ANESTHESIOLOGY

Recombinant Thrombomodulin on Neutrophil Extracellular Traps in Murine Intestinal Ischemia–Reperfusion

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Intestinal ischemia–reperfusion causes multiple-organ dysfunction syndrome
- Neutrophil extracellular traps and histone activation may be important in the development of multiple-organ dysfunction
- Recombinant thrombomodulin binds to circulating histones and may modulate their activation

What This Article Tells Us That Is New

- In male mice, recombinant thrombomodulin administration after intestinal ischemia–reperfusion increased survival rate
- Histones and neutrophil extracellular traps were found in the livers and intestines after ischemia–reperfusion, and recombinant thrombomodulin reduced these accumulations only in the liver
- Recombinant thrombomodulin may attenuate liver injury by suppressing hepatic histone and neutrophil extracellular trap accumulation

ABSTRACT

Background: In multiple-organ dysfunction, an injury affecting one organ remotely impacts others, and the injured organs synergistically worsen outcomes. Recently, several mediators, including extracellular histones and neutrophil extracellular traps, were identified as contributors to distant organ damage. This study aimed to elucidate whether these mediators play a crucial role in remote organ damage induced by intestinal ischemia–reperfusion. This study also aimed to evaluate the protective effects of recombinant thrombomodulin, which has been reported to neutralize extracellular histones, on multiple-organ dysfunction after intestinal ischemia–reperfusion.

Methods: Intestinal ischemia was induced in male C57BL/6J mice *via* clamping of the superior mesenteric artery. Recombinant thrombomodulin (10 mg/kg) was administered intraperitoneally with the initiation of reperfusion. The mice were subjected to a survival analysis, histologic injury scoring, quantitative polymerase chain reaction analysis of tumor necrosis factor-α and keratinocyte-derived chemokine expression, Evans blue dye vascular permeability assay, and enzyme-linked immunosorbent assay analysis of histones in the jejunum, liver, lung, and kidney after 30- or 45-min ischemia. Neutrophil extracellular trap formation was evaluated by immunofluorescence staining.

Results: Recombinant thrombomodulin yielded statistically significant improvements in survival after 45-min ischemia (ischemia–reperfusion without *vs.* with 10 mg/kg recombinant thrombomodulin: 0% *vs.* 33%, n = 21 per group, $P = 0.001$). Recombinant thrombomodulin reduced the histologic injury score, expression of tumor necrosis factor-α and keratinocyte-derived chemokine, and extravasation of Evans blue dye, which were augmented by 30-min ischemia–reperfusion, in the liver, but not in the intestine. Accumulated histones and neutrophil extracellular traps were found in the livers and intestines of 30-min ischemia–reperfusion–injured mice. Recombinant thrombomodulin reduced these accumulations only in the liver.

Conclusions: Recombinant thrombomodulin improved the survival of male mice with intestinal ischemia–reperfusion injury. These findings suggest that histone and neutrophil extracellular trap accumulation exacerbate remote liver injury after intestinal ischemia–reperfusion. Recombinant thrombomodulin may suppress these accumulations and attenuate liver injury.

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Multiple-organ dysfunction syndrome is the leading cause of death in intensive care units, and the gut has been recognized as a crucial organ in the development of this condition in critically ill patients.[1] Intestinal ischemia, a life-threatening abdominal emergency, is encountered in various surgical settings and is associated with mortality rates of greater than 50%.[2] In addition to ischemia, reperfusion can also trigger intense inflammatory injuries locally and then remotely, leading to multiple-organ dysfunction and death in clinical intensive care settings.

The existing experimental evidence from intestinal ischemia–reperfusion models suggests that platelets in combination with complements and Paneth cell–derived interleukin-17A exert detrimental effects on distant organs, including the liver, lung, and kidney.[3,4] Despite scientific advances, no promising novel treatment targets for intestinal

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ischemia–reperfusion–induced multiple-organ dysfunction syndrome have been identified.

Recent experimental studies showed that extracellular histones could induce cytotoxicity as damage-associated molecular patterns triggering the inflammatory cascade *via* Toll-like receptors.[5,6] Extracellular histones are released not only from damaged tissues but also from neutrophils during the formation of neutrophil extracellular traps.[7,8] Neutrophil extracellular traps comprise DNA studded with histones and proteases, including neutrophil elastases. Increasing evidence from basic research studies has elucidated the mechanism by which neutrophil extracellular traps produce numerous histones. In response to various pathogens and damage-associated molecular patterns, histone citrullination is induced in activated neutrophils *via* peptidyl arginine deiminase-4, leading to the decondensation of chromatin and the release of DNA and cytotoxic proteins to the extracellular space.[9] In turn, the histones activate neutrophils *via* Toll-like receptors to form neutrophil extracellular traps, which results in histone autoamplification.[10] Histones and neutrophil extracellular traps were reported to associate with the pathogenesis of acute lung injury in experimental animal models of acute kidney injury and severe trauma.[11,12] However, it remains uncertain whether histones and neutrophil extracellular trap formation contribute to the development of extraintestinal organ damage after intestinal ischemia–reperfusion.

