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Involvement of high mobility group box 1 and the therapeutic effect of recombinant thrombomodulin in a mouse model of severe acute respiratory distress syndrome

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Summary

Acute respiratory distress syndrome (ARDS) is accompanied by severe lung inflammation induced by various diseases. Despite the severity of the symptoms, therapeutic strategies have been ineffective. High mobility group box 1 (HMGB1), which was identified originally as a DNA binding protein, has been proposed as a mediator of acute lung injury. In addition to its anticoagulant activity, recombinant thrombomodulin (rTM) possesses an ability to suppress the inflammatory response through neutralizing HMGB1. T regulatory (T_{reg}) cells in the lungs are reported to modify innate immune responses during resolution of acute lung injury. In the present study, we investigated the therapeutic effect of rTM, and the contribution of T_{reg} cells to this effect, in a mouse model of severe ARDS. C57BL/6 mice received sequential intratracheal administration of α-galactosylceramide (α-GalCer) and lipopolysaccharide (LPS), which resulted in the development of severe ARDS. HMGB1 levels in the lungs increased to a higher level in ARDS mice compared to those in mice treated with LPS alone. HMGB1 was expressed in the infiltrating neutrophils and macrophages in lungs. T_{reg} cells were reduced significantly in the lungs of ARDS mice compared to those in mice treated with LPS alone. rTM administration prolonged the survival time and ameliorated the development of ARDS, which was associated with increased T_{reg} cells and synthesis of interleukin (IL)-10 and transforming growth factor (TGF)-β in the lungs. These results suggest that HMGB1 is involved in the development of severe ARDS and rTM shows therapeutic effects through promoting the accumulation of T_{reg} cells at the inflammatory sites.

Keywords: ARDS, HMGB1, IL-10, regulatory T cells, TGF-β, thrombomodulin

Introduction

Acute respiratory distress syndrome (ARDS) is characterized by diffuse lung injury with severe hypoxaemia, and under various pathogenic conditions it is associated with a high mortality of approximately 25–30% [1]. The lung injury triggered by inhalation of the causative agents via the airborne route is designated as direct lung injury, and that developed in the context of systemic disorders, such as sepsis, is designated as indirect lung injury [2]. The direct lung injury type of ARDS was reported to occur during infection with the severe acute respiratory syndrome (SARS) virus [3] or the highly pathogenic avian influenza A/H5N1 virus [4,5], and following chemical or physical hazards such as bronchial aspiration of gastric contents or drowning [6]. The serum levels of interferon (IFN)-γ, tumour necrosis factor (TNF)-α and interleukin (IL)-6 were elevated in fatal human A/H5N1 influenza virus infections [7], and several cytokine and chemokine levels including TNF-α, IL-1β, IL-6 and IL-8 were increased in bronchoalveolar lavage (BAL) fluids in cases of acute-phase ARDS [8–10].

In animal models of direct lung injury induced by intratracheal administration of lipopolysaccharide (LPS), an acute and robust influx of inflammatory cells into the lungs, was observed; however, these inflammatory changes were resolved completely within 48 h, and neither alveolar epithelial injury nor vascular leakage, which are typical

histological features of ARDS [11,12], was observed. Recently, we reported an animal model of severe ARDS that was established by sensitizing mice with α-galactosylceramide (α-GalCer), a potent activator of natural killer T (NK T) cells, followed by a challenge with lipopolysaccharide (LPS) [13]. In this model, all mice died rapidly and had diffuse alveolar damage in their lungs, which was accompanied by a large increase in IFN-γ and TNF-α production.

High mobility group box 1 (HMGB1), identified originally as a DNA binding protein, also has potent proinflammatory properties. Exposure of neutrophils or macrophages to HMGB1 induces nuclear translocation of nuclear factor-kappa B (NF-κB) and enhanced production of proinflammatory cytokines, including TNF-α and IL-1β, at least in part through the interaction of HMGB1 with Toll-like receptor (TLR)-2, TLR-4 and the receptor for advanced glycation end products (RAGE) [14–16]. HMGB1 has been proposed recently to be one of the late mediators in LPS-induced sepsis lethality [17–20]. Intratracheal HMGB1 injection results in the development of acute pulmonary inflammation, and HMGB1 blockade decreases the severity of LPS-induced acute lung injury (ALI) [17,21] and haemorrhage-induced ALI [22,23].

Thrombomodulin (TM), a thrombin receptor on the endothelial cell surface, is an important player in the natural anti-coagulant and anti-inflammatory system. TM has unique anti-inflammatory properties in its N-terminal lectin-like domain, through which it binds HMGB1 and thereby suppresses HMGB1-induced inflammation [24]. Recombinant TM (rTM) is a human soluble TM with an extracellular domain that includes the active site [25]. Similar to TM, rTM possesses an ability to suppress the inflammatory response in addition to its anti-coagulant activity [24,26]. Recently, Ogawa and co-workers reported that a soluble rTM improved respiratory dysfunction and survival in patients with sepsis [27].

Regulatory T (T_{reg}) cells are a subset of $CD4^+$ T cells that express the surface marker CD25 and the transcription factor forkhead box protein 3 (FoxP3), and these T_{reg} cells have been implicated in controlling autoreactive T cells *in vivo* [28]. T_{reg} cells exert suppressive effects in an increasing array of pathophysiological events [29,30], including regulation of immune responses in some inflammatory conditions [31–33]. Recently, D'Alessio and co-workers reported that T_{reg} cells modify the innate immune responses during resolution of ALI caused by LPS treatment [34].

In the present study, we hypothesized that HMGB1 was involved in the inflammatory response and development of severe ARDS in our model. We also questioned whether or not rTM treatment improved the inflammatory response and clinical course in ARDS mice and, if so, how T_{reg} cells contributed to the beneficial effect of this treatment. To address the hypothesis and questions, we examined the kinetics of HMGB1 secretion in the BAL fluids and assessed histologically the localization of HMGB1-expressing cells in the lungs of ARDS mice. We also examined the effect of rTM treatment on the survival and inflammatory responses in these mice. We found that pulmonary HMGB1 levels increased more in ARDS mice than in mice treated with LPS alone, and that rTM administration ameliorated the development of this pathological condition through increasing $CD4^+CD25^+FoxP3^+$ T_{reg} cells in the lungs of ARDS mice.

