Inhibiting S100A8/A9 attenuates airway obstruction in a mouse model of heterotopic tracheal transplantation

Dai Shimizu[a], Mikio Okazaki[a], Seiichiro Sugimoto[a], Rie Kinoshita[b], Kentaro Nakata[a, c], Shin Tanaka[a], Kohei Hashimoto[a], Kentaroh Miyoshi[a], Masaomi Yamane[a, d], Akihiro Matsukawa[e], Masakiyo Sakaguchi[b], Shinichi Toyooka[a]

[a]Department of General Thoracic Surgery and Breast and Endocrinological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

[b]Department of Cell Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

[c]Division of Cardiovascular and Thoracic Surgery, Department of Surgery, Duke University Medical Center, Durham, USA

[d]Department of Surgery, Division of Thoracic Surgery, Shimane University Faculty of medicine

[e]Department of Pathology and Experimental Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

2-5-1 Shikata-cho Kitaku, Okayama 700-8558, Japan

Coauthor email addresses:

Dai Shimizu: me19036@s.okayama-u.ac.jp; Seiichiro Sugimoto: sugimo-s@cc.okayama-u.ac.jp; Rie Kinoshita: rie-k@okayama-u.ac.jp; Kentaroh Nakata: kentaronakata555@gmail.com; Shin Tanaka: p2764qg0@okayama-u.ac.jp; Kohei Hashimoto: pxpe9n72@s.okayama-u.ac.jp; Kentaroh Miyoshi: kmiyoshi@okayama-u.ac.jp; Masaomi Yamane: myamane@pc5.so-net.ne.jp; Akihiro Matsukawa: amatsu@md.okayama-u.ac.jp; Masakiyo Sakaguchi: masa-s@md.okayama-u.ac.jp; Shinichi Toyooka: toyooka@md.okayama-u.ac.jp

Corresponding author: Mikio Okazaki, MD, PhD, 2-5-1 Shikata-cho Kitaku, Okayama 700-8558, Japan, Tel: +81-86-235-7265, Fax: +81-86-235-7269, E-mail: mikiookazaki@okayama-u.ac.jp

Abbreviations

BOS, bronchiolitis obliterans syndrome; Ccl2, C-C motif chemokine 2; CLAD, chronic lung allograft dysfunction; Col1a1, type-I collagen alpha 1 chain; Col3a1, type-III collagen alpha 1 chain; DAMP, damage-

associated molecular pattern; H&E, hematoxylin and eosin; HMGB1, high-mobility group box 1; Il-1β, interleukin-1β; Il-6, interleukin-6; MT, Masson's trichrome; NF-κB, nuclear factor-κB; OB, obliterative bronchiolitis; qPCR, quantitative reverse transcriptase polymerase chain reaction; RAGE, receptor for advanced glycation end products; S100, S100 calcium binding protein; SEM, standard error of the mean; SMA, smooth muscle actin; Tgf-β1, tumor growth factor-β1; Tnf-α, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

Abstract

Although bronchiolitis obliterans syndrome (BOS) is a major cause of death after lung transplantation, an effective drug therapy for BOS has not yet developed. Here, we assessed the effectiveness of a neutralizing anti-S100 calcium binding protein (S100) A8/A9 antibody against BOS. A murine model of heterotopic tracheal transplantation was used. Mice were intraperitoneally administered control IgG or the S100A8/A9 antibody on day 0 and twice per week until they were sacrificed. Tissue sections were used to evaluate the obstruction ratio, epithelium-preservation ratio, α-smooth muscle actin (SMA)-positive myofibroblast infiltration, and luminal cell death. Quantitative reverse transcriptase-polymerase chain reaction analysis was performed to analyze the mRNA-expression levels of collagen, inflammatory cytokines, and chemokines on days 7, 14, and 21. The anti-S100A8/A9 antibody significantly improved the obstruction ratio and epithelium-preservation ratio, with less α-SMA-positive myofibroblast infiltration compared to the control group. Antibody treatment reduced the type-III collagen: type-I collagen gene-expression ratio. The antibody also significantly suppressed the number of dead cells in the graft lumen. The expression levels of tumor growth factor β1 and C-C motif chemokine 2 on day 21, but not those of interleukin-1β, interleukin-6, and tumor necrosis factor α, were significantly suppressed by S100A8/A9 antibody treatment. These findings suggest that S100A8/A9 may be a potential therapeutic target for BOS after lung transplantation.

