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<th>Title</th>
<th>High serum levels of thrombospondin-1 in patients with idiopathic interstitial pneumonia.</th>
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<td>Author(s)</td>
<td>Ide, Mioko; Ishii, Hiroshi; Mukae, Hiroshi; Iwata, Atsuko; Sakamoto, Noriho; Kadota, Jun-ichi; Kohno, Shigeru</td>
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HIGH SERUM LEVELS OF THROMBOSPONDIN-1 IN PATIENTS WITH
IDIOPATHIC INTERSTITIAL PNEUMONIA

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Summary

Thrombospondin-1 (TSP-1), a multifunctional matricellular glycoprotein, can activate transforming growth factor-β, an important profibrotic cytokine involved in various fibrotic diseases. TSP-1 is expressed in the lung tissue of animal models of bleomycin-induced pulmonary fibrosis and in patients with some interstitial lung diseases. The present study investigated the serum and bronchoalveolar lavage fluid (BALF) levels of TSP-1 in the idiopathic interstitial pneumonias (IIPs) and the relationship between these levels and other clinical factors. The TSP-1 in the serum and BALF was measured in 45 patients with pathologically diagnosed IIPs [22 with usual interstitial pneumonia (UIP), 23 patients with nonspecific interstitial pneumonia (NSIP)], as well as in 28 patients with pulmonary sarcoidosis and 15 healthy volunteers using a competitive enzyme immunoassay. The expression and localization of TSP-1 in the lungs was analyzed by immunohistochemical staining. The serum TSP-1 levels were significantly higher in patients with IIPs than in either those with sarcoidosis or the controls. These levels correlated well with those of an angiogenic cytokine vascular endothelial growth factor while correlating inversely with the %VC. Positive immunostaining of TSP-1 was predominantly observed in the regenerated alveolar epithelium and alveolar macrophages in the lung. Our findings suggest that the circulating TSP-1 levels are associated with the presence of interstitial pneumonia, but further studies are required before we can definitively conclude that TSP-1 plays a role in the pathogenesis of these diseases.
Running title: Thrombospondin-1 in interstitial pneumonia

Key words: thrombospondin-1, idiopathic interstitial pneumonias, transforming growth factor-β
Introduction

Emerging evidence supports a major role for a thrombospondin-1 (TSP-1) and transforming growth factor-β (TGF-β) axis in fibrotic disease. TGF-β is a potent and tightly regulated cytokine known to affect growth, differentiation, and gene expression. An excessive amount of active TGF-β causes enhanced tumour progression, as well as progressive fibrosis in multiple organ systems and suppression of the immune system. Studies of animal models of pulmonary fibrosis and lung sections from patients with idiopathic interstitial pneumonias (IIPs) suggest an important role for TGF-β1 in pulmonary fibrosis. This profibrotic cytokine is secreted by most cell types as a latent, biologically inactive complex that has to be activated extracellularly for receptor binding. Although high levels of immunoreactivity for TGF-β1 were observed in patients with idiopathic pulmonary fibrosis (IPF), especially in alveolar macrophages, the autocrine or paracrine effects of TGF-β1 can therefore only manifest themselves upon the activation of secreted TGF-β1.