Thrombomodulin is a transmembrane glycoprotein expressed on the surfaces of various cells. Previous experimental studies revealed that thrombomodulin forms a complex with thrombin *via* its epithelial growth factor-like domain to activate protein C and inhibit thrombin activity.[13,14] Additionally, thrombomodulin exerts anti-inflammatory effects by interacting with high morbidity group box-1 *via* its N-terminal lectin-like domain.[15] A recent basic study reported that recombinant thrombomodulin inhibits histone-induced lethal thromboembolism by binding to circulating histones in histone-challenged mice.[16]

Based on the above-described experimental studies, we hypothesized that histones and neutrophil extracellular trap formations would contribute to the development of remote organ damage induced by intestinal ischemia–reperfusion and that recombinant thrombomodulin would attenuate multiple-organ dysfunction progression by blocking histones and neutrophil extracellular trap formations, leading to improved survival after intestinal ischemia–reperfusion. In this animal experimental study, we evaluated whether recombinant thrombomodulin would improve 12-h survival after intestinal ischemia–reperfusion, the primary endpoint. Furthermore, we assessed the effects of ischemia–reperfusion injury and recombinant thrombomodulin administration on histone content and neutrophil extracellular trap formation in local and extraintestinal organs.

Materials and Methods

Mice and Surgical Procedures

All experiments used 8- to 10-week old male C57BL/6J mice (body weight: 20 to 25 g) obtained from Tokyo Laboratory Animal Science (Japan). The mice were maintained under specific pathogen-free conditions and a 12-h light/dark cycle, with free access to food and water. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services Public Health Services, National Institutes of Health, Bethesda, Maryland; publication No. 86-23, 1985) and approved by the University of Tokyo Institutional Review Board.

The mice were anesthetized by the intraperitoneal injection of ketamine hydrochloride (Daiichi-sankyo, Japan) and xylazine hydrochloride (Bayer, Germany). All procedures were conducted on anesthetized, spontaneously breathing mice whose body temperatures were monitored by a rectal thermometer and maintained at 37°C using a controlled heating table. After midline laparotomy, the superior mesenteric artery was clamped with a small nontraumatic vascular clip (Muromachi Kikai, Japan) to deliver approximately 85 g of pressure for 30 or 45 min (ischemic phase). Subsequently, the clip was removed, and the intestine was allowed to reperfuse. Sham-operated mice underwent an identical surgical procedure without arterial clamping. The laparotomy incision was sutured with 3-0 Emsorb (Matsuda Kogyo, Japan), and 1.0 ml of sterile saline was infused subcutaneously. All surgical procedures were performed by a single surgeon. Recombinant thrombomodulin (Asahi Kasei Pharma, Japan) or an equivalent volume of saline was injected intraperitoneally to mice in the ischemia–reperfusion plus recombinant thrombomodulin or ischemia–reperfusion group, respectively. Preparation of recombinant thrombomodulin or saline and their injection to the operated animals were conducted in a blind fashion. During the survival analysis, the mice were monitored every hour up to 12 h postreperfusion or death. In separate experiments, the animals were euthanized at 3 h postreperfusion, and various organs (jejunum, liver, lung, and kidney) were harvested for further analyses.

Experimental Design

This study comprised six experiments, which were conducted during the daytime.

Survival Analysis after Intestinal Ischemia–Reperfusion. First, the impact of the intestinal ischemic time on 12-h survival was evaluated using two experimental groups: the 30- and 45-min ischemia groups. Twenty-three mice were allocated to each group. Next, we examined the relationship between the recombinant thrombomodulin dose and 12-h survival in the 45-min ischemia group. This experiment comprised four experimental groups: the ischemia–reperfusion,

ischemia–reperfusion plus 5 mg/kg recombinant thrombomodulin, ischemia–reperfusion plus 10 mg/kg recombinant thrombomodulin, and ischemia–reperfusion plus 20 mg/kg recombinant thrombomodulin groups. Twenty-one mice were allocated to each group. Furthermore, we compared 12-h survival outcomes after 30-min ischemia in two experimental groups: the ischemia–reperfusion and ischemia–reperfusion plus 10 mg/kg recombinant thrombomodulin groups. Twenty-one mice were allocated to each group.

