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HMGB1 blockade significantly improves luminal fibrous obliteration in a murine model of bronchiolitis obliterans syndrome

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ABSTRACT

Background: Although high-mobility group box-1 (HMGB1), which is a nuclear protein, was reported to enhance the allogeneic responses in transplantation, the effect of HMGB1 on bronchiolitis obliterans syndrome (BOS) is unknown.

Methods: A murine heterotopic tracheal transplantation model was used. Protein concentrations of HMGB1, interferon-γ (IFN-γ), interleukin (IL)-10, and IL-17 were analyzed in the isografts, allografts, controls, and HMGB1-neutralizing antibody administered allografts ($n = 6$; Days 1, 3, 5, 7, 14, 21, and 28). The luminal fibrous occlusion was analyzed (n = 6; Days 7, 14, 21, and 28). Infiltrating CD8 and CD4 T lymphocytes around the allografts and serum levels of IFN-γ and IL-10 were evaluated ($n = 6$; Day 7).

Results: The HMGB1 levels in the allografts were significantly increased compared with the isografts at Day 7. HMGB1 blockade did not change the IL-17 level, but decreased the IFN-γ/IL-10 ratio in the early phase (Days 5 and 7) and significantly improved the fibrous occlusion in the late phase (Days 14, 21, and 28). HMGB1 blockade significantly suppressed the CD8 T lymphocytes infiltration and decreased the serum IFN-γ/IL-10 ratio compared with the control at Day 7.

Conclusions: HMGB1 may be a trigger of the BOS pathogenesis and candidate target for the treatment of the disease.

1. Introduction

Lung transplantation is one therapeutic option for patients with end-stage pulmonary diseases, but long-term survival is still poor because of chronic lung allograft dysfunction (CLAD) [1]. Bronchiolitis obliterans syndrome (BOS) is a form of CLAD that prevents long-term survival after lung transplantation. An estimated 50–80% of lung transplant recipients are affected by BOS within 5 years after transplantation [2], and 5-year survival after BOS is only 30–50% [3]. BOS was reported to arise from a disease process dependent on alloimmune responses (i.e., rejection) and other alloimmune-independent functions; however, the underlying mechanisms are still unknown [4].

High-mobility group box-1 (HMGB1) is a nuclear protein that functions as an architectural chromatin-binding factor. HMGB1 protein released from necrotic cells functions similar to cytokines, by sending warning signals to neighboring cells [5]. Previous studies reported that HMGB1 is passively released from necrotic or damaged cells and actively secreted by activated dendritic cells (DCs) and macrophages [6]. Extracellular HMGB1 contributes to the pathogenesis of many inflammatory or respiratory diseases [7–9]. In addition, it was recently suggested that HMGB1 has an important role in complications that occur after organ transplantation by enhancing allogeneic responses [10]. However, the relationship between HMGB1 and the pathogenesis of BOS after lung transplantation is unknown.

According to the previous reports, T helper type 1 (Th1) cytokines, including interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α), have a pivotal role in allograft rejection [11]. A significant increase in IFN-γ and TNF-α in the blood of BOS patients compared with stable patients has been reported [12–15]. On the other hand, it was reported that one of the T helper type 2 (Th2) cytokines, IL-10, was significantly downregulated in serum from BOS patients [14–16]. In murine BOS models, IL-10 expression was shown to improve allograft rejection and tracheal luminal obstruction after orthotropic and heterotopic transplantation [17,18]. Regarding Th17 cytokine, some studies suggested

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the significant association between IL-17 and CLAD [15,19], but others reported that IL-17 had little influence on the degree of fibrosis of the lung graft [20,21]. Given that HMGB1 has been reported to change the Th1/Th2/Th17 polarization in peripheral blood mononuclear cells isolated from human blood [6,22,23], we analyzed not only tracheal luminal obliteration but also the levels of several cytokines, including IFN-γ, TNF-α, IL-10, and IL-17. Thus, the current study investigated the effect of HMGB1 on murine BOS using a heterotopic bronchiolitis obliterans model.

2. Methods

2.1. Induction of the murine bronchiolitis obliterans model

Male 6-week-old C57BL/6 J and BALB/c mice (KBT Oriental, Tokyo, Japan) were obtained from pathogen-free inbred colonies. All mice were housed with humane care 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' prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health. Our institutional review board approved the animal studies (No. A27-315-0). A well-established heterotopic tracheal transplantation model was used in this study [24,25]. In brief, donor mice were anesthetized with isoflurane, euthanized by cervical dislocation, and placed in a supine position. The tracheae were exposed by midline incision and blunt dissection. The grafts were harvested from the first tracheal ring to the carina so that each length of the tracheae was longer than 8 mm. The harvested grafts were placed into 0.9% sodium chloride with antibiotics (penicillin). Recipient mice were anesthetized with isoflurane and the grafts were placed into subcutaneous pouches on the posterior back area of mice. To investigate the role of HMGB1 in this model, we first conducted an analysis of HMGB1 and cytokine concentrations in untreated isografts and allografts. The experimental schema is shown in Fig. 1A.