TSP-1 is a multifunctional matricellular glycoprotein expressed by a variety of cell types including platelets, macrophages, fibroblasts, vascular smooth muscle cells, and endothelial cells. It is often found at the sites of inflammation and wound healing. In addition, TSP-1 can inhibit and stimulate angiogenesis and tumour progression, as well as affect wound healing, platelet aggregation, and arterial remodelling through its various domains and binding sites. TSP-1 expression in vitro is regulated by various cytokines such as platelet-derived growth factor, fibroblast growth factor-2, and TGF-β. TSP-1 has also
been identified as a major activator of latent TGF-β \textit{in vitro} and during mouse postnatal development \textit{in vivo}.\textsuperscript{13,14} The activation of alveolar macrophage-derived latent TGF-β \textit{in vitro} requires a complex interaction of plasmin with TSP-1 and its receptor CD36.\textsuperscript{5} This is likely to be important \textit{in vivo} for bleomycin-induced pulmonary fibrosis, where a synthetic CD36 peptide abrogated TGF-β activation and tissue fibrosis.\textsuperscript{5,6} Kuhn \textit{et al.}\textsuperscript{15} demonstrated TSP-1 in the extracellular matrix immediately beneath the reactive epithelium in both organizing pneumonia and IPF, thus suggesting that it might be synthesized by the regenerating epithelium. In addition, Idell \textit{et al.}\textsuperscript{16} reported that the bronchoalveolar lavage fluid (BALF) levels of TSP in patients with adult respiratory distress syndrome correlated with the composite injury scores that were used to quantitate the degree of lung injury. Collectively, these findings suggest that TSP-1 thus plays a role in the development of pulmonary alveolitis leading to fibrosis.

In this study, we measured the serum and BALF levels of TSP-1 and examined the localization of TSP-1 in patients with IPF and nonspecific interstitial pneumonia (NSIP), the two largest subsets in IIPs. The role of TSP-1 in the pathogenesis of these diseases is discussed in the context of our findings.
Materials and methods

Study population

The study protocol was approved by the Human Ethics Review Committees of Nagasaki University School of Medicine and Oita University Faculty of Medicine. Seventy-three patients and healthy volunteers were enrolled in this study. A signed consent form was obtained from each subject. The subjects included 22 patients with IPF who were pathologically diagnosed to have usual interstitial pneumonia (UIP), 23 with idiopathic NSIP, 28 with pulmonary sarcoidosis as a control disease and 15 healthy volunteers (Table 1). In all patients with IIPs, the diagnosis was pathologically confirmed using surgical lung biopsy specimens obtained from at least two different sites. The NSIP patients included 18 fibrosing NSIP cases and 5 cellular and fibrosing NSIP cases. None of the enrolled patients had received either steroids or immunosuppressive therapy at the time of clinical sample collection. Any patients with cancer in any organ or those suspected of having a malignancy were excluded from the study. Any patients with UIP and NSIP associated with collagen vascular diseases were also excluded. All healthy volunteers had normal chest radiographs, were free of symptoms, and were not taking any medications.

BAL procedure and blood sampling

After obtaining informed consent, BAL was performed as described previously using a flexible fiberoptic bronchoscope (Olympus P-20, Olympus, Tokyo, Japan). The BALF was
passed through BD Falcon™ Cell Strainers and then centrifuged at 500 × g for 10 min at 4°C. The remaining fluid was centrifuged at 500 × g for 5 min, and the supernatant was stored at –80°C for further quantification of non-cellular components including TSP-1. Peripheral venous blood samples were obtained from all subjects on the day of BAL sampling and the serum was stored at –80°C until analysis.

*Measurements of TSP-1, KL-6, SP-A, SP-D, and VEGF*

We measured the levels of each marker using specific kits according to the protocols provided by the manufacturers. The TSP-1 levels in serum and BALF were measured by a competitive enzyme immunoassay (EIA) using a commercially available kit (CHEMICON International, Temecula, CA). The KL-6 levels were measured using a sandwich-type electrochemiluminescence immunoassay kit (Sanko Junyaku Co., Tokyo). The surfactant protein A (SP-A) and SP-D levels were measured using sandwich-type EIA kits (Sysmex Corp., Hyogo and Yamasa Shoyu Co., Tokyo, respectively). We also measured the serum levels of vascular endothelial growth factor (VEGF) in each subject using an EIA kit (Pierce, Rockford, IL) to test for angiogenesis. All assays were performed in duplicate.