Materials and methods

Animals

$Vα14^+$ NK T cell-deficient mice [Jα18 knock-out (KO) mice], established through targeted deletion of the Jα18 gene segment [35] and back-crossed for eight generations to C57BL/6, were kindly provided by Dr Toshinori Nakayama (Graduate School of Medicine, Chiba University, Chiba, Japan) and Dr Masaru Taniguchi (Riken Research Center for Allergy and Immunology, Yokohama, Japan). C57BL/6 mice and Jα18KO mice were bred in a pathogen-free environment at the institute for Animal Experimentation, Tohoku University Graduate School of Medicine. All mice used in the experiments were 7–8 weeks of age. All experimental protocols described in the present study were approved by the Ethics Review Committee for Animal Experimentation of Tohoku University.

Reagents

α-GalCer, purchased from Funakoshi (Tokyo, Japan), was dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA) at a concentration of 5 mg/ml and then diluted with phosphate-buffered saline (PBS) to a final concentration of 0·4% DMSO for *in-vivo* use. LPS from *Escherichia coli* (O111: B4) was purchased from Sigma-Aldrich.

Severe ARDS model

To induce severe ARDS, mice were anaesthetized using an intraperitoneal injection of 70 mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL, USA), restrained on a small board, and 50 μl of α-GalCer (1 μg) was injected into the trachea of each mouse using a 24-gauge catheter (Terumo, Tokyo, Japan). Twenty-four h later, 50 μl LPS (50 μg) was administered via the same route. Control mice received 50 μl 0·4% DMSO-containing PBS (dPBS) instead of α-GalCer into the trachea, and/or 24 h later 50 μl PBS instead of LPS was administered using the same route.

Treatment with rTM

rTM was provided by Asahi Kasei Pharma (Chiba, Japan). Mice were administered rTM at a dose of 3 mg/kg or

Table 1. Lung injury scoring system.

Parameters	Score per field		
	0	1	2
A. Neutrophils in the alveolar space	None	1–5	>5
B. Neutrophils in the interstitial space	None	1–5	>5
C. Hyaline membranes	None	1	>1
D. Proteinaceous debris filling the airspaces	None	1	>1
E. Alveolar septal thickening	<2×	2×–4×	>4×

Score = $[(20 \times A) + (14 \times B) + (7 \times C) + (7 \times D) + (2 \times E)]$/ (number of fields × 100). Reference: American Thoracic Society [36].

10 mg/kg intraperitoneally every 12 h, beginning 12 h before LPS injection. The time–course of rTM administration is illustrated in Fig. S1.

Histological examination

The lung specimens obtained from mice were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Sections were cut and stained with haematoxylin and eosin (H&E) using standard staining procedures at Biomedical Research Core, Animal Pathology Platform of Tohoku University Graduate School of Medicine. We measured histological evidence of tissue injury using the lung injury scoring system, recommended by the American Thoracic Society (ATS) [36]. Lung injury scoring system parameters include neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspaces and alveolar septal thickening. At least 20 random high-power fields (×400 total magnification) were scored independently in a blinded fashion. Each of five histological findings was graded using a three-tiered scheme, as summarized in Table 1. The sum of each of the five independent variables shown in Table 1 were weighted according to the relevance ascribed to each feature by the ATS Committee, and were then normalized to the number of fields evaluated.

Immunohistochemical analysis

Lung tissues from α-GalCer/LPS-treated mice were fixed in 10% neutral buffered formalin. After paraffin-embedded blocks had been cut into 5-μm sections and mounted onto slides, the specimens were deparaffinized and rehydrated. High-temperature antigen retrieval involved boiling the slides in citrate buffer (10 mM per litre, pH 6·0) for 5 min followed by blocking with 2% rabbit serum. The samples were incubated with a primary antibody [polyclonal chicken immunoglobulin (Ig)Y anti-HMGB1 antibody, dilution 1:200] (Shino-Test, Sagamihara, Japan) overnight at 4°C. Endogenous peroxidase activity was blocked by treatment with 0·3% H_2O_2 blocking solution for 20 min. After washing, slides were incubated with biotinylated rabbit anti-chicken IgY antibody (Acris Antibodies GmbH, Herford, Germany) diluted 1:1000 with PBS and were then incubated with horseradish peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan) and washed. The slides were incubated with diaminobenzidine substrate and counterstained with Carrazzi's haematoxylin solution (Wako, Osaka, Japan). Chicken IgY (Shino-Test) diluted with PBS was used as a non-immune control.

Preparation of BAL fluids and lung homogenates

Mice were killed at various intervals after LPS exposure. Their chests were opened, their tracheae were cannulated [22 G intravenous (i.v.) catheter] and 1 ml of PBS was infused intratracheally and withdrawn. This procedure was performed three times. BAL fluids were centrifuged at 450 ***g*** for 10 min at 4°C, and the supernatants were stored at −80°C until cytokines were measured. After collecting BAL fluids, the pulmonary circulation was rinsed by injecting 3 ml PBS into the right ventricle. Next, lungs were harvested and stored, either in 1·5 ml PBS for analysis of cytokines in the interstitial lung area or in the same volume of RPMI-1640 (Nipro, Osaka, Japan) medium supplemented with 10% fetal calf serum (FCS) (BioWest, Nuaillé, France) for analysis of the lung interstitial leucocytes. The lungs that were stored in PBS were homogenized and centrifuged at 450 ***g*** for 10 min at 4°C, and the supernatants were stored at −80°C until the cytokines were measured. For surface antigen expression analysis, the homogenized lung cells were incubated for 60 min at 37°C with vigorous shaking in RPMI-1640 medium supplemented with 10 mM HEPES (Sigma-Aldrich) and 10% FCS containing 20 U/ml collagenase and 1 μg/ml DNase I (Sigma-Aldrich). Tissue fragments and dead cells were removed by passing the solution through a 40-μm nylon mesh. After centrifugation at 450 × g for 5 min at 4°C, each cell pellet was re-suspended in 4 ml of 40% (v/v) Percoll (Pharmacia, Uppsala, Sweden) and layered onto 4 ml of 80% (v/v) Percoll. After centrifugation at 450 ***g*** for 20 min at room temperature, the cells at the interface were collected, washed three times and counted using a haemocytometer.