2.2. HMGB1-neutralizing antibody

HMGB1-neutralizing antibody (isotype chicken immunoglobulin Y (IgY)) was purchased from Shino-test (Kanagawa, Japan). To evaluate the effect of HMGB1, 3 mg/kg HMGB1-neutralizing antibody was administered intraperitoneally to recipient mice on the day before transplantation and then twice a week on days −1, 3, 7, 10, 14, 17, 21, 24, and 28 [26]. For controls, an equal amount of chicken IgY was injected intraperitoneally on the same days as the experimental group. Therefore, we evaluated four groups in total: control isograft, control allograft, treatment control (IgY) allograft and treatment allograft ($n = 6$ for all arms of each group).

2.3. Sample collection

The grafts were obtained after the euthanasia of mice by cervical dislocation on days 1, 3, 5, 7, 14, 21, and 28 after transplantation ($n = 6$ for all arms of each group). All grafts were cut in half for histopathologic evaluation and enzyme-linked immunosorbent assays (ELISAs). Half of the tracheae were fixed in 4% paraformaldehyde at room temperature for 24 h for histopathologic assessment. The other half of the grafts were stored at −80 °C for ELISAs. Blood samples were collected from the inferior vena cava and the serum was separated by centrifugation ($n = 6$ for all arms of each group). All blood samples were stored at −80 °C until ELISAs were performed.

2.4. Histopathologic evaluation

Formalin-fixed tissues were paraffin embedded and cut into 5-μm sections. The slides were stained with hematoxylin-eosin (HE) and Masson's trichrome stain. The luminal obstruction of the trachea was assessed using Masson's trichrome stain according to the following equation: (area with abnormal proliferative fibrotic tissues)/(area within cartilage) × 100% [27]. The obstruction ratio was evaluated with ImageJ 1.50 software (National Institutes of Health, Bethesda, MD, USA).

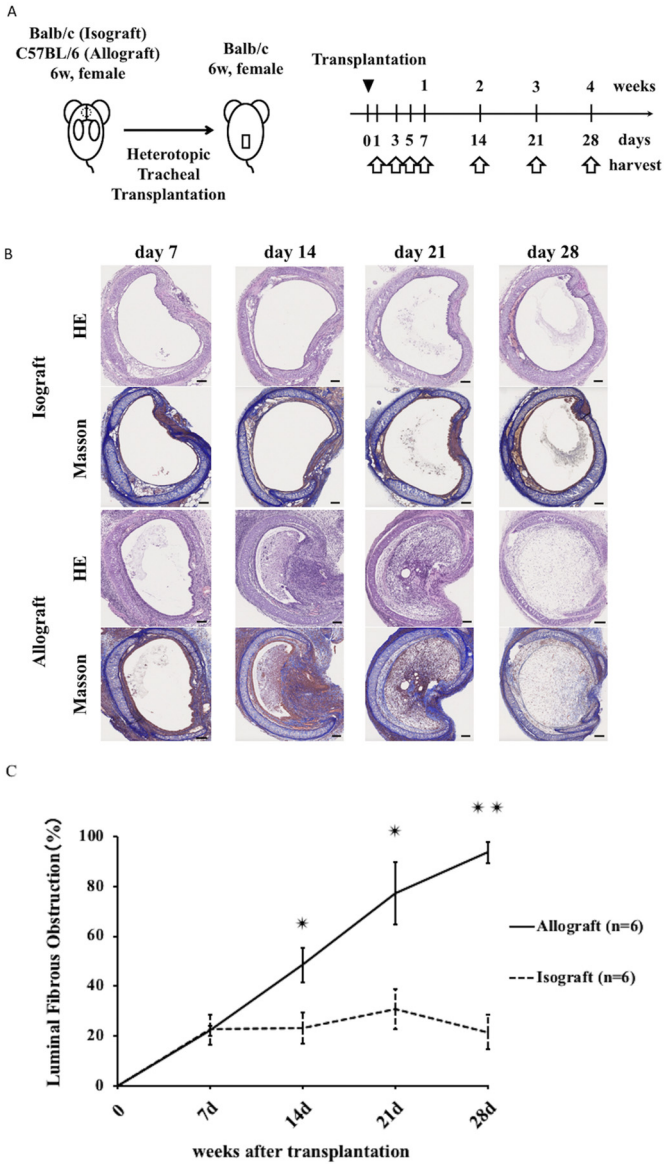

Fig. 1. (A) The experimental schema is shown. Untreated tracheae samples were harvested at the indicated times after transplantation. (B) Representative pathological images of isografts or allografts after transplantation (original magnification, ×100). Hematoxylin-eosin stain. Scale bar = 100 μm. (C) Blinded quantitative analysis of the luminal fibrous occlusion ($n = 6$ for all arms of each group). Data represent the means ± standard error of the mean. $^{*}p < 0.05$; $^{**}p < 0.01$.