Histologic Evaluation of the Harvested Organs and Assessment of Biochemical Markers of Tissue Injury. We assessed the jejunum, liver, lung, and kidney histology and biochemical markers in three experimental groups: the sham-operated, ischemia–reperfusion (30 min), and ischemia–reperfusion (30 min) plus recombinant thrombomodulin (10 mg/kg) groups. For each experiment, seven mice were allocated to each group.

Expression of Cytokine and Chemokine Genes. The expression of cytokine and chemokine messenger RNAs (mRNAs) was quantified in the following three experimental groups: the sham-operated, ischemia–reperfusion (30 min), and ischemia–reperfusion (30 min) plus recombinant thrombomodulin (10 mg/kg) groups. Seven mice were allocated to each group.

Vascular Permeability Assay. Vascular leakage was quantified in each harvested organ from three experimental groups: the sham-operated, ischemia–reperfusion (30 min), and ischemia–reperfusion (30 min) plus recombinant thrombomodulin (10 mg/kg) groups. Eight, ten, and ten mice were allocated to the respective groups.

Histone Measurement in Plasma and Organs. First, 56 mice were divided into two groups: the sham-operated and ischemia–reperfusion (30 min) groups (n = 28 each). These mice were then divided into four groups to compare the increases in histone levels in target organs: intestine, liver, lung, and kidney groups. Seven intestinal ischemia–reperfusion–injured and seven sham-operated mice were allocated to each group. The change in the histone level in each organ was calculated by subtracting the average histone measurements in the sham animals from histone contents in the ischemia–reperfusion animals. Second, we assessed the effect of recombinant thrombomodulin on the histone level in each organ in three experimental groups: the sham-operated, ischemia–reperfusion (30 min), and the ischemia–reperfusion (30 min) plus recombinant thrombomodulin (10 mg/kg) groups. Seven mice were allocated to each group.

Immunofluorescence Assay. First, we assessed potential changes in the citrullinated histone H3- and Ly6G/C-positive areas in the harvested organs after intestinal ischemia–reperfusion. This experiment comprised two experimental groups for each organ: the sham-operated and ischemia–reperfusion (30 min) groups. Seven mice were allocated to each group. Second, we compared the neutrophil extracellular trap component-positive areas in the following three experimental groups: the sham-operated, ischemia–reperfusion (30 min), and ischemia–reperfusion (30 min) plus recombinant thrombomodulin (10 mg/kg) groups. Seven mice were allocated to each group.

Histology and Tissue Injury Scoring

The harvested organs were perfused with phosphate-buffered saline and fixed overnight in 10% phosphate-buffered formalin. Subsequently, the tissues were embedded in paraffin, sectioned at a 5-μm thickness, and stained with hematoxylin-eosin. To evaluate intestinal injuries, 50 to 100 villi per tissue section were graded on a six-tiered scale as previously described.[17] We also graded the degree of liver injury using a previously described semiquantitative score.[18] Briefly, liver injuries were evaluated using three indices: sinusoidal congestion, vacuolization, and hepatocyte necrosis. Each index was scored from 0 to 4, and the total scores ranged from 0 to 12 points based on the proportion of involvement. The liver injury score was determined in 10 randomly selected nonoverlapping fields from respective individual mouse liver sections at ×400 magnification. Furthermore, lung and renal damage were assessed using previously described lung injury and tubular injury scores, respectively.[19,20] The scores were averaged for respective organs (intestine, liver, lung, and kidney) and individual animals. All histology analyses were conducted by a third-party in a blinded manner.

Immunofluorescence Staining

Harvested jejunum, liver, lung, and kidney tissues were frozen in Tissue-Tek OCT compound (Sakura, Japan) and cryosectioned at a thickness of 5 μm. The sections were fixed with cold acetone for 15 min and blocked with 1% bovine serum albumin and 1% goat serum albumin in phosphate-buffered saline for 1 h at room temperature. Subsequently, the tissues were incubated overnight at 4°C in 1 μg/ml rabbit anti-mouse citrullinated histone H3 antibody (Abcam, United Kingdom) and 2 μg/ml rat anti-mouse Ly6G/C (neutrophil marker) antibody (Santa Cruz Biotechnology, USA) as primary antibodies. After washing, the sections were incubated with Alexa 488–goat anti-rabbit IgG (4 μg/ml) and Alexa 555–goat anti-rat IgG (4 μg/ml; both Invitrogen, USA) for 1 h at room temperature. DNA was stained with 1 μg/ml TOPRO-3 (Invitrogen). Confocal images were acquired using a Leica TCS SP5 II microscope (Leica, Germany). The percentages of citrullinated histone H3–positive and Ly6G/C-positive areas in each field were calculated in 10 randomly selected nonoverlapping fields from respective sections of the individual mouse jejunum, liver, lung, and kidney at ×400 magnification. The percentages of positive areas were averaged in respective organs and individual animals. The imaging analysis was conducted in a blinded manner using MCID software (MCIC, United Kingdom).