Analysis of cell surface antigens and intracellular cytokines

The lung interstitial leucocytes were preincubated with anti-FcγRII/III monoclonal antibodies (mAbs), which were prepared from the culture supernatants of hybridoma cells (clone 2.4G2) using a protein G column kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) on ice for 15 min in PBS containing 1% FCS and 0·1% sodium azide. The cells were stained using fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (clone GK1.5; BD Biosciences, Franklin Lakes, NJ, USA) and Pacific Blue-conjugated CD25 (clone PC61; BioLegend, San Diego, CA, USA) or FITC-conjugated control rat IgG2aκ (clone RTK2758; Biolegend)

and Pacific Blue-conjugated control rat IgG1κ (clone RTK2071; Biolegend). After the cell surfaces were stained, the cells were stained for intracellular FoxP3; the cells were fixed and permeabilized with FoxP3 staining buffer (e-Bioscience, San Diego, CA, USA), then stained with phycoerythrin (PE)-conjugated FoxP3 mAb (clone FJK-16S; eBioscience) or control rat IgG2aκ (clone RKT2758; Biolegend). The stained cells were analysed using a flow cytometer (FACS Canto II; BD Biosciences). Data were collected from 30 000 individual cells using forward- (FSC) and side-scatter (SSC) parameters to limit the lymphocyte population.

Measurement of cytokine concentrations

The IFN-γ and TNF-α concentrations in the lung homogenate supernatant and BAL fluids were measured using enzyme-linked immunosorbent assay (ELISA) with capture and biotinylated developing antibodies (BD Biosciences). The detection limits were 15 pg/ml and 5 pg/ml, respectively. The IL-1β, IL-6, IL-10, transforming growth factor (TGF-β) and HMGB1 concentrations were measured using ELISA kits (BioLegend for IL-1β, IL-6 and IL-10, eBioscience for TGF-β and Shino-Test for HMGB1). The detection limits were 30 pg/ml, 8 pg/ml, 16 pg/ml, 30 pg/ml and 2·5 ng/ml, respectively. The cytokine concentrations in BAL fluids were normalized to the protein content measured by detergent-compatible (DC) protein assay (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data analysis was conducted using JMP Pro® version 9.0.2 software (SAS Institute Inc., Cary, NC, USA) on a Macintosh computer. Data are expressed as median and interquartiles. Statistical analysis between groups was performed using analysis of variance with *post-hoc* analysis (Fisher's protected least significant difference test). Survival data were analysed using the log-rank test. A P-value $< 0{\cdot}05$ was considered significant.

Results

Pulmonary HMGB1 secretion kinetics in severe ARDS mice

It has been reported recently that HMGB1 play a critical role in LPS-induced ALI [17,21]. Therefore, to address the possible involvement of HMGB1 in the pathogenesis of severe ARDS, we first measured HMGB1 secretion in lungs. As shown in Fig. 1a, the HMGB1 concentration in BAL fluids was increased threefold at 6 h and sixfold at 12 h in mice treated with α-GalCer/LPS compared to HMGB1 levels before LPS treatment, and then reduced slightly at 24 h in mice treated with α-GalCer/LPS. In contrast, dPBS/LPS-treated mice showed a gradual increase in the HMGB1 levels at 6, 12 and 24 h, and the HMGB1 levels were lower at 6 and 12 h in dPBS/LPS-treated mice than in those treated with α-GalCer/LPS. In α-GalCer/PBS-treated mice, the HMGB1 levels were increased at 6 h at an equivalent level to those in α-GalCer/LPS-treated mice, but thereafter these levels were reduced at 12 and 24 h. In addition, the HMGB1 BAL fluid levels in α-GalCer/LPS-treated mice were reduced at 12 h in Jα18KO mice compared to those in wild-type (C57BL/6) mice, indicating that $V\alpha14^{+}$ NK T cells were essential in the secretion of HMGB1 in the lungs of severe ARDS mice (Fig. 1b).

Cellular source of HMGB1 in severe ARDS mice

To determine which cells contribute to HMGB1 secretion in the lungs, an immunohistochemical analysis was performed on the lung sections from α-GalCer/LPS-treated mice that received LPS after 12 h. As shown in Fig. 1c, HMGB1 expression was detected in the cytoplasm of neutrophils and macrophages infiltrating into alveolar spaces and intra-alveolar septa. In contrast, HMGB1 was stained positively in the bronchial and alveolar epithelial cell nuclei in α-GalCer/LPS-treated mice and in all the other groups (data not shown). These results suggest that neutrophils and macrophages accumulating at the inflammatory sites may contribute to the secretion of HMGB1 during the development of severe ARDS.

IFN-γ and TNF-α production in severe ARDS mice

In our earlier study [13], IFN-γ and TNF-α were shown to be critical cytokines in the development of severe ARDS. Therefore, in this study we measured the concentrations of these cytokines in the BAL fluids and lung homogenates. As shown in Fig. 1d, IFN-γ production in BAL fluid began to increase at 24 h after LPS administration in α-GalCer/LPS-treated mice, and its production in the lung homogenates increased to a peak level at 6 h, decreased at 12 h and then increased again at 24 h. In contrast, IFN-γ production in dPBS/LPS-treated mice was at the pre-LPS treatment level or at lower levels than that in α-GalCer/LPS-treated mice at each time-point. TNF-α production in the BAL fluid began to increase at 6 h after LPS treatment, reached a peak level at 12 h and then decreased at 24 h in α-GalCer/LPS-treated mice, and its production in the lung homogenates increased to a peak level at 6 h and then decreased at 12 h and 24 h. In addition, dPBS/LPS-treated mice showed similar TNF-α production kinetics in the BAL fluid and lung homogenate, although the levels were much lower than those in α-GalCer/LPS-treated mice.

Effect of rTM on the development of severe ARDS

The anti-inflammatory effect of rTM, an anti-coagulant drug, occurs potentially through neutralization and

inhibition of HMGB1. In order to determine the effect of rTM on the development of severe ARDS, α-GalCer/LPS-treated mice received rTM intraperitoneally, and their mortality was observed. rTM administration at a dose of 10 mg/kg, but not 3 mg/kg, increased the survival of α-GalCer/LPS-treated mice significantly compared to those that received PBS treatment (Fig. 2a). We also performed a histological analysis to examine the effect of this treatment on the inflammatory changes in the lungs of severe ARDS mice. There was less infiltration of the inflammatory cells into alveolar spaces and intra-alveolar septa and fewer oedematous changes in the interstitial area in rTM-treated mice compared to control mice at 48 h after LPS administration (Fig. 2b). For an objective evaluation of the inflammatory changes, we quantified the histological changes of the tissue injuries using the lung injury scoring system. As shown in Fig. 2c, the lung injury scores were significantly lower in rTM-treated mice than those in control mice. These results indicate that rTM effectively inhibited the development of severe ARDS caused by α-GalCer sensitization and LPS challenge.