2.5. Enzyme-linked immunosorbent assays (ELISAs)

Frozen grafts were homogenized in radio-immunoprecipitation assay buffer containing sodium dodecyl sulfate buffer (Nacalai Tesque, Kyoto, Japan). Concentrations of cytokines were assessed in duplicate from plasma samples or graft samples using standard sandwich ELISA kits as follows: HMGB1 (Shino-test, Tokyo, Japan), IFN-γ, TNF-α, IL-10, and IL-17 (R&D Systems, Minneapolis, USA), with sensitivities of 2.5 ng/ml, 9.4 ng/ml, 10.9 pg/ml, 15.6 ng/ml, and 10.9 pg/ml, respectively. Control cytokine concentrations were measured from tracheae

harvested from normal BALB/c mice ($n = 6$). The IFN-γ/IL-10 ratio was investigated as a surrogate of Th1/Th2 balance by calculating IFN-γ protein concentration divided by IL-10 protein concentration [28].

2.6. Immunohistochemical staining

Immunohistochemical staining for CD8 and CD4 was performed on 4-μm formalin-fixed and paraffin-embedded sections. Sections were first deparaffinized, and then blocked with 10% normal goat serum. The tissue sections were then incubated with following primary polyclonal antibodies at 4°C overnight: rabbit anti-mouse CD8 (1:200 dilution, Bioss, Woburn, MA) and anti-mouse CD4 (1:200 dilution, Bioss, Woburn, MA). The sections were then incubated with a horseradish peroxidase polymer-conjugated goat anti-rabbit secondary antibody (Dako, Glostrup, Denmark). The sections were reacted in 3,3′-diaminobenzidine, and counterstained with hematoxylin. The infiltration of CD8- and CD4-positive cells was evaluated by counting the number of CD8- and CD4-positive-staining cell per high-powered field (×1000) over 10 fields, then averaging the cell counts ($n = 6$ for each group) [10].

2.7. Statistical analysis

Data are expressed as the means ± standard error of the mean. One-way analysis of variance (ANOVA) was used to compare multiple groups. A p-value < 0.05 was considered statistically significant. All analyses were performed with JMP® 13.0 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Luminal occlusion in untreated allografts after heterotopic tracheal transplantation

Tracheae subcutaneously transplanted from B6 mice into BALB/c mice began to occlude at 14 days after transplantation and were completely obstructed at 28 days after transplantation (Fig. 1B). Masson's trichrome staining showed that the luminal fibrous obstruction ratio in the allografts was significantly elevated at 28 days after transplantation compared with the isografts ($n = 6$ for all arms of each group; 93.5% vs 21.5%, $p < 0.01$; Fig. 1C).

3.2. HMGB1 and cytokine levels in untreated allografts after heterotopic tracheal transplantation

The highest level of HMGB1 protein concentration in the allografts was observed at 7 days after transplantation (Fig. 2A). The protein concentration of HMGB1 in the allografts was significantly increased compared with isografts at 7 days after transplantation ($n = 6$ for all arms of each group; 5.6 ng/ml vs 2.1 ng/ml, $p < 0.05$; Fig. 2A). The concentration of IFN-γ in allografts tended to be higher than that in isografts, but did not reach statistical significance (n = 6 for all arms of each group; 2.6 ng/ml vs 0.7 ng/ml at Day 7, $p = 0.15$; Fig. 2B). The protein concentration of another Th1 cytokine, TNF-α, in tracheal grafts was also assessed; however, all the data were under the detection limit of the ELISA. The protein concentration of IL-10 in the allografts was significantly decreased compared with that in the isografts at 28 days after transplantation ($n = 6$ for all arms of each group; 3.0 ng/ml vs 4.0 ng/ml, $p < 0.01$; Fig. 2C). The concentrations of IL-17 in allografts were significantly higher than those in controls at 1 and 3 days after transplantation ($n = 6$ for all arms of each group; 1.8 vs 3.1 at Day 1, $p < 0.05$; 1.8 vs 2.9 at Day 3, $p < 0.05$; Fig. 2D). The IFN-γ/IL-10 ratio of the allografts at 5 days after transplantation was significantly higher than that in control ($n = 6$ for all arms of each group; 0.7 vs 0.1, $p < 0.01$; Fig. 2E) [28].

3.3. Tracheal luminal fibrous occlusion was improved by HMGB1-neutralizing antibody

To investigate whether HMGB1 is associated with BOS, HMGB1 antibody was administered to recipient mice (Fig. 3A). The time course of the protein concentration of HMGB1 in serum is shown in Supplementary Fig. 1 ($n = 6$ for all arms of each group). Although the control grafts, similar to the allografts, began to occlude at 14 days after transplantation and resulted in complete obstruction at 28 days after transplantation (Fig. 3B), HMGB1-neutralizing antibody significantly inhibited tracheal luminal fibrous formation at 14–28 days after transplantation ($n = 6$ for all arms of each group; 69.8% vs 94.2%, $p < 0.05$ at Day 28; Fig. 3C). However, HMGB1-neutralizing antibody did not prevent late phase tracheal luminal fibrous formation ($n = 6$ for all arms; 69.8 ± 8.3% at Day 28; Fig. 3C).