Vascular Permeability Assay using Evans Blue Dye

Vascular permeability was evaluated using Evans blue dye, as previously described.[21] The results are expressed as the number of micrograms of Evans blue dye per gram of organ tissue (wet weight).

Measurement of Myeloperoxidase Activity in the Lung

Lung myeloperoxidase activity was measured as previously reported.[21] The results were normalized to the protein content as determined by a BCA protein assay (Pierce Biochemistry, USA).

Quantification of Histones in Plasma and Organs

The histone levels in plasma and organs were measured using a sandwich enzyme-linked immunosorbent assay kit (Cell Death Detection ELISA^{plus}; Sigma–Aldrich, USA). Here, monoclonal mouse antibodies specific for single- and double-stranded DNA and histones (H1, H2A, H2B, H3, and H4) were used to detect histone-associated DNA fragments. To assess the histone content, tissue specimens of the jejunum, liver, lung, and kidney were weighed, mechanically homogenized in 1 ml of lysis buffer (containing 4-nonylphenyl-polyethylene glycol, pH 6.8 to 7.4; Roche Diagnostics, USA) using a MicroSmash with 5-mm zirconia beads (TOMY, Japan) at 3,800 rpm for 4 min, incubated for 30 min at room temperature, and centrifuged at 8,000*g* for 20 min. The histone content in the supernatant was quantified as directed by the manufacturer. The results are presented as absorbance units per milligram of organ tissue (wet weight).

Real-time Polymerase Chain Reaction Assay of Keratinocyte-derived Chemokine and Tumor Necrosis Factor-α Expression in Harvested Organs

Total RNA was extracted from homogenates of the jejunum, liver, lung, and kidney using TRIzol (Invitrogen). A high-capacity cDNA reverse-transcription kit (Applied Biosystems, USA) and random primers were used to synthesize complementary DNA from total RNA according to the manufacturer's protocol. The level of keratinocyte-derived chemokine mRNA was assessed *via* real-time quantitative polymerase chain reaction with the FAST SYBR Green master mix (Applied Biosystems). The following keratinocyte-derived chemokine-specific primers were used: sense, 5′-GGCTGGGATTCACCTCAAGAAC-3′ and antisense, 5′-TGTGGCTATGACTTCGGTTTGG-3′. The levels of tumor necrosis factor-α mRNA and 18s ribosomal RNA were examined using TaqMan gene expression assays (Applied Biosystems) and TaqMan primers for tumor necrosis factor-α (sample ID number Mm00443258_m1) and 18s ribosomal RNA (sample ID number 4310893E). Quantitative gene expression levels were calculated relative to the 18s ribosomal RNA level. Amplification data were analyzed using ViiA 7 Software ver.1.2 (Applied Biosystems).

Measurement of Aspartate Aminotransferase, Alanine Aminotransferase, and Blood Urea Nitrogen

Aspartate aminotransferase, alanine aminotransferase, and blood urea nitrogen were measured as previously described.[22]

Measurement of High Morbidity Group Box-1 in Plasma

Plasma high morbidity group box-1 levels were analyzed using an established enzyme-linked immunosorbent assay kit (Fuso Pharmaceutical, Japan), according to the manufacturer's protocol.

Outcomes

The primary outcome measure was the effect of recombinant thrombomodulin on 12-h survival of mice after intestinal ischemia–reperfusion. The secondary outcome measures were the impacts of ischemia–reperfusion injury and recombinant thrombomodulin on histone accumulation and neutrophil extracellular trap formation in the jejunum, liver, lung, and kidney.

Sample Size Estimation

In our pilot study, we compared the 12-h survival rates of five mice treated with saline and five treated with 10 mg/kg recombinant thrombomodulin *via* the 45-min ischemia model. We estimated respective survival rates of 0 and 40%. Based on this observation, we calculated a sample size of 10 mice per group, assuming a type I error rate of 0.05 and power of 0.8. To further confirm our results, we added 10 to 12 mice to each experimental group in the survival analysis.

Implication for the Replacement, Refinement, or Reduction of Animal Subjects

Organ crosstalk cannot be evaluated using *in vitro* assays. Therefore, we could not avoid the use of murine experimental models in our investigation of the interactions between the intestines and extraintestinal organs. To refine the experiment, we anesthetized all mice *via* ketamine hydrochloride and xylazine hydrochloride injection during surgery. Furthermore, all mice were euthanized by cervical dislocation after an overdose of the above anesthetic agents. To reduce the number of animals, we statistically calculated the minimum sample size with reference to previous studies before the experiments.