Keywords: Lung transplantation, Chronic lung allograft dysfunction, Bronchiolitis obliterans syndrome, S100A8, S100A9, Fibroblast

1. Introduction

Lung transplantation is the only therapeutic option available for end-stage respiratory diseases. Although survival outcomes have improved in recent years, bronchiolitis obliterative syndrome (BOS), a form of chronic lung allograft dysfunction (CLAD), has been the leading cause of post-transplant mortality for over three decades [1,2]. Pathologically, BOS is characterized by obliterative bronchiolitis (OB) in small airways and is associated with inflammation and tissue fibrosis. OB may result from both innate and adaptive immunity. Recent data have indicated that both CD4+ and CD8+ T cells and neutrophils play key roles in BOS development [3]. Profibrotic cytokines and chemokines released from damaged airway epithelial cells or infiltrating inflammatory cells promote fibroblast activation and extracellular matrix production, resulting in airway remodeling and eventual obstruction [3,4]. Although some aspects of the underlying mechanism of BOS have been discovered, no therapy is available to cure BOS.

S100 calcium binding protein (S100) A8/A9 (also known as calprotectin) is a heterodimer complex of S100A8 and S100A9. It acts as damage-associated molecular patterns (DAMPs), which mediate the activation of the inflammatory response. S100A8/A9 is mainly released from neutrophils, monocytes, and damaged cells [5]. In addition to their pro-inflammatory roles, S100 proteins can elicit profibrotic effects [6–8]. S100A8/A9 is closely associated with a wide range of inflammatory and fibrotic conditions of the lungs [5,8]. We have investigated the roles of S100A8/A9 in cancer metastasis with a newly developed S100A8/A9 neutralizing antibody (clone #45) that suppressed metastasis and lung inflammation [9]. We recently demonstrated that clone #45 inhibited bleomycin-induced inflammation and fibrosis in a mouse model [8].

DAMPs are closely associated with CLAD. DAMP-expression levels are higher in bronchoalveolar lavage samples from patients with CLAD (including those with BOS) than in recipients without CLAD [10]. Furthermore, in a murine model of heterotopic tracheal transplantation, high-mobility group box 1 (HMGB1), a DAMP, exacerbated OB via the receptor for advanced glycation end products (RAGE), which is a common receptor for S100A8/A9 [11].

The heterotopic tracheal transplantation model was the first established model of OB [12]. In this model, OB-like lesions with luminal obstruction can readily and reproducibly develop in the dorsally engrafted trachea [3]. Because of its high reproducibility, this model is widely used to analyze the therapeutic effects of new drugs, which has contributed expedited BOS research.

Here, we investigated the therapeutic potential of S100A8/A9 neutralizing antibodies for BOS after lung

transplantation. We tested the hypothesis that the S100A8/A9 neutralizing antibody clone #45 can attenuate both inflammation and luminal fibrosis in a murine heterotopic tracheal transplantation model.

2. Materials and methods

2.1. Animals

Pathogen-free male C57BL/6 J mice (CLEA Japan, Tokyo, Japan), weighing 25–30 g, were used as recipients. Pathogen-free male BALB/c mice (CLEA Japan, Tokyo, Japan) weighing 25–30 g were used as donors. For isogenic transplantations, C57BL/6 J mice were used as both the donors and recipients. All mice were housed in isolator cages under hygienic conditions with free access to food and water at a constant temperature of 23°C with a 12-h light-dark cycle. All mice were cared for in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996). The study protocol was approved by the relevant ethics committee and institutional review board (approval number OKU-2020842).