3.4. Change in HMGB1 protein and cytokine levels in allografts by HMGB1-neutralizing antibody

The administration of HMGB1-neutralizing antibody tended to decrease HMGB1 protein production in the allografts, but did not reach statistical significance from 5 to 14 days after transplantation (n = 6 for all arms of each group; 2.2 ng/ml vs 3.5 ng/ml, $p = 0.09$ at Day 7; Fig. 4A). The levels of IFN-γ protein were significantly decreased in HMGB1-neutralizing antibody treated allografts in comparison to the controls at 5 and 14 days after transplantation ($n = 6$ for all arms of each group; 1.1 ng/ml vs 8.4 ng/m, $p < 0.05$ at Day 5; 1.4 ng/ml vs 2.5 ng/ml, $p < 0.05$ at Day 14; Fig. 4B). The protein concentrations of TNF-α in tracheal grafts were all under the detection limit of the ELISA. The administration of HMGB1-neutralizing antibody significantly increased IL-10 protein levels in the allografts compared with the controls at 28 days after transplantation ($n = 6$ for all arms of each group; 4.2 ng/ml vs 2.0 ng/m, $p < 0.05$; Fig. 4C). However, the IL-17 levels were not significantly changed by HMGB blockade (Fig. 4D). The IFN-γ/IL-10 ratios of the allografts were significantly decreased by HMGB1-neutralizing antibody than those in the controls at 5 and 7 days after transplantation ($n = 6$ for all arms of each group; 0.4 vs 2.1 at Day 5, $p < 0.01$; 0.5 vs 1.7 at Day 7, $p < 0.01$; Fig. 4E).

3.5. Change in T lymphocytes infiltration and cytokine levels in serum by HMGB1-neutralizing antibody

The pathological conditions of the control allografts were characterized by the infiltration of inflammatory cells at 7 days after transplantation (Fig. 3B). The number of recruited CD8 T lymphocytes around the allografts was significantly decreased by HMGB1-neutralizing antibody in comparison to those in the control group ($n = 6$ for each group; 19/HPF vs 48/HPF, $p < 0.01$ at Day 7; Fig. 5A). Given the change in the profile of T lymphocytes, we also analyzed the systemic levels of IFN-γ and IL-10, and IFN-γ/IL-10 ratio in serum at 7 days after transplantation (Fig. 5B). Although the protein concentration of IFN-γ in serum was not changed ($n = 6$ for all arms of each group; 9.9 ng/ml vs 11.0 ng/ml at Day 7, $p = 0.52$), the IL-10 protein level in serum was significantly increased (n = 6 for all arms of each group; 49.6 ng/ml vs 22.9 ng/ml at Day 7, $p < 0.05$). The IFN-γ/IL-10 ratios of the plasma were significantly decreased by HMGB1 blockade than those in the control (n = 6 for all arms of each group; 0.2 vs 0.9 at Day 7 at Day 7, $p < 0.01$). The time courses of the protein concentrations of IFN-γ and IL-10, and IFN-γ/IL-10 ratio in serum are shown in Supplementary Figs. 2, 3, and 4, respectively (n = 6 for all arms of each group).

4. Discussion

In the present study, we demonstrated that HMGB1 protein had increased in the allografts before the fibrous luminal obstruction