Effect of rTM on IFN-γ and TNF-α production in severe ARDS mice

In our earlier study [13], IFN-γ and TNF-α were shown to be critical cytokines in the development of severe ARDS caused by α-GalCer/LPS-treatment. Therefore, in the present research, we determined the effect of rTM on the production of IFN-γ and TNF-α in lungs by measuring the concentrations of these cytokines in the BAL fluids and lung homogenates at 6, 12 and 24 h in α-GalCer/LPS-treated mice that received either rTM or PBS. rTM treatment did not show any significant effect on the production of IFN-γ and TNF-α in the BAL fluids and lung homogenates at 6, 12 and 24 h after LPS treatment (Table 2). These results suggest that rTM attenuated the development of severe ARDS in a different manner than by suppressing IFN-γ and TNF-α.

Effect of rTM on T_{reg} cells in the lungs of severe ARDS mice

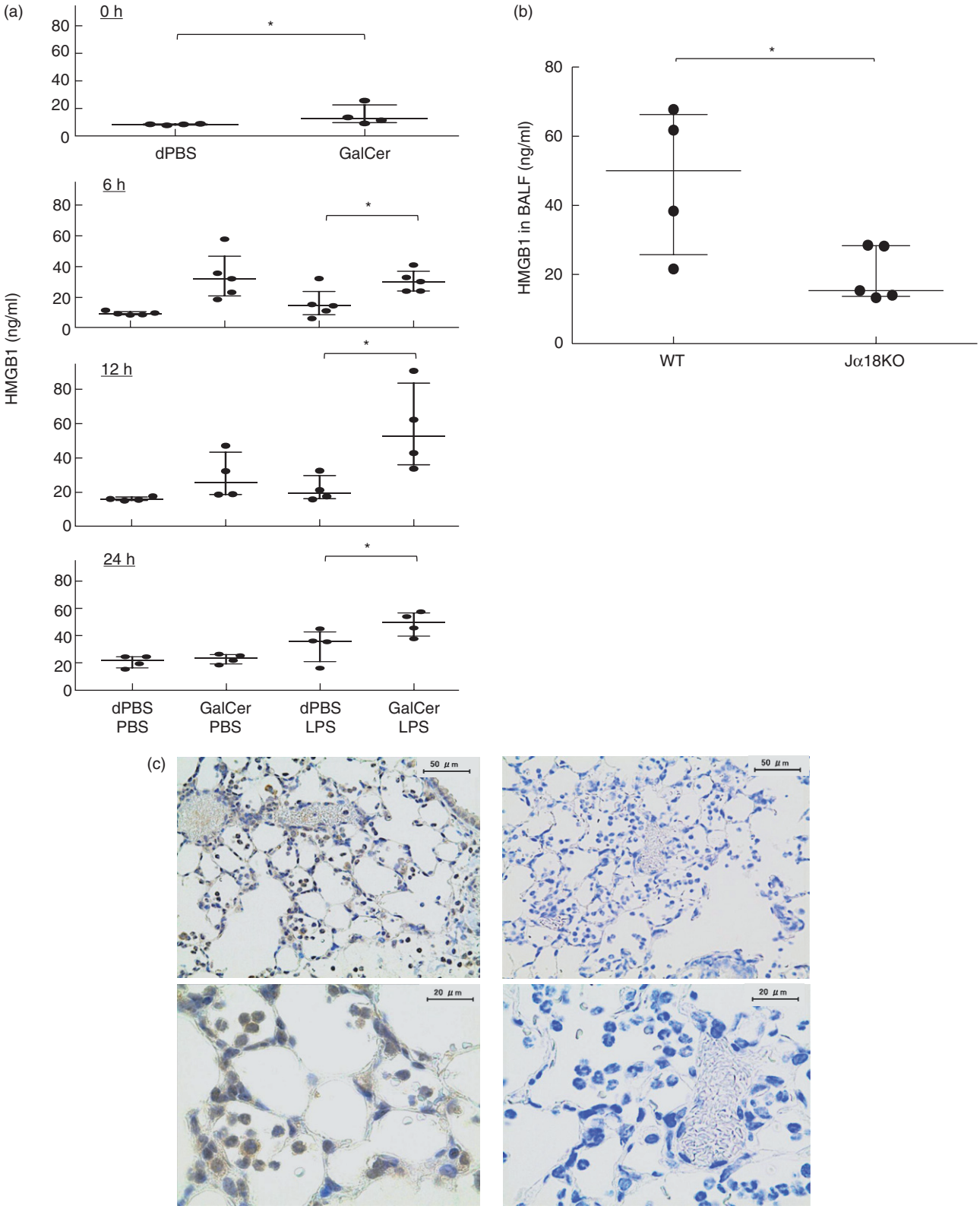
In previous studies [34], T_{reg} cells were shown to be involved in the resolution of ALI caused by intratracheal LPS administration. If this occurs in our severe ARDS model, T_{reg} cells, identified as $CD4^+CD25^+FoxP3^+$ cells, were hypothesized to be reduced in α-GalCer/LPS-treated mice compared to those in dPBS/LPS-treated mice. As shown in Fig. 3, the proportion of T_{reg} cells was significantly lower in the lung lymphocytes from α-GalCer/LPS-treated mice than in those from dPBS/LPS-treated mice. In addition, administration of rTM resulted in a significant increase in T_{reg} cells in the lung lymphocytes from α-GalCer/LPS-treated mice compared to mice that received PBS. These results suggest that recovery of the reduced T_{reg} cells in the lungs of severe ARDS mice may be related to the beneficial effect of rTM treatment.

We also compared the effect of rTM on the IL-10 and TGF-β synthesis in the lungs of severe ARDS mice, because these cytokines are known to play a key role in the anti-inflammatory effect of T_{reg} cells [37,38]. As shown in Fig. 4, rTM administration increased the level of these cytokines significantly in the lung homogenates from α-GalCer/LPS-treated mice compared to that of mice that received PBS. These results suggest that the beneficial effects of rTM treatment in severe ARDS mice may occur through the increased production of the anti-inflammatory cytokines, IL-10 and TGF-β.