Statistical Analysis

The Shapiro–Wilk test and Levene's median test were used to assess the normality of distribution and equal variance of all continuous variables, respectively. If these tests rejected the null hypothesis, a nonparametric test was applied for comparison. The data are expressed as means ± SD when normally distributed and as medians (interquartile ranges)

when nonnormally distributed. The statistical significance of differences between groups were analyzed using the independent *t* test (parametric values) or Wilcoxon rank-sum test (nonparametric values). Multiple pairwise comparisons were conducted using a one-way ANOVA followed by the Tukey–Kramer test for parametric values and the Kruskal–Wallis test followed by the Steel–Dwass test for nonparametric values. The survival rates (primary variable) were compared using a log-rank test. A two-tailed *P* value of less than 0.05 was considered statistically significant for all tests. In response to peer review, additional animals were added to the experiment. However, no *P* value adjustments were made for the repeated analysis of the data after adding sample size. Outliers that could not be invalidated for technical reasons are reported as dots in the whisker box plots and were included in the statistical analyses. In this study, each experimental group was considered a unit of analysis. All calculations were conducted using JMP 13.0 software (SAS Institute, USA).

Results

Effect of Recombinant Thrombomodulin on Survival after Intestinal Ischemia–Reperfusion

We initially compared survival relative to the severity of intestinal ischemia–reperfusion injury and observed reduced mortality with a 30-min intestinal ischemia duration, compared to a 45-min duration (26% *vs.* 4%, n = 23 mice per group, $P = 0.009$; fig. 1A). Treatment with 10 or 20 mg/kg of recombinant thrombomodulin (but not 5 mg/kg) improved survival in mice with severe (45-min) intestinal ischemia–reperfusion injury (ischemia–reperfusion *vs.* ischemia–reperfusion plus 5 mg/kg recombinant thrombomodulin, 0% *vs.* 10%, n = 21 mice per group, $P = 0.281$; ischemia–reperfusion *vs.* ischemia–reperfusion plus 10 mg/kg recombinant thrombomodulin, 0% *vs.* 33%, n = 21 per group, $P = 0.001$; ischemia–reperfusion *vs.* ischemia–reperfusion plus 20 mg/kg recombinant thrombomodulin, 0% *vs.* 32%, n = 21 in ischemia–reperfusion and n = 19 in ischemia–reperfusion plus 20 mg/kg recombinant thrombomodulin, $P = 0.001$). No incremental improvement was observed with 20 mg/kg recombinant thrombomodulin relative to 10 mg/kg (fig. 1B). The baseline characteristics of each experimental group are reported in Supplemental Digital Content 1 (http://links.lww.com/ALN/C16; see table, baseline characteristics of experimental groups in survival analyses). Two mice allocated to the 20 mg/kg recombinant thrombomodulin group were excluded because major bleeding occurred during surgery.

In the severe intestinal ischemia–reperfusion model, the protective effect of recombinant thrombomodulin (10 and 20 mg/kg) was observed after 3 h of reperfusion. Previous studies reported increased inflammatory cytokine expression and prominent tissue damage in local and remote organs at 3 to 5 h after intestinal ischemia–reperfusion.[3,4,23] However, some untreated animals died after 2 h of reperfusion. Therefore, elucidation of the mechanism by which recombinant thrombomodulin improved survival in intestinal ischemia–reperfusion–injured mice required another model. In this study, we collected organs at 3 h of reperfusion using the less severe (30-min ischemia) model treated with 10 mg/kg of recombinant thrombomodulin (ischemia–reperfusion plus recombinant thrombomodulin group) or saline (ischemia–reperfusion group). In this latter model, the ischemia–reperfusion plus recombinant thrombomodulin group had a higher survival rate, although the intergroup difference did not reach statistical significance