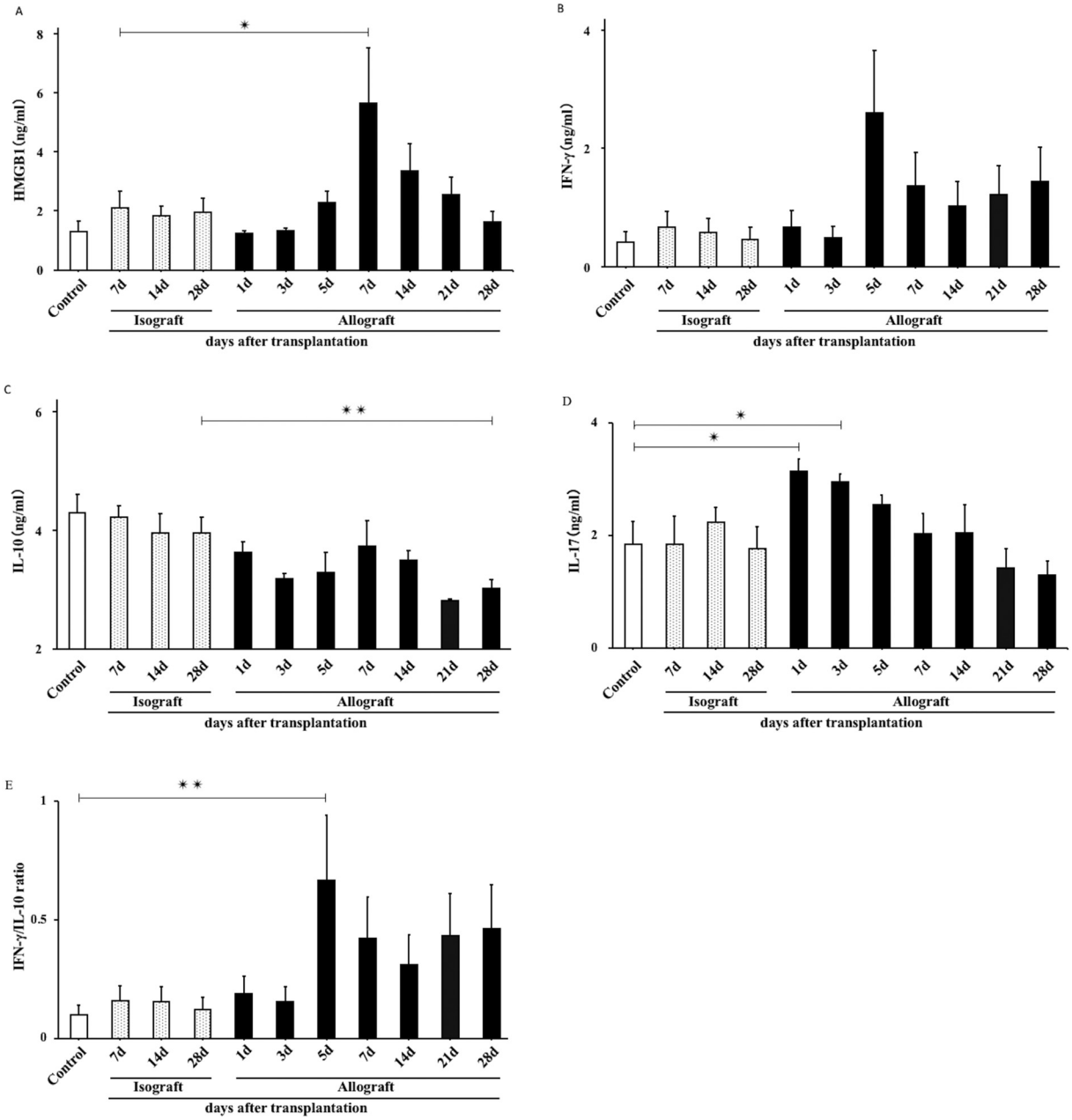

Fig. 2. Protein concentrations of HMGB1 (A), IFN-γ (B), IL-10 (C), and IL-17 (D) in isografts and allografts after heterotopic tracheal transplantation were analyzed (n = 6 for all arms of each group). (E) The IFN-γ/IL-10 ratios in isografts and allografts were calculated (n = 6 for all arms of each group). Data represent the means ± standard error of the mean. $^{*}p < 0.05$; $^{**}p < 0.01$.

started, and that HMGB1-neutralizing antibody significantly improved fibrous luminal occlusion in a murine heterotopic BOS model. We also showed that HMGB1-neutralizing antibody significantly decreased IFN-γ/IL-10 ratio, although no change was observed in the IL-17 level in the allograft. This is the first study that showed HMGB1 blockade significantly improved the luminal obstruction by changing Th1/Th2 polarization in a murine heterotopic tracheal transplantation model.

To elucidate whether HMGB1 neutralization would effectively improve BOS, we used a well-established murine heterotopic tracheal transplantation model [24,25]. The advantages of this model are as follows: 1) The pathogenesis of allografts is quite similar to human BOS in that migration and proliferation of fibroblasts, which lead to airway fibrosis by producing substantial extracellular matrix, are followed by peri-airway and subepithelial monocyte infiltration. The alloantigen-dependent fibroproliferative remodeling in murine allografts remarkably resembles fibrotic occlusion in small airways in human BOS. Accordingly, the significant influence of TGF-β signaling in myofibroblast differentiation in both murine and human BOS has been reported [29]. The histopathological changes of murine allografts have been believed to be indistinguishable from those of human BOS lesions [30]. Because of the similarity, this model is suitable for immunological studies. 2) Surgical procedures of this model are simpler compared with other models including the orthotopic lung transplantation model. This model utilizes trachea, which is relatively large airway and easy to manipulate. Because of the high reproducibility, this model is ideal for scientific studies. On the other hand, the disadvantages of this model are as follows: 1) Immune responses in mice may be different from those in humans. The luminal fibrous occlusion in the murine heterotopic BOS model develops within 4 weeks without immunosuppressive agents, although the obliterative airway disease in patients develops