2.2 S100A8/A9 antibody treatment

A mouse monoclonal anti-S100A8/A9 neutralizing antibody (clone #45) was produced as described previously [9]. Mouse IgG (#0107-01, Southern Biotech, AL, USA) was used as a control. The antibody dose (500 μg/dose) was determined in our previous study using a mouse model of bleomycin-induced pulmonary fibrosis [8].

2.3. Subcutaneous heterotopic tracheal transplantation

Heterotopic tracheal transplantation was performed as described [13,14]. Briefly, the donor mice were euthanized in 100% CO_2. Each trachea was separated from the esophagus by blunt dissection and excised from the cricoid cartilage to the bifurcation point via a midline incision. The tracheal grafts were rinsed and placed in 0.9% sodium chloride solution until transplantation. The recipient mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) in 0.9% sodium chloride. Each tracheal graft was engrafted in a 1.5 cm × 1.5 cm subcutaneous pouch placed via a 5 mm dorsal skin incision and blunt

subcutaneous dissection. The graft was placed away from the skin incision and the skin was closed with a 6-0 proline suture. Thirty minutes elapsed between harvesting and transplantation. An anti-S100A8/A9 antibody (500 μg/dose) or a control mouse IgG (500 μg/dose) was administered intraperitoneally immediately following skin closure. The day of transplantation was defined as day 0. The S100A8/A9 antibody or control IgG was administered twice per week until the recipient mice were sacrificed.

2.4. Histological examinations of grafts

The grafts were removed from the recipient mice via blunt dissection immediately after CO_2-induced euthanasia. The excised grafts were fixed in 10% buffered formalin and divided in the middle. The graft pieces were embedded in paraffin to obtain a cross section of the middle part of the trachea. Paraffin blocks were sectioned at a 5 μm thickness and stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT). All specimens were examined in a blinded manner. The H&E-stained graft sections were photographed under a BZ-X710 light microscope (Keyence, Osaka, Japan). The intra-cartilage and free-lumen areas were measured by analyzing the digital images of each section from the center of each tracheal graft, using ImageJ software. The luminal-obstruction ratio was calculated as follows: (1 − free lumen/intra-cartilage area) × 100. The epithelium-preservation ratio on day 21 was calculated as follows: (length of the basal membrane covered with epithelial cells/circumferential length of basal membrane) × 100.

2.5. Immunohistochemistry

Formalin-fixed and paraffin-embedded sections were deparaffinized, and antigens were retrieved by heating the slides in antigen unmasking solution (Vector Laboratories: #H-3301, CA, USA). Endogenous peroxidase activity was quenched with 3% H_2O_2. Normal horse serum (2.5%; Vector Laboratories, #S-2012) was used to block the sections. The sections were incubated with anti-α-smooth muscle actin (α-SMA) antibody (1:100, #19245, Cell Signaling Technology, MA, USA) overnight at 4°C. Immunocomplexes were detected using the ImmPACT DAB Substrate Kit (Vector Laboratories: #SK-4105) and counter-stained with hematoxylin. Digital images were obtained using a BZ-X710 light microscope (Keyence) and analyzed in ImageJ software using the color deconvolution plug-in. The α-SMA-positive area was measured with an image of a graft section at 10× magnification.

2.6. RNA isolation and quantitative reverse transcriptase polymerase chain reaction (qPCR) analysis

The entire piece of the tracheal graft excised from each recipient's subcutaneous pocket was immersed in RNAlater (Qiagen, Inc., CA, USA) and stored at -20°C until they were processed for RNA extraction. The day 0 samples excised from donor BALB/c mice were prepared for transplantation and immersed in RNAlater just before engraftment. Total RNA from the tracheal graft was extracted using an RNeasy Mini Kit (Qiagen, Inc.). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. qPCR analysis was performed using PowerSYBR Green PCR Master Mix (Thermo Fisher Scientific). The forward and reverse primer pairs are provided in the Supplementary Methods section. The mRNA-expression levels were determined using an ABI StepOnePlus Real-Time PCR instrument (Thermo Fisher Scientific). The gene encoding the TATA box-binding protein was detected as a reference gene. The data were quantified using the comparative ΔΔ-CT method, and the relative expression levels of target genes were calculated by setting the values of the day 0 samples or isografts to 1. The primer sequences are provided in the Supplementary Methods section.