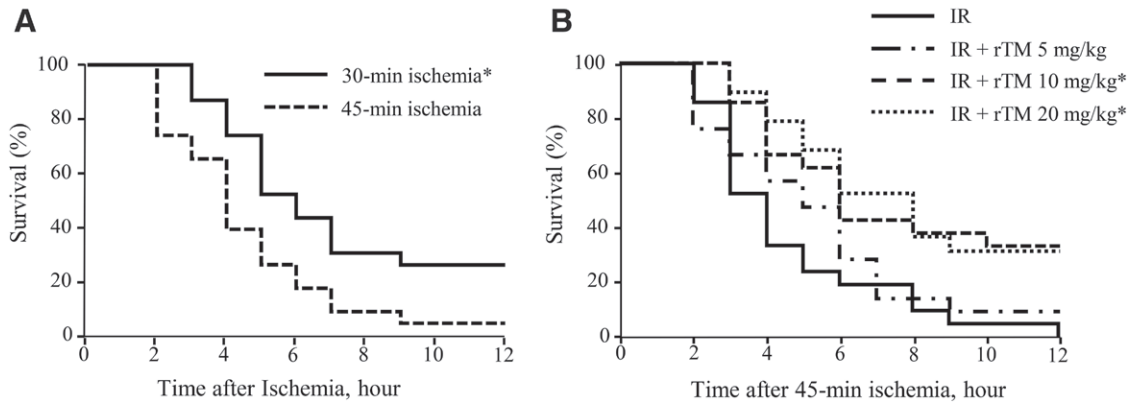

Fig. 1. Survival analysis of a 45-min intestinal ischemia–reperfusion (IR) model and the protective effect of recombinant thrombomodulin. (*A*) Mice subjected to 30- or 45-min intestinal ischemia were analyzed ($P = 0.009$ by log-rank test; n = 23 mice per group, n = 46 total).*$P < 0.05$ *versus* 45-min ischemia. (*B*) Treatment with recombinant thrombomodulin (rTM) at 10 and 20 mg/kg significantly improved survival after intestinal ischemia–reperfusion (IR *vs.* IR + 5 mg/kg rTM group, $P = 0.281$ by log-rank test; IR *vs.* IR + 10 mg/kg rTM group, $P = 0.001$; IR *vs.* IR + 20 mg/kg rTM group, $P = 0.001$; n = 21 mice in the IR, the IR + 5 mg/kg rTM, and IR + 10 mg/kg rTM groups and n = 19 in the IR + 20 mg/kg rTM group; n = 82 total). *$P < 0.05$ *versus* the IR group.

(ischemia–reperfusion *vs.* ischemia–reperfusion plus 10 mg/kg recombinant thrombomodulin, 24% *vs.* 38%, n = 21 mice per group, $P = 0.165$; Supplemental Digital Content 2, http://links.lww.com/ALN/C17; the figure shows the impact of recombinant thrombomodulin on survival in 30-min intestinal ischemia–reperfusion models).

Administration of Recombinant Thrombomodulin Did Not Protect against Intestinal Injury

Locally, intestinal ischemia–reperfusion induced devastating jejunal injury characterized by epithelial sloughing, denudation of villi, and hemorrhage in the lamina propria. Treatment with recombinant thrombomodulin failed to attenuate these local injuries (fig. 2, A to C). There were no statistically significant differences in the intestinal injury scores between the ischemia–reperfusion and ischemia–reperfusion plus recombinant thrombomodulin groups (fig. 2D).

Treatment with Recombinant Thrombomodulin Attenuated Intestinal Ischemia–Reperfusion–induced Tissue Damage in the Liver but Not in the Lung and Kidney

Intestinal ischemia–reperfusion induced severe liver injury with diffuse sinusoidal congestion and vacuolization. Remarkably, recombinant thrombomodulin mitigated this liver damage (fig. 3, A to C). A semiquantitative evaluation of liver injury scores revealed the amelioration of intestinal ischemia–reperfusion–induced liver injury by recombinant thrombomodulin treatment (fig. 3D). Similarly, liver enzymes were elevated in response to intestinal ischemia–reperfusion and attenuated by recombinant thrombomodulin (fig. 3, E and F).

Intestinal ischemia–reperfusion had no impact on histologic images of the lung (Supplemental Digital Content 3, http://links.lww.com/ALN/C18; fig. 3A shows representative images of lung histology) and kidney (Supplemental Digital Content 3, http://links.lww.com/ALN/C18; fig. 3D shows representative images of kidney histology) in comparison with tissues from sham-operated mice. The lung and kidney injury scores were consistent with the histologic findings (Supplemental Digital Content 3, http://links.lww.com/ALN/C18; fig. 3B shows lung injury score, and fig. 3E shows tubular injury score). Myeloperoxidase activity in the lung was also consistent with the histologic findings (Supplemental Digital Content 3, http://links.lww.com/ALN/C18; fig. 3C shows pulmonary myeloperoxidase