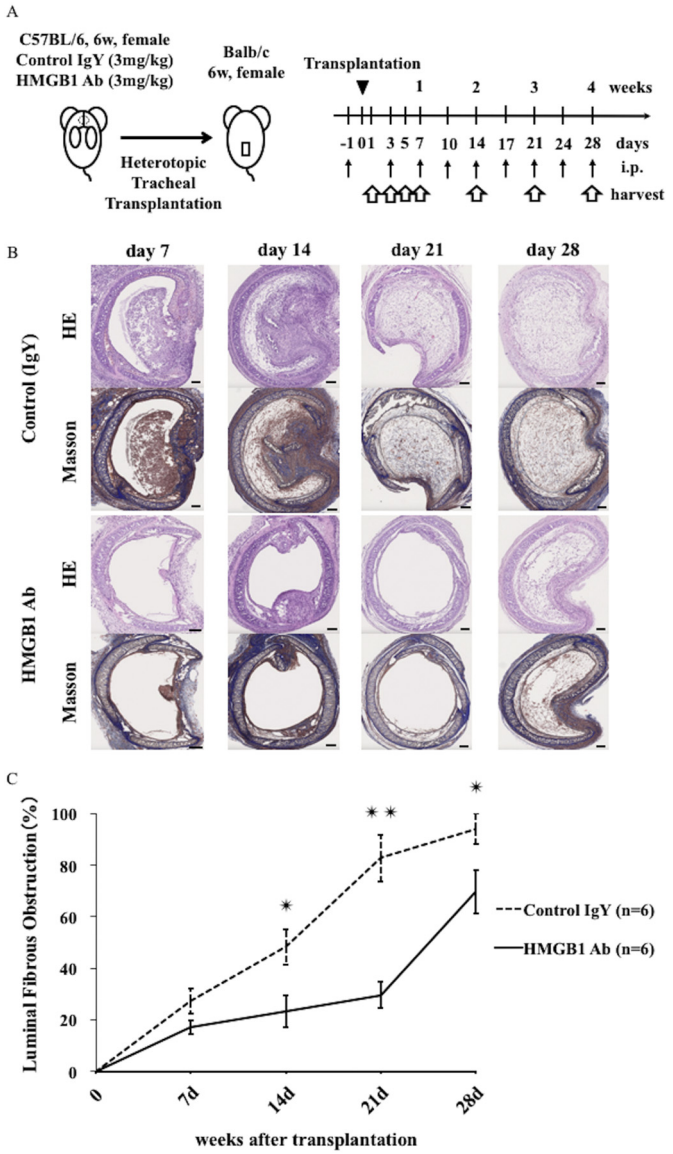

Fig. 3. (A) The experimental schema is shown. HMGB1-neutralizing antibody or control mouse IgY (both 3 mg/kg) was injected intraperitoneally into recipients at the indicated times. (B) Representative pathological features of control allografts or HMGB1-neutralizing antibody administered allografts after transplantation (original magnification, ×100). Hematoxylin-eosin stain. Scale bar = 100 μm. (C) Blinded quantitative analysis of the luminal fibrous occlusion (n = 6 for all arms of each group). Data represent the means ± standard error of the mean. $^{*}p < 0.05$; $^{**}p < 0.01$.

over months to years in the presence of immunosuppression. 2) In the murine BOS model, the tracheal graft is placed into subcutaneous pouches, which makes it impossible to assess systemic immunity after transplantation. 3) This model utilizes large airway, although human BOS affects small airways of transplanted lung. Given the advantage and disadvantage discussed above, validation of the results observed in the current study with another experimental model may be necessary. For example, transvenous administration of both immunosuppressive drugs and HMGB1 blockade into the recipient mice using orthotopic lung transplantation model would make it up for the limitations of the current study, because the orthotopic model utilizes vascularized lung graft with interface with air.

According to the previous reports, the association between HMGB1 protein and CLAD has already been suggested in patients who underwent lung transplantation [31,32]. However, in these previous studies, the relationship between HMGB1 protein and CLAD was investigated in a cross-sectional time, and their causal relationship remained unclear. Therefore, we adopted a longitudinal study design using murine BOS model and analyzed the HMGB1 level at 7 points postoperatively (Days 1, 3, 5, 7, 14, 21, and 28). As shown in Fig. 2A, the increase of HMGB1 level was observed at Day 7, although the tracheal luminal obstruction ratio was significantly elevated in the late phase (Fig. 1C). This time course indicates that HMGB1 may be a trigger to provoke the pathogenesis of BOS.

Subsequently, we demonstrated that HMGB1 inhibition significantly improved the tracheal luminal obstruction in comparison to the control at Days 14, 21 and 28 (Fig. 3C), which supports the hypothesis that HMGB1 up-regulation leads to the pathogenesis of BOS. He L et al. has also shown a similar attenuating effect of HMGB1 blockade on airway fibrosis [33]. However, they analyzed the airway fibrosis only at Day 28 after transplantation. Our study investigated airway obstruction at Days 7, 14, 21, and 28 after transplantation. Because we focused on the time course of the airway fibrosis, our study elucidated that HMGB1-neutralizing antibody could not prevent tracheal fibrous formation only in the late phase (Fig. 3C). This time course made it clear that HMGB1 did not have a significant role once the pro-fibrotic responses were initiated.

With regard to the production source of HMGB1, previous studies have suggested that HMGB1 is released from necrotic cells (passive secretion) and/or immune cells (active secretion) [5]. Given that the vascularization of the grafts begins at 3–7 days after heterotopic tracheal transplantation [33,34], and that the HMGB1 level in isografts did not increase at Day 7, active secretion from the recruited immune cells may play a pivotal role in HMGB1 elevation in allografts. Thus, the high level of HMGB1 derived from immune cells may contribute to the fibrous luminal obstruction in a murine heterotopic BOS model [35].