Discussion

In the present study, we determined the involvement of HMGB1 in the pathogenesis of severe ARDS using a mouse model established by sensitization with α-GalCer and challenged with LPS. The main findings are as follows: (i) HMGB1 level in BAL fluids began to increase at 6 h and peaked at 12 h after exposure to LPS in ARDS mice, and the HMGB1 level was higher in ARDS mice than that in mice with ALI caused by LPS treatment alone; (ii) neutrophil and

Fig. 1. High mobility group box 1 (HMGB1) and cytokine production in the lungs of severe acute respiratory distress syndrome (ARDS) mice. Mice received intratracheal administration of lipolysaccharide (LPS) or phosphate-buffered saline (PBS) 24 h after administration of α-galactosylceramide (α-GalCer) or 0·4% dimethylsulphoxide (DMSO)-containing phosphate-buffered saline (dPBS) via the same route. (a) The HMGB1 concentration in the bronchoalveolar lavage (BAL) fluids was measured at 0, 6, 12 and 24 h after treatment with LPS or PBS. The data are shown as the median and interquartiles and each dot represents an individual mouse. (b) Jα18KO and wild-type mice received intratracheal administration of LPS 24 h after administration of α-GalCer via the same route. The HMGB1 concentration in the BAL fluids was measured 12 h after treatment with LPS. The data are shown as the median and interquartiles and each dot represents an individual mouse. $^*P < 0{\cdot}05$. (c) Mice received intratracheal administration of LPS 24 h after administration of α-GalCer via the same route. The lung sections, prepared 12 h after LPS treatment, were stained with anti-HMGB1 antibody (left) or control chicken immunoglobulin (Ig)Y (right). Representative pictures are shown at magnifications of ×400 (top) and ×1000 (bottom). (d,e) Mice received intratracheal administration of LPS or PBS 24 h after administration of α-GalCer or dPBS via the same route. The interferon (IFN)-γ (d) and tumour necrosis factor (TNF)-α (e) concentrations in the BAL fluids and lung homogenates were measured at 0, 6, 12 and 24 h after treatment with LPS or PBS. The measurements in BAL fluids are normalized to the respective protein content. The data are shown as the median and interquartiles and each dot represents an individual mouse. $^*P < 0{\cdot}05$.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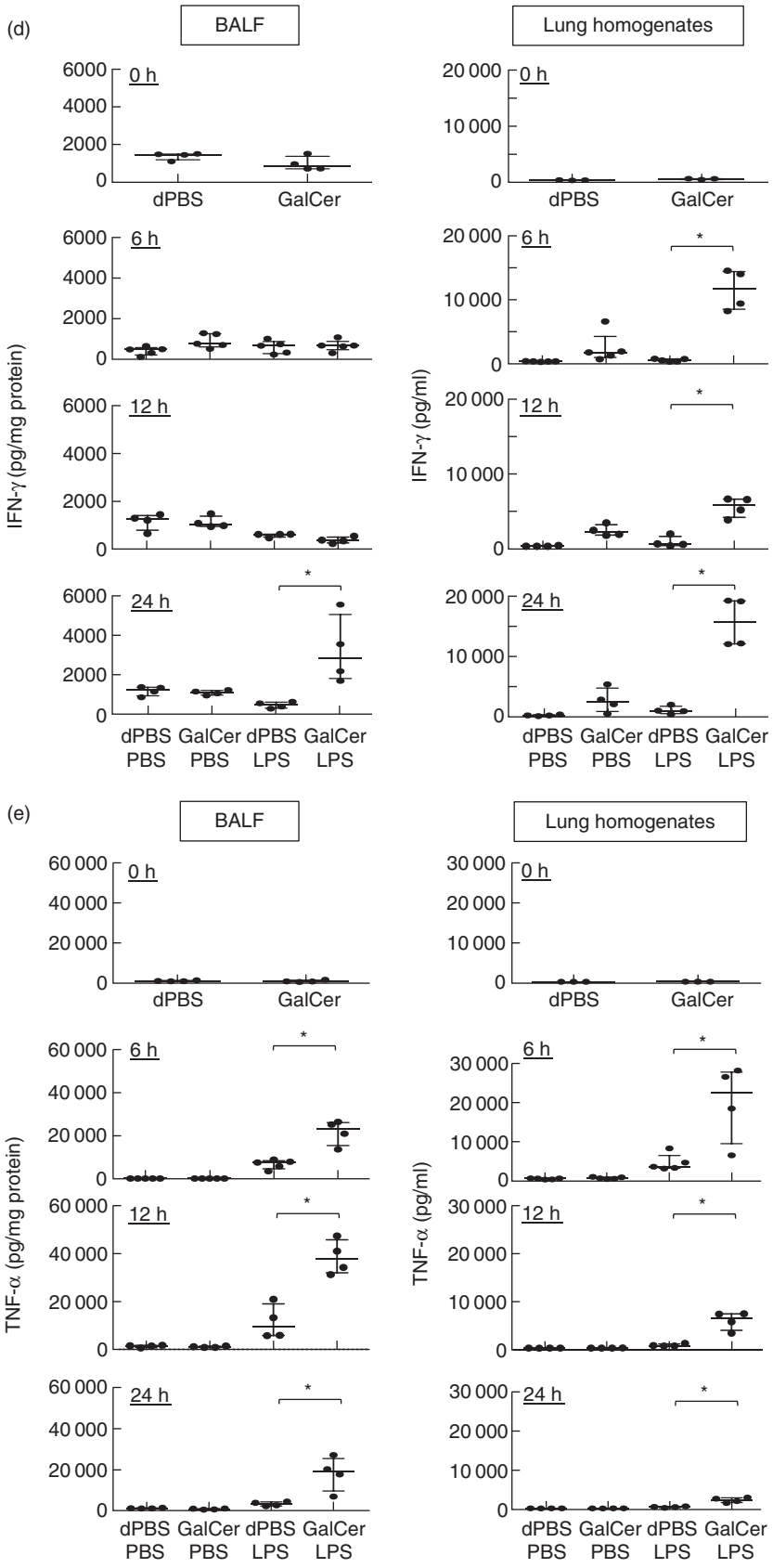