*Immunohistochemistry of lung tissues*

To confirm the localization of TSP-1 in the lungs, an immunohistochemical analysis was performed for TSP-1 (Clone A6.1, Lab Vision Corporation, CA), SP-A (VisionBioSystems
Novocastra™, UK), and CD68 (DakoCytomation, Denmark) using paraffin-embedded sequential lung sections obtained from 5 UIP cases and 5 NSIP cases. The control specimens were obtained from the normal part of the lung removed for lung cancer. Briefly, after deparaffinization and rehydration, the tissue sections (4-μm thick) were soaked in 0.3% H₂O₂ with absolute methanol for 20 min to inactivate endogenous peroxidases. The lung sections were incubated overnight at 4°C with primary antibodies in a moist chamber. After washing in phosphate buffered saline, the sections were incubated for 30 min with EnVision+™ (Peroxidase, Mouse, DakoCytomation, Denmark), and then developed with 3,3’-diaminobenzidine for TSP-1 and AEC+ Substrate-Chromogen® (DakoCytomation, Denmark) for SP-A and CD68. The primary antibody was replaced by an irrelevant immunoglobulin G1 as a negative control. The number of SP-A positive type II pneumocytes and CD68 positive alveolar macrophages that expressed TSP-1 in the active fibrotic areas of UIP and NSIP were counted at ×400 magnification in 5 randomly selected fields in the lung specimen obtained from each individual. The results were expressed as the median (range) of the total number of 5 fields.

**Statistical analysis**

All values were expressed as the median (range). The differences between groups were examined using the ANOVA test with a post-hoc analysis (Bonferroni/Dunn test). The
correlations between two variables were determined using the Spearman's rank correlation analysis. A $p$ value below 0.05 denoted a statistically significant difference.

**Results**

**BALF differential cell count**

The differential cell counts in BALF for the four subject groups are listed in Table 2. In the patients with idiopathic NSIP and pulmonary sarcoidosis, the percentages of alveolar macrophages were significantly lower in comparison to the healthy volunteers ($p<0.01$), while there were more lymphocytes in patients with NSIP and sarcoidosis in comparison to the patients with UIP and the healthy volunteers ($p=0.005$, respectively). The percentages of neutrophils and eosinophils were significantly higher in the patients with UIP, while the CD4/CD8 ratio was significantly higher in the patients with sarcoidosis, in comparison to the healthy volunteers ($p<0.01$, respectively).

**Serum and BALF levels of TSP-1**

The serum levels of TSP-1 in the patients with UIP and NSIP were significantly higher than those in the patients with sarcoidosis (Fig. 1A, $p<0.0001$, each) and the healthy volunteers ($p<0.0001$, each), but the levels were not significantly different between the UIP and NSIP patients. The BALF levels of TSP-1 in the patients with UIP and NSIP were significantly lower than in those with sarcoidosis (Fig. 1B, $p<0.0001$, each) or the healthy volunteers.
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(p<0.005, each). The TSP-1 levels in BALF were slightly lower in the patients with UIP than in those with NSIP (p<0.05; Fig. 1B).

**Correlations between TSP-1 levels and clinical data**

We examined the correlation between the serum levels of TSP-1 and the various clinical parameters of the patients with IIPs (UIP plus NSIP). The serum TSP-1 levels correlated inversely with the %VC (r=−0.37, p<0.05), but there was no correlation between the serum TSP-1 level and the other clinical parameters, such as the other pulmonary function indicators, PaO₂, BALF cell proportions, the well-known serum markers for interstitial pneumonia (Table 2; KL-6, SP-A and SP-D), and patient survival. There was a strong correlation, however, between the serum levels of TSP-1 and VEGF in the patients with IIPs (r=0.66, p=0.0004), which were significantly higher than those in the control subjects (Table 2).