Regarding the mechanisms of HMGB1 blockade on the pathogenesis of BOS, previous studies reported that HMGB1 blockade suppresses inflammatory cell migration via HMGB1 and CXC chemokine ligand 12 complex formation [36,37]. We found that the profile of the suppressed inflammatory cells in murine BOS model was CD8 T lymphocyte (Fig. 5B), which is in line with the results of a previous report in vitro [35]. The decrease in the number of CD8 T lymphocytes at least partly contributes to the decrease in the IFN-γ/IL-10 ratio in the allografts (Fig. 4E). In addition, HMGB1 blockade inhibits proinflammatory cytokine production via receptor for advanced glycation end products/toll-like receptor 4, and the downstream transcription factor NF-κB [6,22], which also causes the decrease in the IFN-γ/IL-10 ratio. Consistent with the change in the profile of T lymphocytes, serum IFN-γ/IL-10 ratio was also significantly decreased in the HMGB1-neutralizing antibody administered allografts (Fig. 5C). HMGB1 blockade is reported to down-regulate production of IFN-γ in recipient's spleen in murine heart transplantation model, and our findings were in line with the previous report [10]. According to the previous report, the increase in IFN-γ and decrease in IL-10 are both observed in serum of patients with BOS [14]. Thus, it was suggested that HMGB1 blockade improved luminal fibrous obstruction by changing the Th1/Th2 polarization. Regarding Th17 cytokine, no change in IL-17 levels was observed between HMGB1-neutralizing antibody administered allografts and the controls (Fig. 4D), although IL-17 was significantly elevated in the allografts compared with the isografts at Days 1 and 3 (Fig. 2D).

From the viewpoint of clinical translation, we hypothesize that alveolar macrophage-produced HMGB1 by ischemia-reperfusion (IR) after lung transplantation activates natural killer T cells and results in BOS in reference to a previous report [38]. In lung transplantation, organ ischemia and subsequent reperfusion is unavoidable, and inflammation occurs despite non-infectious conditions [39]. The lung IR injury has been reported to contribute to the risk for development of BOS in a clinical setting [40]. The alveolar macrophage-produced HMGB1 is known to an independent mediator for activation of natural killer T cells, which initiates lung IR injury [38]. Thus, further studies