Fig. 1. *Continued*

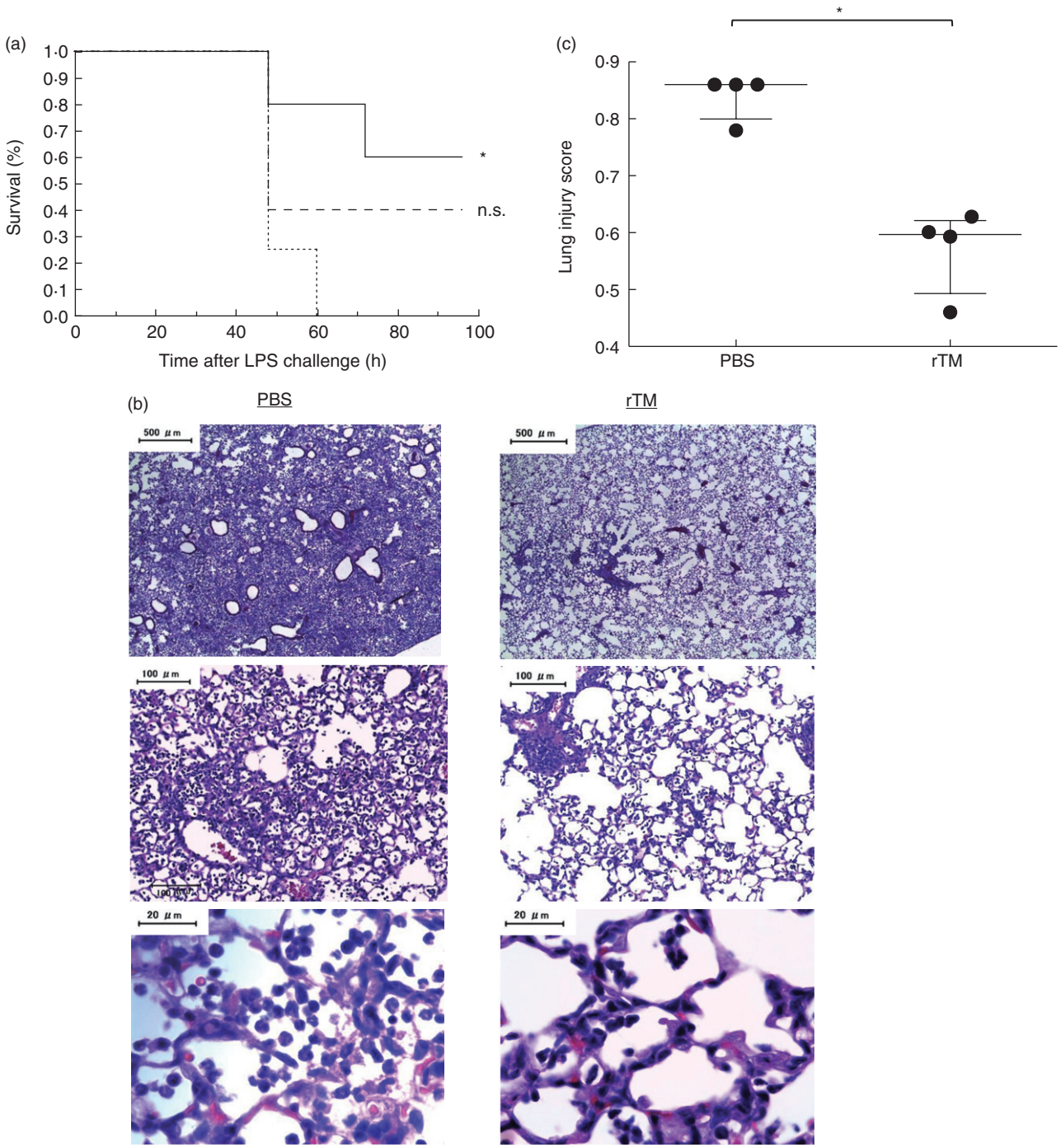

Fig. 2. Effect of recombinant thrombomodulin (rTM) on the development of severe acute respiratory distress syndrome (ARDS). (a) Mice received intratracheal administration of lipolysaccharide (LPS) 24 h after administration of α-galactosylceramide (α-GalCer) via the same route. These mice were treated intraperitoneally with 3 mg/kg or 10 mg/kg rTM or phosphate-buffered saline (PBS) every 12 h, beginning 12 h before LPS administration. The number of live mice was noted every 12 h. Each group consists of five mice. Dotted line, treated with PBS; solid line, treated with 10 mg/kg rTM; broken line, treated with 3 mg/kg rTM. (b,c) Mice received intratracheal administration of LPS 24 h after administration of α-GalCer via the same route. These mice were treated intraperitoneally with 10 mg/kg rTM or PBS every 12 h, beginning 12 h before LPS administration. The lung sections, prepared 48 h after LPS treatment, were stained with haematoxylin and eosin and observed using light microscopy. (b) Representative pictures are shown at magnifications of ×40 (upper) and ×200 (middle) ×1000 (bottom). (c) The lung injury scores of these sections were evaluated according to the scoring system described in Table 1. The data are shown as the median and interquartiles and each dot represents an individual mouse. *$P < 0{\cdot}05$; n.s.: not significant, compared with mice treated with PBS.

Table 2. Effect of recombinant thrombomodulin on interferon (IFN)-γ and tumour necrosis factor (TNF)-α production in the lungs of severe acute respiratory distress syndrome (ARDS) mice.

		IFN-γ			TNF-α		
		PBS	rTM	*P*-value	PBS	rTM	*P*-value
6 h	BAL fluids	952 (605–1203)	595 (382–871)	0·095	5263 (3094–6794)	2871 (1571–4328)	0·143
	Lung homogenates	2152 (495–2324)	3016 (197–4327)	0·111	1389 (758–2038)	1561 (1337–1962)	0·279
12 h	BAL fluids	660 (584–758)	847 (724–1638)	0·095	2914 (2271–3301)	3405 (2505–4574)	0·206
	Lung homogenates	7814 (5951–13 157)	11 082 (2398–15 135)	0·548	1345 (1261–1436)	934 (569–1589)	0·302
24 h	BAL fluids	2882 (1927–3787)	3533 (1994–5427)	0·365	2879 (1391–3703)	1672 (1422–3061)	0·373
	Lung homogenates	25 882 (5872–32 074)	23 905 (19 980–36 641)	0·274	422 (259–554)	406 (363–480)	0·500

Mice received α-galactosylceramide (α-GalCer)/lipopolysaccharide (LPS) treatment and were injected intraperitoneally with recombinant thrombomodulin (rTM) or phosphate-buffered saline (PBS) every 12 h, beginning 12 h before LPS challenge. IFN-γ and TNF-α concentrations in the bronchoalveolar lavage (BAL) fluids (pg/mg protein) and lung homogenates (pg/ml) were measured at 6, 12 and 24 h after LPS treatment. The measurements in BAL fluids are normalized to the respective protein content. Each value represents the median (interquartile range) of four or five mice.

macrophage infiltration in the lungs were stained with anti-HMGB1 antibody in ARDS mice; (iii) IFN-γ and TNF-α production was detected at higher levels in ARDS mice than that in ALI mice; (iv) rTM prolonged the survival and ameliorated the inflammatory changes in the lungs of ARDS mice; and (v) rTM increased the proportion of T_{reg} cells and the production of IL-10 and TGF-β in the lungs of ARDS mice. These results suggested that HMGB1 may be involved in the pathogenesis of severe ARDS; amelioration of ARDS by rTM probably occurred through reversal of the reduction in both T_{reg} cells and anti-inflammatory cytokine production.