**Localization of TSP-1**

Immunohistochemical staining for TSP-1 demonstrated high levels of immunoreactivity predominantly in the SP-A positive type II pneumocytes (Fig. 2a-c), CD68 positive alveolar macrophages (Fig. 2e-g), and weakly in the infiltrating mononuclear cells. There was almost no detectable TSP-1 signal in the bronchial epithelial cells and fibroblasts. A stronger TSP-1 signal was detected in the active fibrotic areas in the lungs of the patients with both NSIP and UIP (Fig. 2a, e). The number of TSP-1/SP-A double positive cells were 312.0 (179—621) in
the UIP patients and 272.5 (97–470) in the NSIP patients (not significantly different). The number of TSP-1/CD68 double positive cells were 61.0 (35–118) in the UIP patients and 68.5 (43–216) in the NSIP patients (not significantly different). Minimal staining was seen in the airway epithelium in the control lung tissue (Fig. 2i).

**Discussion**

The role of TSP-1 in pulmonary inflammation/fibrosis and connective tissue synthesis has been previously demonstrated in an experimental rat model. The activated alveolar macrophages obtained from rat lungs after bleomycin administration released increased amounts of active TGF-β1 as well as plasmin and TSP-1. Previous reports indicate that the activation of latent TGF-β1 by plasmin may occur at the cell surface of the activated alveolar macrophages and requires a TSP-1/CD36 (a receptor for TSP-1) interaction. In turn, a decreased availability of the cell-derived active TGF-β1 or a disruption of the TSP-1/CD36 interaction might lead to reduced inflammation and fibrosis after bleomycin-induced lung injury. Azuma et al. also showed a limited amount of TSP-1/2 in the platelets of control mice, but it was observed in the foamy cells of the fibrotic lesions induced by bleomycin. These observations implicated TSP-1 in the pathogenesis of lung injury and fibrosis. However, the relationship between TSP-1 and the interstitial pneumonias in humans has not yet been fully evaluated.
The major finding of the present study is that the patients with IIPs had elevated levels of TSP-1 in the serum and low levels in the BALF in comparison to the sarcoidosis patients and the healthy control patients. In addition, in the lung sections with IIPs, TSP-1 was prominently expressed in the regenerated alveolar epithelium and alveolar macrophages in active fibrotic lesions. This suggests that the local production of TSP-1 by the alveolar epithelium or alveolar macrophages is accelerated and that this elevated production may reflect the circulating TSP-1 levels. This is the first report of elevated TSP-1 levels in the serum of patients with IIPs. However, this study has several limitations. The study population was heterogeneous, and the participants were at different stages of their disease. These methodological compromises were necessary to compile a study cohort of sufficient size. In addition, the patients and controls were not well matched for age and this reflects our difficulty in recruiting non-diseased elderly controls. In addition, we did not clearly elucidate whether age and smoking affect either the serum or BALF concentrations of TSP-1. Furthermore, careful interpretation should be applied to such findings regarding TSP-1, because the serum levels of TSP-1 correlate with the cancer stage in certain cancer types including non-small-cell lung cancer, while also increasing in patients with other diseases such as systemic sclerosis and dermatomyositis. In this study, the elevated levels of TSP-1 in the patients with IIPs correlated significantly with those of an angiogenic cytokine VEGF. TSP-1 is also known to affect angiogenesis. Simler et al. showed that the circulating levels of other angiogenic cytokines, such as VEGF, endothelin-1 (ET-1), and
interleukin-8 were high and were significantly related to the HRCT fibrosis score in the patients with IIPs. In our study, although the plasma ET-1 levels [3.48 (2.78–4.46) pg/ml in the UIP patients, 3.32 (2.68–4.54) pg/ml in the NSIP patients] did not correlate with the serum or BALF levels of TSP-1 in the patients with IIPs (data not shown), our data suggest that TSP-1 might thus play a similar role to VEGF-related angiogenesis in interstitial pneumonia.