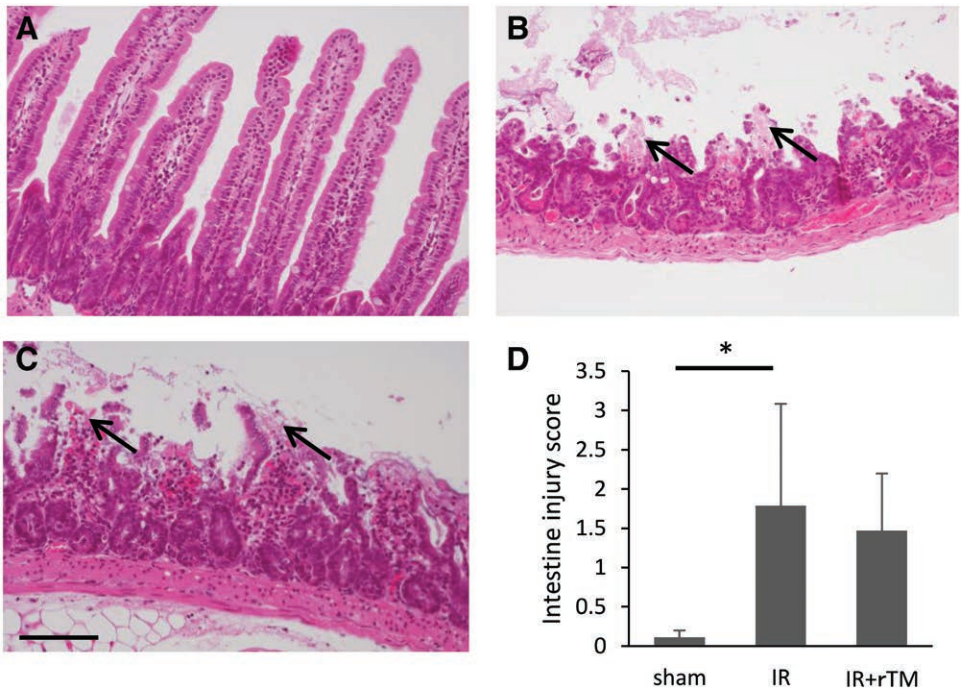

Fig. 2. Intestinal damage after intestinal ischemia–reperfusion. Intestinal ischemia–reperfusion (IR)-injured mice (30-min ischemia followed by 3 h of reperfusion) were injected with saline (IR group) or 10 mg/kg recombinant thrombomodulin (IR + rTM group) at the beginning of reperfusion. Representative images of hematoxylin and eosin stained jejunal tissues from the sham-operated (*A*), IR (*B*), and IR + rTM (*C*) groups are shown. *Arrows* indicate denudation of the villi and exudation of lamina propria. The *scale bar* indicates 100 μm. (*D*) The extent of intestinal damage was evaluated using intestinal injury scores (sham *vs.* IR group, $P = 0.019$; IR *vs.* IR + rTM group, $P = 0.857$; n = 7 mice per group, n = 21 total). *$P < 0.05$ *versus* respective control.