Previous research has revealed that HMGB1 is one of the key mediators in the pathophysiology of lung injury [17,21–23]. Ueno and co-workers reported that HMGB1 levels in BAL fluids were higher in mice treated with LPS than those

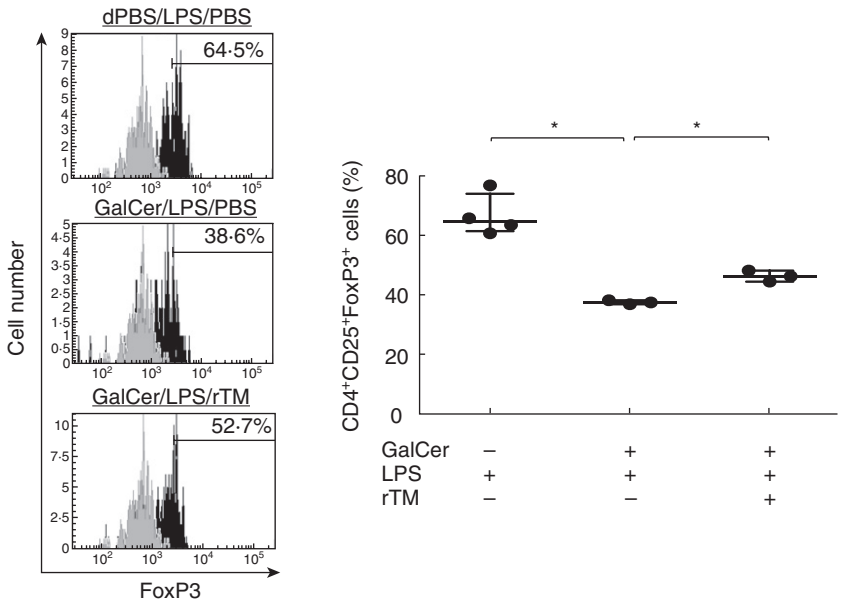

Fig. 3. Effect of recombinant thrombomodulin (rTM)on the proportion of regulatory T (T_{reg}) cells in the lungs of severe acute respiratory distress syndrome (ARDS) mice. Mice received intratracheal administration of lipolysaccharide (LPS) 24 h after administration of α-galactosylceramide (α-GalCer) or 0·4% dimethylsulphoxide (DMSO)-containing phosphate-buffered saline (dPBS) via the same route. These mice were administered 10 mg/kg rTM or PBS 12 h before and at the time of LPS treatment. The lung leucocytes obtained 6 h after LPS treatment were stained with fluorescein isothiocyanate (FITC) anti-CD4, Pacific Blue anti-CD25 and phycoerythrin (PE) anti- forkhead box protein 3 (FoxP3) monoclonal antibodies (mAb) and analysed for the proportion of $CD4^+CD25^+FoxP3^+$ cells in the lymphocyte population using flow cytometry. Representative histograms of FoxP3 expression in $CD4^+CD25^+$ cells are shown (left). Grey, isotype-matched control immunoglobulin (Ig)G; black, anti-FoxP3 mAb. The data are shown as the median and interquartiles and each dot represents an individual mouse (right). dPBS/LPS/PBS, treated with PBS in dPBS/LPS-treated mice; GalCer/LPS/PBS, treated with PBS in α-GalCer/LPS-treated mice; GalCer/LPS/rTM, treated with rTM in α-GalCer/LPS-treated mice. $^*P < 0·05$.

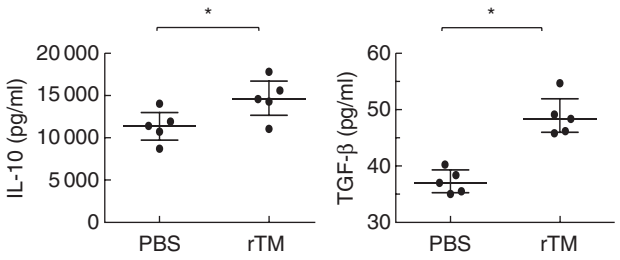

Fig. 4. Effect of recombinant thrombomodulin (rTM)on the production of interleukin (IL)-10 and transforming growth factor (TGF)-β in the lungs of severe acute respiratory distress syndrome (ARDS) mice. Mice received intratracheal administration of lipolysaccharide (LPS) 24 h after administration of α-galactosylceramide (α-GalCer) or 0·4% dimethylsulphoxide (DMSO)-containing phosphate-buffered saline (dPBS) via the same route. The IL-10 and TGF-β concentrations in the lung homogenates, prepared 6 h after LPS treatment, were measured. The data are shown as the median and interquartiles and each dot represents an individual mouse. $^*P < 0{\cdot}05$.

in control mice [21]. In the present study, HMGB1 levels in BAL fluids were higher in ARDS mice than those in mice with ALI caused by LPS alone, suggesting that robust secretion of HMGB1 may contribute to the severe lung injury and fatal outcome. It is known that activated NK T cells rapidly produce a large amount of IFN-γ [39–41]. Recently, we have shown that IFN-γ is secreted by NK T cells and that it plays a critical role during the development of severe lung injury in our model [13]. In addition, IFN-γ was reported to induce HMGB1 release in *in-vitro* experiments [42]. Thus, IFN-γ secreted by NK T cells may be an upstream mediator of HMGB1 release. In agreement with this possibility, our results showed that a large amount of IFN-γ was secreted in the lungs before HMGB1 levels increased in α-GalCer/LPS-treated mice compared to mice in other groups, and HMGB1 release was reduced to the basal level in NK T cell-deficient mice.