Similar to previous findings for the BALF levels of SP-A in IIP patients,18,27 the present study showed lower levels of TSP-1 in the BALF, even in the presence of high serum levels in the patients with IIPs. This could reflect reduced access to the alveolar compartment, reduced production of TSP-1 by the damaged alveolar epithelium, or an increased uptake and degradation by alveolar macrophages and airway epithelial cells. In addition, Yehualaeshet et al. 5 reported an increased TSP-1 secretion from explanted rat alveolar macrophages that reached maximal levels 7 days after bleomycin administration, declining rapidly thereafter to the control levels of alveolar macrophages from normal rats. This suggests the possibility of relatively low levels of TSP-1 in the lung alveoli of patients in the chronic phase of IIPs. However, the exact mechanism underlying this clearance of TSP-1 remains unclear, and we could not clarify this point in this study.

Pulmonary fibrosis remains a devastating clinical disorder for which there are limited therapeutic options. The pathogenesis of IPF also remains largely unknown, but observations based on animal models of pulmonary fibrosis 2–6 and lung tissue specimens from patients
with IPF \(^7,8\) suggest that TGF-\(\beta\) plays a key role in pulmonary inflammation/fibrosis. TSP-1 was identified as a major activator of TGF-\(\beta\) in pulmonary fibrosis models \textit{in vivo}.\(^5\) The contribution of other TGF-\(\beta\) activators cannot be excluded, however, the specific and localized inhibition of TGF-\(\beta\) activation by TSP-1 blocking is thus considered to provide a potential anti-TGF-\(\beta\) strategy for the treatment of inflammatory/fibrotic diseases.\(^{28}\) In particular, targeting TSP-1-mediated activation of TGF-\(\beta\) as a therapeutic intervention for fibrotic lung diseases could be a promising alternative because the TGF-\(\beta\) protein expression and alternate activation pathways of TGF-\(\beta\) are not affected. In addition, the therapeutic long-term strategies focusing on the non-specific and systemic blockade of TGF-\(\beta\) ligand receptor interactions is considered problematic due to the complex functions of TGF-\(\beta\).

In summary, we herein demonstrated the presence of high serum levels of TSP-1 in patients with IIPs in comparison to the control patients, and the expression of TSP-1 in the alveolar epithelium and macrophages in active fibrotic lesions. The serum levels of TSP-1 did not closely correlate with the clinical biomarkers or patient survival apart from the %VC and the serum levels of VEGF. Our findings suggest an association between the serum TSP-1 levels and the presence of interstitial fibrosis, however, further studies are required to prove a link between an increased production of TSP-1 and the pathogenesis of the pulmonary fibrotic responses seen in IIPs, and to elucidate the potential of TSP-1 as a molecular target for therapeutic intervention in the treatment of fibrotic lung diseases.
Conflict of Interest

None of the authors have a conflict of interest to declare in relation to this work.

Acknowledgments

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References


Figure Legends

Figure 1

Thrombospondin-1 (TSP-1) levels in serum (A) and bronchoalveolar lavage fluid (BALF) (B) of patients with idiopathic usual interstitial pneumonia (UIP), idiopathic nonspecific interstitial pneumonia (NSIP), pulmonary sarcoidosis, and healthy volunteers. The P values for the overall comparison of all four subject groups are given.

Figure 2

Photomicrographs of immunohistochemical stain of TSP-1, surfactant protein A (SP-A), and CD68 in sequential lung sections of NSIP (a-d), UIP (e-h), and normal lung (i). Note the positive TSP-1 staining (brown; a, b, e, f) in the SP-A positive type II pneumocytes (red; c) and CD68 positive alveolar macrophages (red; g). No staining with isotype control (d, h). The TSP-1 signal in normal lung was quite low (i). Magnification; ×40 (a, e, i), ×200 (b, c, d), ×100 (f, g, h).
**Table 1** Patient characteristics