2.7 Cell-death analysis

Cell death was analyzed by performing terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays with the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) per the manufacturer's instructions. Slides were mounted using ProLong™ Gold Antifade Mountant with DAPI staining (Thermo Fisher Scientific). Both TUNEL- and DAPI-positive nuclei were regarded as dead. The number of dead cells in the lumen was counted in images taken with a BZ-X710 fluorescence microscope (Keyence) at 10× magnification using ImageJ software.

2.8. Statistical analysis

Our data were analyzed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). A two-tailed Student's *t*-test was used to compare two groups, and one-way analysis of variance followed by Bonferroni's post-hoc test was used for multiple comparisons. The data are presented as the mean ± standard error of the mean.

$p < 0.05$ was considered to reflect a statistically significant difference.

3. Results

3.1. Changes in S100A8- and S100A9-expression levels after tracheal transplantation

First, we quantified the expression levels of *S100A8* and *S100A9* in the grafts by qPCR. On day 3, the expression levels of *S100A8* and *S100A9* were both significantly upregulated compared with those on day 0. Their expression levels tended to be elevated on day 14, although the difference compared with those on days 0, 7, and 21 was not significant (Fig. 1).

3.2. The S100A8/A9 neutralizing antibody ameliorated luminal obstruction after heterotopic tracheal transplantation

To evaluate the anti-fibrotic effect of the S100A8/A9 neutralizing antibody on luminal obstruction after allogeneic transplantation, we evaluated the luminal-obstruction ratio on day 21. First, we investigated the optimal frequency for S100A8/A9 antibody administration. We found that administering the antibodies twice per week had a greater antifibrotic effect than using less frequent dosing schedules at day 21 (Fig. S1). Based on this result, a 500 μL/dose of anti-S100A8/A9 antibody or control IgG was administered twice per week in subsequent experiments. No fibroproliferative tissue was observed in the lumen on days 7 or 14. The luminal surface epithelium was thinner on day 14 (versus that on day 7) or was lost. The debris in the lumen of the graft on day 14 comprised detached epithelial cells and infiltrated inflammatory cells (Fig. 2A). Less fibrotic tissue was observed on day 21 in the S100A8/A9 antibody group than that in the control IgG group. On the surface of the lumen, epithelial cells remained in some specimens of the S100A8/A9 antibody-treatment group at day 21; however, epithelial cells were completely lost in all specimens of the control IgG group (Fig. 2A and 2B). The obstruction ratio on day 21 was significantly higher in the IgG group than that in the antibody group (65.4% versus 27.7%, respectively; $p = 0.0007$; Fig. 2C). The proportions of the luminal surface covered with residual epithelial cells in the antibody group were significantly higher than those in the control group (31.2% versus 0%, respectively; $p = 0.0487$; Fig. 2D).

3.3. The S100A8/A9 antibody reduced the α-SMA positive myofibroblasts and type-III collagen alpha 1 chain (Col3a1) gene-expression levels after tracheal transplantation

Previously, we demonstrated that S100A8/A9 neutralizing antibodies downregulated α-SMA (a myofibroblast marker) in activated fibroblasts in vitro [8]. Therefore, we hypothesized that the anti-fibrotic effect of S100A8/A9 neutralizing antibodies on luminal obstruction after tracheal transplantation is related to myofibroblast reduction. To test this hypothesis, we immunostained for α-SMA using grafts harvested on day 21. The α-SMA-positive luminal area was smaller in the S100A8/A9 antibody group than in the IgG group (8,143 μm^2 versus 31,142 μm^2, respectively, $p = 0.0230$; Figs. 3A and 3B). The α-SMA-positive area in the submucosal area was significantly smaller in the antibody group than in the IgG group (22,456 μm^2 versus 53,166 μm^2, respectively, $p = 0.0357$; Fig. 3A and 3C).

qPCR analysis showed that, on day 14, the expression levels of type-I collagen alpha 1 chain (*Col1a1*) and *Col3a1* were not upregulated in both IgG group and the S100A8/A9 antibody group compared with those in the isogenic transplantation group. Antibody administration did not reduce the expressions of either collagen. However, the expression levels of both *Col1a1* and *Col3a1* were significantly upregulated on day 21, and S100A8/A9 antibody treatment significantly reduced *Col3a1* expression but not *Col1a1* expression (Fig. 3D).

