 181: 4780-4790.

26. Dunsmore SE, Roes J, Chua FJ, Segal AW, Mutsaers SE, Laurent GJ. Evidence that neutrophil elastase-deficient mice are resistant to bleomycin-induced fibrosis. *Chest*. 2001; 120: 35S-36S.

27. Gasse P, Mary C, Guenon I, Noulin N, Charron S, Schnyder-Candrian S, Schnyder B,1 Akira S, Quesniaux V, Lagente V, Ryffel B, Isabelle Couillin I. IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice. *J Clin Invest.* 2007; 117: 3786-3799.

28. Townsend MJ, Fallon PG, Matthews DJ, Jolin HE, McKenzie AN. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J Exp Med.* 2000; 191: 1069-1076.