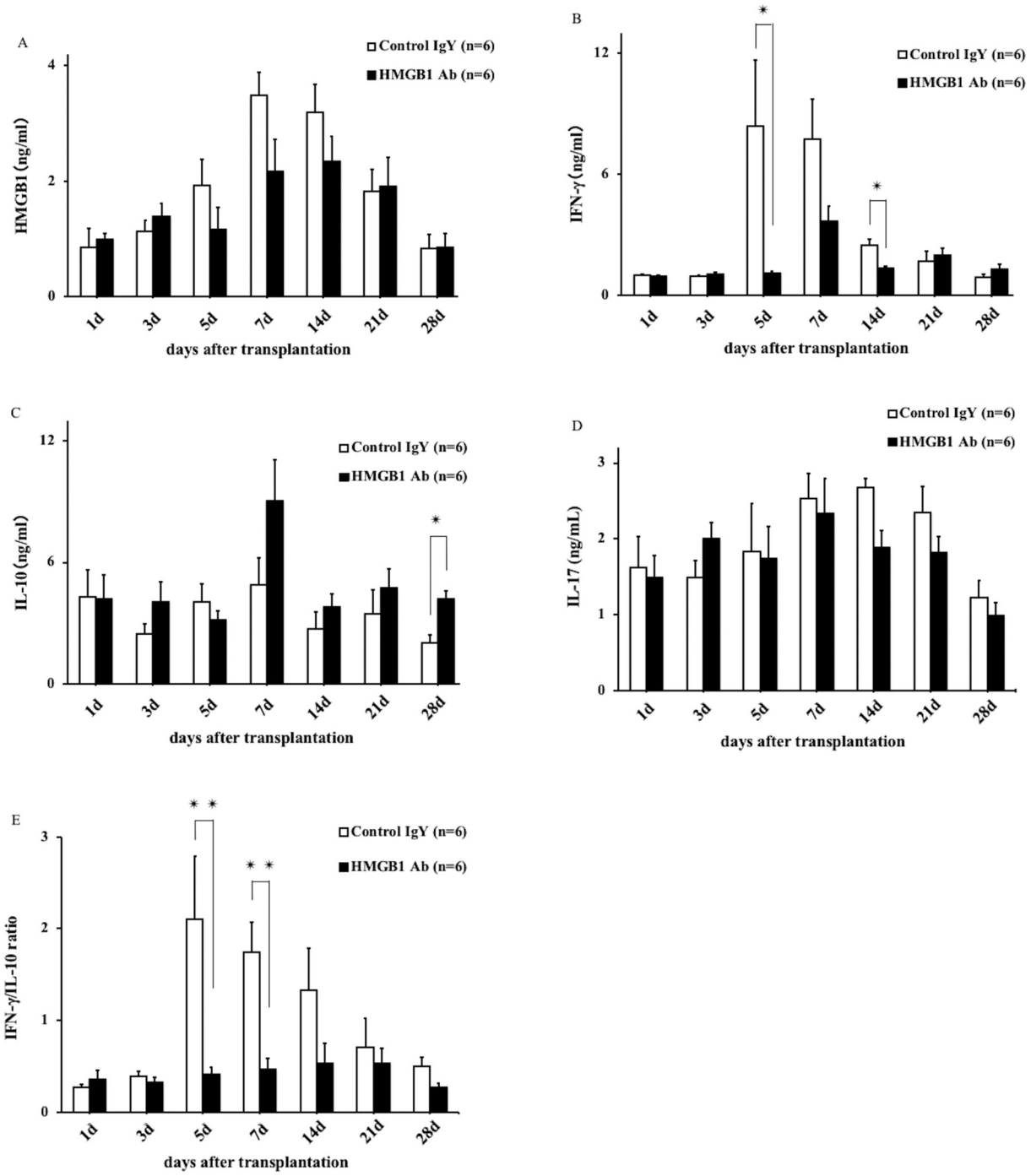

Fig. 4. Protein concentration analyses of HMGB1 (A), IFN-γ (B), IL-10 (C), and IL-17 (D) in allografts of control and HMGB1-neurtalizing antibody administered groups after transplantation (n = 6 for all arms of each group). (E) The IFN-γ/IL-10 ratios in control allografts and HMGB1-neurtalizing antibody administered allografts were calculated (n = 6 for all arms of each group). Data represent the means ± standard error of the mean. $^{*}p < 0.05$; $^{**}p < 0.01$.

investigating the relationship between HMGB1 and lung IR injury via natural killer T cells using murine orthotopic lung transplantation model may lead to a better understanding of the clinical impact of HMGB1 on BOS pathogenesis.

The present study had several limitations. First, we only analyzed IFN-γ, IL-10, and IL-17 cytokines due to the small sample size of the murine tracheae. It is important to analyze other Th1, Th2, and Th17 cytokines to reach the definitive conclusions. Second, this experimental model does not completely reflect clinical human BOS because the murine heterotopic model lacks ischemia-reperfusion injury, and the route of drug administration was intraperitoneal. Further studies with the orthotopic lung transplantation model, and/or other routes of administration (how to deliver the HMGB1 inhibitor to the target) are necessary.

In conclusion, HMGB1 plays an important role in the pathogenesis of BOS in a murine heterotopic tracheal transplantation model. The HMGB1 blockade ameliorates the fibrous tracheal obliteration in murine BOS. Further studies are necessary to confirm the therapeutic approaches using HMGB1 inhibitory drugs for human BOS.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.trim.2018.11.007.

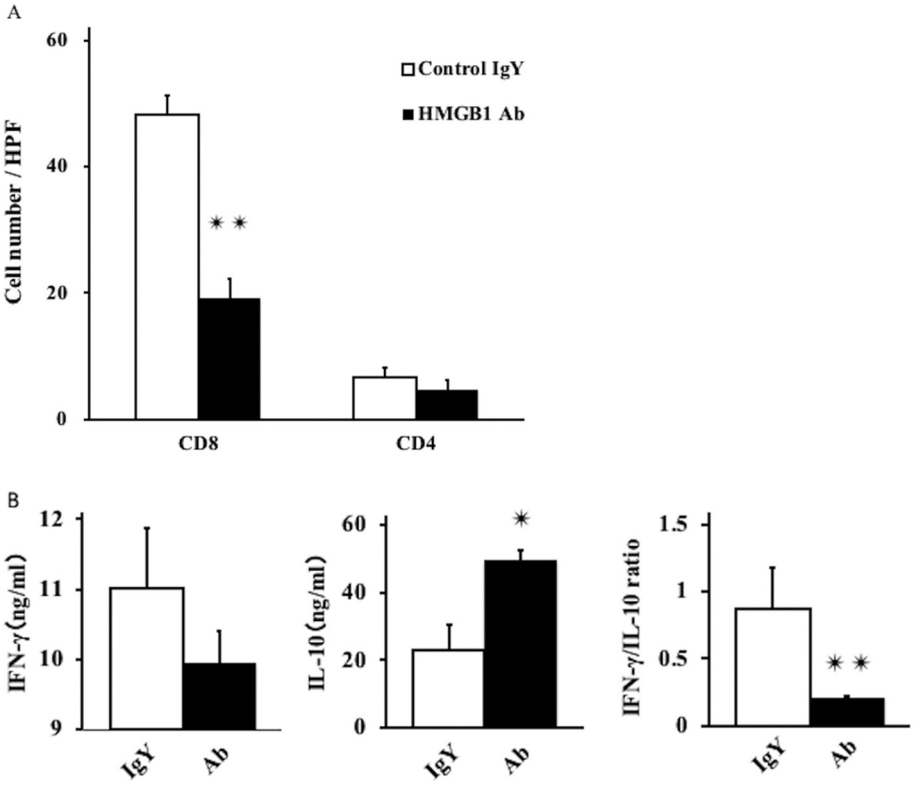

Fig. 5. (A) The average CD8- and CD4-positive cell counts per high-powered field (original magnification, ×1000) over 10 fields (n = 6 for each group). (B) Protein concentration analyses of IFN-γ and IL-10 in plasma of control and HMGB1-neurtalizing antibody administered groups after transplantation (n = 6 for all arms of each group). The IFN-γ/IL-10 ratios in plasma of control group and HMGB1-neurtalizing antibody administered group were calculated (n = 6 for all arms of each group). Data represent the means ± standard error of the mean. $^{*}p < 0.05$; $^{**}p < 0.01$.

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Conflicts of interest

All the authors declare no conflicts of interest.

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