3.4. The S100A8/A9 antibody reduced tumor growth factor-β1 (Tgf-β1) and C-C motif chemokine 2 (Ccl2) expression after tracheal transplantation, but did not reduce the expression other BOS-associated inflammatory cytokines and chemokines

S100A8/A9 are closely related to inflammation in various diseases [5], and the S100A8/A9 antibody decreased inflammatory cytokine expression in bleomycin-induced murine lung fibrosis [8]. Therefore, we hypothesized that the anti-fibrotic effect of the anti-S100A8/A9 antibody on luminal obstruction after tracheal transplantation was attributable to its anti-inflammatory effect. We investigated the expression levels of inflammatory cytokines and chemokines associated with acute rejection and BOS [3,15,16] on days 7, 14, and 21 by qPCR. Anti-S100A8/A9 antibody treatment significantly reduced *Tgf-β1* and *Ccl2* expression at day 21, but not at earlier time points. However, contrary to our hypothesis, the expression levels other proinflammatory cytokines, interleukin-1β (*Il-1β*), interleukin-6 (*Il-6*), and tumor necrosis factor α (*Tnf-α*), were not reduced by antibody treatment on days 7, 14, and 21 (Fig. 4).

3.5. S100A8/A9 antibody reduced cell death after tracheal transplantation

Because the S100A8/A9 antibody reduced epithelial loss on day 21 (Fig. 2B and 2D), we investigated the effect of S100A8/A9 antibody on cell death in TUNEL assays. On day 14, a TUNEL signal was detected in the lumen (Fig. 5A and 5B). The proportion of nuclei double-positive for TUNEL and DAPI staining in the graft lumen to the total number of DAPI-positive nuclei was calculated (Fig. 5B). The relative proportion of double-positive cells was significantly lower in the antibody-treatment group than that in the IgG group (7.16% and 16.2%, respectively; $p = 0.0391$; Fig. 5C).

4. Discussion

The main finding of this study was that the S100A8/A9 neutralizing antibody inhibited luminal fibrosis after allogeneic tracheal transplantation. The anti-fibrotic effect of the antibody was associated with the inhibition of fibroblast activation and reduced cell death, but it was not accompanied by the reduction of common proinflammatory cytokines due to rejection.

This antifibrotic effect may reflect the direct suppression of fibroblast activation. Previously, we demonstrated that S100A8/A9 promoted both mouse- and human-derived fibroblast proliferation and differentiation into α-SMA-positive myofibroblasts in vitro by stimulating the RAGE–nuclear factor-κB (NF-κB) axis. Furthermore, S100A8/A9-induced α-SMA and collagen upregulation, as well as NF-κB activation, were reversed by S100A8/A9 antibody treatment in both mouse and human fibroblasts [8]. These data suggest that, in our tracheal-transplantation model, S100A8/A9 neutralizing antibodies directly prevented S100A8/A9 binding to RAGE on fibroblasts and suppressed differentiation into myofibroblasts (Fig. 6).

In this study, the areas of α-SMA-positive fibrotic tissues and the ratio of *Col3a1* to *Col1a1* gene-expression levels were suppressed by S100A8/A9 antibody treatment on day 21. Col1a1 has been used as a fibroblast marker [4]. Sato et al. demonstrated that in a rat model of heterotopic tracheal transplantation model, switching from a fibroblast-dominant state to a myofibroblast-dominant state occurred between day 14 and day 21 as a maturation step of allograft fibrosis. They also reported that switching from type-I to type-III collagen coincided with fibrosis maturation between day 14 and day 35 [4]. These facts suggest that the S100A8/A9 antibody suppressed fibroblast differentiation into myofibroblasts and, consequently, class switching from type-I to type-III collagen in our

murine model of tracheal transplantation.