TNF-α is reported to contribute partially to the IFN-γ-induced release of HMGB1 in *in-vitro* experiments [19,42], and TNF-α alone also induced the HMGB1 release [19]. In the current study, a robust amount of TNF-α was secreted in the lungs of α-GalCer/LPS-treated mice before HMGB1 reached a peak level, which was a higher level than that in mice with ALI caused by LPS treatment alone. However, administration of anti-TNF-α mAb did not decrease the HMGB1 concentration in both BAL fluids and lung homogenates (data not shown). Thus, TNF-α may not be involved greatly in HMGB1 release in the lungs of ARDS mice. In addition, we measured the concentrations of IL-1β and IL-6 in BAL fluids in our model, because these cytokines were reported to increase in cases of acute-phase ARDS [8–10]. However, in contrast to IFN-γ and TNF-α, no significant increase was detected in these levels in α-GalCer/LPS-treated mice compared to those in dPBS/LPS-treated mice, except for IL-1β at 12 h (Fig. S1). Further investigation is necessary to define the precise role of these inflammatory cytokines in the development of ARDS.

Under stable conditions, HMGB1 exists mainly in the nucleus as a DNA binding protein, but nuclear HMGB1 is translocated to the cytoplasm before release into the extracellular milieu when the cells receive a threat stimulus [43]. Ueno and co-workers demonstrated that HMGB1 is present in the nucleus and the cytoplasm of alveolar macrophages in mice with ALI caused by LPS [21]. In the present study, an immunohistochemical analysis revealed that HMGB1 was detected in the cytoplasm of neutrophils and macrophages infiltrating into alveolar spaces and intra-alveolar septa, indicating that both neutrophils and macrophages are the cellular sources of HMGB1 in mice with severe ARDS caused by α-GalCer/LPS treatment. Thus, these leucocytes accumulating at the inflammatory sites may play an important role in the secretion of HMGB1 during the development of ARDS.

rTM is known to bind HMGB1 at an N-terminal lectin-like domain and to suppress the inflammatory response [24]. In a previous study by Abeyama *et al.*, rTM was shown to decrease the local leucocyte accumulation and TNF-α production in the ultraviolet (UV)-irradiated skin [24]. Similarly, Nagato and co-workers reported that rTM reduced the serum IL-1β and TNF-α levels in a mouse model of LPS-induced systemic inflammatory response syndrome, which is mediated by its antagonistic effect against HMGB1 [26]. Similar to the earlier investigations, results from the current study demonstrated that rTM treatment improved the clinical course and ameliorated the lung inflammatory responses in severe ARDS mice. However, this treatment did not affect the production of IFN-γ and TNF-α, which played a critical role during the development of severe lung injury in our model [13]; this finding was unexpected. Similarly, IL-1β and IL-6 production was not affected by rTM treatment (Table S1). Thus, further research is required to clarify why these inflammatory cytokines were not affected by rTM.

In order to define the mechanism for the beneficial effect of rTM in severe ARDS mice, we addressed the possible involvement of T_{reg} cells, which are known to regulate the immune response after inflammatory conditions by secreting anti-inflammatory cytokines, such as IL-10 and TGF-β [44–46]. In the present study, the proportion of T_{reg} cells in the lungs was significantly lower in severe ARDS mice compared to that in mice treated with LPS alone as early as 6 h after exposure to LPS, and rTM increased T_{reg} cells in the lungs of severe ARDS mice. Thus, these results strongly support the possible involvement of T_{reg} cells in determining the outcome of lung injury; reduced T_{reg} cells lead to development of severe ARDS, whereas such severe lung injury is ameliorated with an increased proportion of T_{reg}

cells. The increased level of T_{reg} cells was detected at an innate phase of the immune response, suggesting the involvement of natural T_{reg} cells [47,48] rather than induced T_{reg} cells, which are developed from naive T cells upon antigenic stimulation [48], in the pathogenic mechanism of severe ARDS. Further research will examine how severe lung injury interferes with T_{reg} cell accumulation and how activation of NK T cells and robust secretion of HMGB1 are related to this phenomenon.

IL-10 and TGF-β possess potent regulatory activities in the immune and inflammatory responses [37,38]. These cytokines have been reported to play a critical role in the suppression of inflammation and immune responses mediated by T_{reg} cells [44–46]. Results from the present study demonstrated that production of both IL-10 and TGF-β was augmented in the lungs of rTM-treated mice compared with that in control mice, which agrees with previous research [37,38,44–46]. These results suggested that rTM may exert beneficial effects against the development of severe lung injury by promoting the production of these anti-inflammatory cytokines from T_{reg} cells.

In conclusion, the present study demonstrated that BAL fluid levels of HMGB1 secreted from the inflammatory leucocytes were associated with the severity of lung injury and that administration of rTM showed clinical effects protecting ARDS mice from severe lung injury and fatal outcome, which was associated with a reversal of the reduced T_{reg} cell accumulation and ameliorated production of IL-10 and TGF-β in the lungs. These findings provide important information for the development of a novel therapeutic strategy through manipulating T_{reg} cells in human ARDS, which has a high mortality rate in hospital intensive care units, and limited available therapeutic agents. However, the precise mechanism of how HMGB1 and T_{reg} cells are involved in the progress of severe lung injury in ARDS has not yet been determined. Moreover, in clinical settings, patients with severe ARDS must receive proper treatments, including drugs and mechanical ventilation, which will affect the pathological condition; without these treatments the animal model may not reflect the pathological mechanism accurately. Further research with a focus on this issue is necessary for translating them into practical medicine.

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Disclosures

The authors state no financial conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Interleukin (IL)-1β and IL-6 production in the lungs of severe acute respiratory distress syndrome (ARDS) mice. Mice received intratracheal administration of lipopolysaccharide (LPS) or phosphate-buffered saline (PBS) 24 h after administration of α-galactosylceramide (α-GalCer) or 0·4% dimethylsulphoxide (DMSO)-containing phosphate-buffered saline (dPBS) via the same route. The IL-1-β and IL-6 concentrations in the bronchoalveolar lavage (BAL) fluids were measured at 0, 6, 12 and 24 h after treatment with LPS or PBS. The measurements in BAL fluids are normalized to the respective protein content. The data are shown as the median and interquartiles and each dot represents an individual mouse. $^*P < 0{\cdot}05$.
Table S1. Effect of recombinant thrombomodulin on interleukin (IL)-1β and IL-6 production in the lungs of severe acute respiratory distress syndrome (ARDS) mice.