<table>
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<tr>
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<th>UIP</th>
<th>NSIP</th>
<th>Sarcoidosis</th>
<th>Healthy volunteers</th>
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<tr>
<td><strong>number</strong></td>
<td>22</td>
<td>23</td>
<td>28</td>
<td>15</td>
</tr>
<tr>
<td><strong>sex (male/female)</strong></td>
<td>16/6</td>
<td>8/15</td>
<td>14/14</td>
<td>12/3</td>
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<tr>
<td><strong>age detection (years)</strong></td>
<td>64 (34-77)</td>
<td>57 (28-77)</td>
<td>55 (26-75)</td>
<td>23 (19-33)</td>
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<tr>
<td><strong>smoking (never/ex./current)</strong></td>
<td>8/10/4</td>
<td>16/5/2</td>
<td>16/8/4</td>
<td>14/0/1</td>
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<td><strong>%VC (%)</strong></td>
<td>80.0 (43.3-117.0)</td>
<td>78.1 (33.0-135.3)</td>
<td>90.6 (71.5-121.6)</td>
<td>N.D.</td>
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<td><strong>%DLCO (%)</strong></td>
<td>51.1 (14.2-118.5)*</td>
<td>62.8 (34.6-109.3)</td>
<td>80.7 (47.8-105.5)</td>
<td>N.D.</td>
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<tr>
<td><strong>PaO₂ (Torr)</strong></td>
<td>85.1 (58.3-103.3)</td>
<td>83.0 (61.4-95.2)</td>
<td>88.2 (64.8-95.2)</td>
<td>N.D.</td>
</tr>
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Data are the median (range). N.D.; not done.
* Compared with sarcoidosis (p<0.01).
Table 2  BAL fluid findings and serum biomarkers

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<tr>
<th></th>
<th>UIP</th>
<th>NSIP</th>
<th>Sarcoïdosis</th>
<th>Healthy volunteers</th>
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<td>Cell differentials (%)</td>
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<td>macrophages</td>
<td>81.9 (45.8-94.0)</td>
<td>49.5 (21.7-89.0)*</td>
<td>57.3 (7.6-86.0)*</td>
<td>88.1 (69.0-93.7)</td>
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<td>lymphocytes</td>
<td>10.0 (2.0-33.2)</td>
<td>41.6 (7.2-69.9)*†</td>
<td>39.4 (9.0-81.5)*†</td>
<td>8.5 (3.4-19.9)</td>
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<td>neutrophils</td>
<td>3.6 (0-21.6)*</td>
<td>1.9 (0-27.0)</td>
<td>0.6 (0-12.0)</td>
<td>0.5 (0-14.6)</td>
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<tr>
<td>eosinophils</td>
<td>2.3 (0-18.3)*</td>
<td>2.0 (0-15.0)</td>
<td>0.5 (0-5.9)†</td>
<td>0 (0-7.0)</td>
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<td>CD4/CD8 ratio</td>
<td>1.4 (0.3-4.7)</td>
<td>0.5 (0.1-2.3)</td>
<td>4.2 (1.1-16.7)*</td>
<td>0.7 (0.3-1.8)</td>
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<td>serum KL-6 (U/ml)</td>
<td>677 (444-2,530)</td>
<td>1,100 (192-3,570)</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>serum SP-A (ng/ml)</td>
<td>85.6 (25.0-118.0)</td>
<td>58.3 (20.3-253.0)</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>serum SP-D (ng/ml)</td>
<td>194.0 (106.0-531.0)</td>
<td>273.0 (17.2-721.0)</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>serum VEGF (pg/ml)</td>
<td>119.1 (23.1-592.6)*§</td>
<td>254.5 (30.8-636.4)*§</td>
<td>63.1 (44.1-630.5)*</td>
<td>11.0 (0-134.2)</td>
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Data are the median (range). N.D.; not done.
* Compared with healthy volunteers (p<0.01),
† Compared with UIP (p<0.01),
§ Compared with sarcoidosis (p<0.05).
Figure 1

A

serum TSP-1 (μg/ml)

UIP
NSIP
Sarcoidosis
Healthy volunteers

B

BALF TSP-1 (μg/ml)

UIP
NSIP
Sarcoidosis
Healthy volunteers

p<0.0001
Figure 2