In our experiments, S100A8/A9 antibody treatment significantly decreased the expression levels of *Tgf-β1* and *Ccl2* by 21 days after tracheal transplantation. We speculate that TGF-β1 and CCL2 helped mediate fibroblast activation in our tracheal transplantation model. TGFβ1 is a key regulator of the transformation from fibroblasts to myofibroblasts. TGF-β1 stimulation can induce α-SMA protein expression [17]. Other data showed that TGF-β inhibited myofibroblast apoptosis [18]. HMGB1, which shares RAGE as a receptor with S100A8/A9, upregulated active TGF-β via the NF-κB signaling pathway and exacerbated fibrosis in separate murine models of lung fibrosis and tracheal transplantation [11,19]. Recent findings demonstrated that high-glucose-induced fibrotic changes in rat-derived heart fibroblasts were mediated by the NF-κB signaling pathway and the TGF-β1/SMAD family member 3 pathway in a RAGE-dependent manner [20]. CCL2 is closely associated with tissue fibrosis. CCL2 regulated macrophage recruitment during inflammation and wound healing [21]. CCL2 was also found responsible for the number of α-SMA-positive cells, *α-SMA*-expression levels, and type-III collagen expression levels during cardiac fibrosis in a mouse model [22]. These findings support our hypothesis that the S100A8/A9 neutralizing antibody suppresses luminal fibrosis by downregulating TGF-β1 (Fig. 6) and CCL2 in the tracheal graft.

Because S100A8/A9 is a DAMPs that induce leukocyte infiltration, we hypothesized that the S100A8/A9 antibody mainly helped suppress inflammation after tracheal transplantation. However, the expression levels of *Il-1β, Il-6,* and *Tnf-α* were not inhibited by antibody treatment. Previous data showed that these three cytokines were upregulated by S100A8/A9 in various in vitro and in vivo disease models [23–28]. In a model of arthritis, anti-S100A9 antibody treatment reduced serum IL-6 and TNFα levels [29]. These discrepancies are partially attributable to different situations that arise during inflammation; with our model of BOS, the elevation of *Il-1β, Il-6*, and *Tnf-α* was independent of S100A8/A9. Alternatively, S100A8/A9 may protect against these inflammatory cytokines. Administering recombinant S100A8 or S100A8/A9 reduced their levels in a murine model of lipopolysaccharide-induced acute lung injury and autoimmune myocarditis [30,31]. Furthermore, the high-affinity binding of S100A8/A9 with IL-1β, IL-6, and TNF-α suggests that S100A8/A9 can sequester cytokines [30]. These data suggest that S100A8/A9 was not responsible for the upregulation of common proinflammatory cytokines during acute rejection and BOS and that S100A8/A9 antibody attenuated luminal fibrosis independently of its anti-inflammatory properties.

Reduced cell death may have also contributed to reduced luminal fibrosis in the antibody-treatment group. The TUNEL assays suggested that the S100A8/A9 antibody inhibited DNA-damage-associated cell death (Fig.

5), and the epithelial layer was preserved after antibody treatment (Fig. 2B and 2D). S100A8/A9 can promote cell death in epithelial cells, including lung epithelial cells [7,32]. These results suggest that, to some extent, the S100A8/A9 antibody suppressed airway epithelial cell damage and blocked desquamation of the epithelium, thereby preventing luminal fibrosis.

The current findings demonstrate that S100A8/A9 neutralizing antibodies have potential clinical applications in treating CLAD. S100A8/A9 was detected at significantly higher levels in patients with BOS than in patients without CLAD. TGF-β was upregulated in patients with CLAD after lung transplantation [33,34]. CCL2 protein levels in bronchoalveolar lavage samples were significantly higher in patients with BOS than in healthy lung-transplant recipients [35]. Type-III collagen deposition and the type-III: type-I collagen ratio were significantly higher in recipients who developed BOS than in patients without CLAD, and the latter finding correlated inversely with respiratory function in patients with BOS [36]. In this study, *Tgf-β1* and *Ccl2* were downregulated by S100A8/A9 antibody treatment. Furthermore, antibody treatment reduced the levels of α-SMA-positive myofibroblasts and prevented collagen class switching. These findings suggest that the S100A8/A9 neutralizing antibody may have a therapeutic effect against fibrosis in clinical BOS after human lung transplantation.

This study has some limitations. Our model of heterotopic tracheal transplantation does not fully represent the clinical lung transplantation scenario. We employed this model because of its high reproducibility and early fibrotic graft responses [37,38]. Further experiments should be performed using murine and porcine lung transplantation models to better evaluate potential clinical applications with the S100A8/A9 antibody.

In conclusion, the anti-S100A8/A9 neutralizing antibody ameliorated luminal obstruction after heterotopic tracheal transplantation. We believe that this antibody is a promising clinical therapeutic candidate for the prevention and reduction of BOS after lung transplantation.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Figure legends

Fig. 1. Upregulation of *S100A8* and *S100A9* after tracheal transplantation. The relative mRNA-expression levels of (A) S100A8 and (B) S100A9 were examined by qPCR. The data shown are expressed as the mean ± standard error of the mean (SEM). ***$p < 0.001$; ****$p < 0.0001$, compared to the data from day 0.

Fig. 2. Luminal obstruction after tracheal transplantation and the anti-fibrotic effect of the anti-S100A8/A9 neutralizing antibody on luminal obstruction. (A) Representative images of graft changes on days 7, 14, and 21. Yellow arrow: luminal obstruction with fibroproliferative tissue. Scale bars, 100 μm. (B) Representative images of the luminal surface on day 21 at 20× magnification. Scale bars, 100 μm. Yellow arrows indicate luminal obstruction with fibroproliferative tissue. Yellow arrowheads indicate the preserved epithelium. (C, D) Obstruction ratio (C) and epithelium-preservation ratio (D) on day 21. The data shown are expressed as the mean ± SEM. IgG, control IgG group; Ab, S100A8/A9 antibody-treatment group.

Fig 3. Reduction of α-SMA-positive myofibroblast proliferation and inhibition of collagen class switching by anti-S100A8/A9 antibody treatment. (A) Representative immunohistochemical images of α-SMA on day 21. Yellow arrowheads indicate the basal membrane. (B, C) Quantification of α-SMA-positive area in the lumen (B) and in the submucosal area (C) at day 21. The area between the basal membrane and the internal surface of the cartilage was defined as the submucosal area. (D) Collagen-expression levels at day 14 (upper row) and day 21 (lower row) were analyzed by qPCR. The *Col3a1: Col1a1* expression ratio was calculated (right column). The data shown are expressed as the mean ± SEM. IgG, control IgG group; Ab, S100A8/A9 antibody group; *$p < 0.05$, **$p < 0.01$; scale bars, 100 μm

Fig. 4. Real-time qPCR analysis of the resected tracheal grafts obtained on days 7, 14, and 21. The data shown are expressed as the mean ± SEM. IgG, control IgG group; Ab, S100A8/A9 antibody-treatment group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

Fig. 5. Reduction of cell death by anti-S100A8/A9 antibody treatment. (A) Representative TUNEL-staining images of the tracheal grafts on day 14. Arrowheads indicate the basal membrane. Dotted squares indicate the areas shown in panel B. (B) Representative images taken at 20× magnification in the luminal area. Arrows indicate both TUNEL- and DAPI-positive cells that were counted in panel C. (C) Quantification of TUNEL- and DAPI-positive cells in the lumen at day 14. The data shown are expressed as the mean ± SEM. IgG, control IgG group; Ab, S100A8/A9 antibody treatment group; scale bars, 200 μm

Fig. 6. Suspected molecular mechanism underlying the anti-fibrotic effect of the anti-S100A8/A9 antibody. (A) S100A8/A9 promotes fibroblast activation, possibly due to TGF-β upregulation via RAGE–NF-κB signaling activation. (B) S100A8/A9 neutralizing antibody suppresses RAGE-NF-κB signaling and reduces TFG-β expression, resulting in the suppression of fibroblast activation and proliferation.