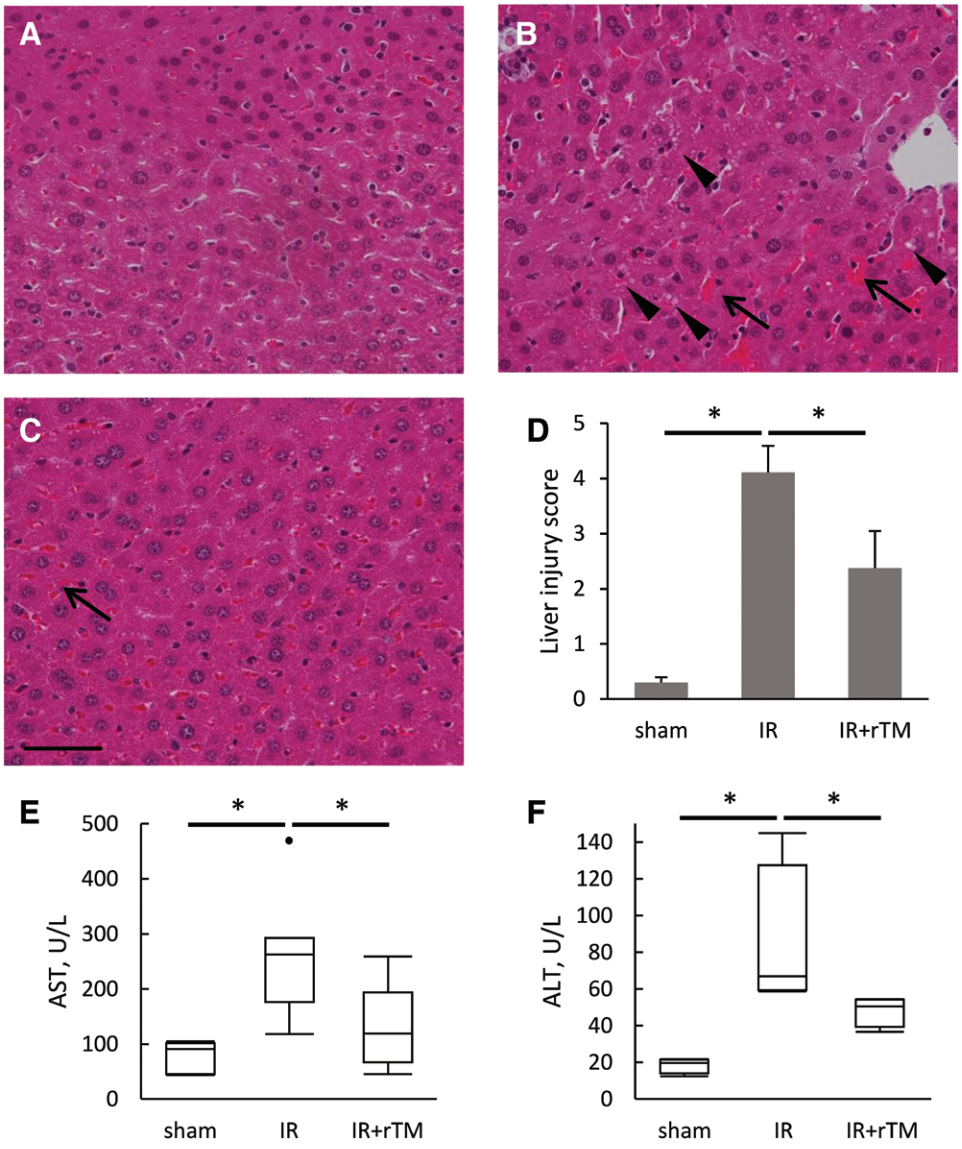

Fig. 3. Liver injury induced by intestinal ischemia–reperfusion. Intestinal ischemia–reperfusion (IR)–injured mice (30-min ischemia followed by 3 h of reperfusion) were treated with saline (IR group) or 10 mg/kg recombinant thrombomodulin (IR + *r*TM group). Representative images of liver histology from the sham-operated (*A*), IR (*B*), and IR + *r*TM (*C*) groups are shown. *Arrows* indicate sinusoidal congestion, and *arrowheads* represent vacuolization. The *scale bar* indicates 50 μm. (*D*) A semiquantitative analysis of remote liver damage was performed using liver injury scores (sham *vs.* IR group, $P < 0.001$; IR *vs.* IR + rTM group, $P < 0.001$; n = 7 mice per group, n = 21 total). (*E*) Aspartate aminotransferase (AST; sham *vs.* IR group, $P = 0.003$; IR *vs.* IR + rTM group, $P = 0.032$). (*F*) Alanine aminotransferase (ALT) levels (sham *vs.* IR group, $P = 0.030$; IR *vs.* IR + rTM group, $P = 0.030$) after intestinal IR (n = 7 mice per group, n = 21 total). The AST and ALT levels are shown as whisker box plots wherein the middle line indicates the median; the upper and lower box lines indicate the third and first quartiles, respectively; and the whiskers extend to the highest and lowest values within 1.5 times the interquartile range from the box edges. Outliers beyond the whiskers are indicated as *dots*. *$P < 0.05$ *versus* respective control.

activity). Although the plasma blood urea nitrogen level increased after intestinal ischemia–reperfusion, treatment with recombinant thrombomodulin did not affect this parameter (Supplemental Digital Content 3, http://links.lww.com/ALN/C18; fig. 3F shows plasma blood urea nitrogen levels).

Recombinant Thrombomodulin Attenuated the Inflammatory Response and Endothelial Dysfunction in the Liver after Intestinal Ischemia–Reperfusion

Previous studies suggested the involvement of inflammatory cytokines and chemokines, including tumor necrosis factor-α and keratinocyte-derived chemokine, in remote

organ injury after an intestinal ischemia–reperfusion insult.[1,4,24,25] To determine which organ would be protected by recombinant thrombomodulin administration after intestinal ischemia–reperfusion, we evaluated the expression of proinflammatory cytokine mRNAs in the jejunum, liver, lung, and kidney at 3 h postreperfusion using quantitative polymerase chain reaction. Intestinal ischemia–reperfusion markedly increased the expression of cytokine mRNAs in the liver and jejunum. Notably, recombinant thrombomodulin induced statistically significant reductions in cytokine mRNA expression in the liver, but not in the jejunum. Although tumor necrosis factor-α mRNA expression in the lung and keratinocyte-derived chemokine mRNA expression in the kidney was elevated after intestinal ischemia–reperfusion, recombinant thrombomodulin did not suppress the expression of either gene in the lung or kidney (fig. 4, A to H).

We next evaluated vascular permeability in the jejunum, liver, lung, and kidney using an Evans blue dye assay. Representative images of jejunum, liver, lung, and kidney from mice injected with Evans blue dye after intestinal ischemia–reperfusion are shown in fig. 5, A to D. Intestinal

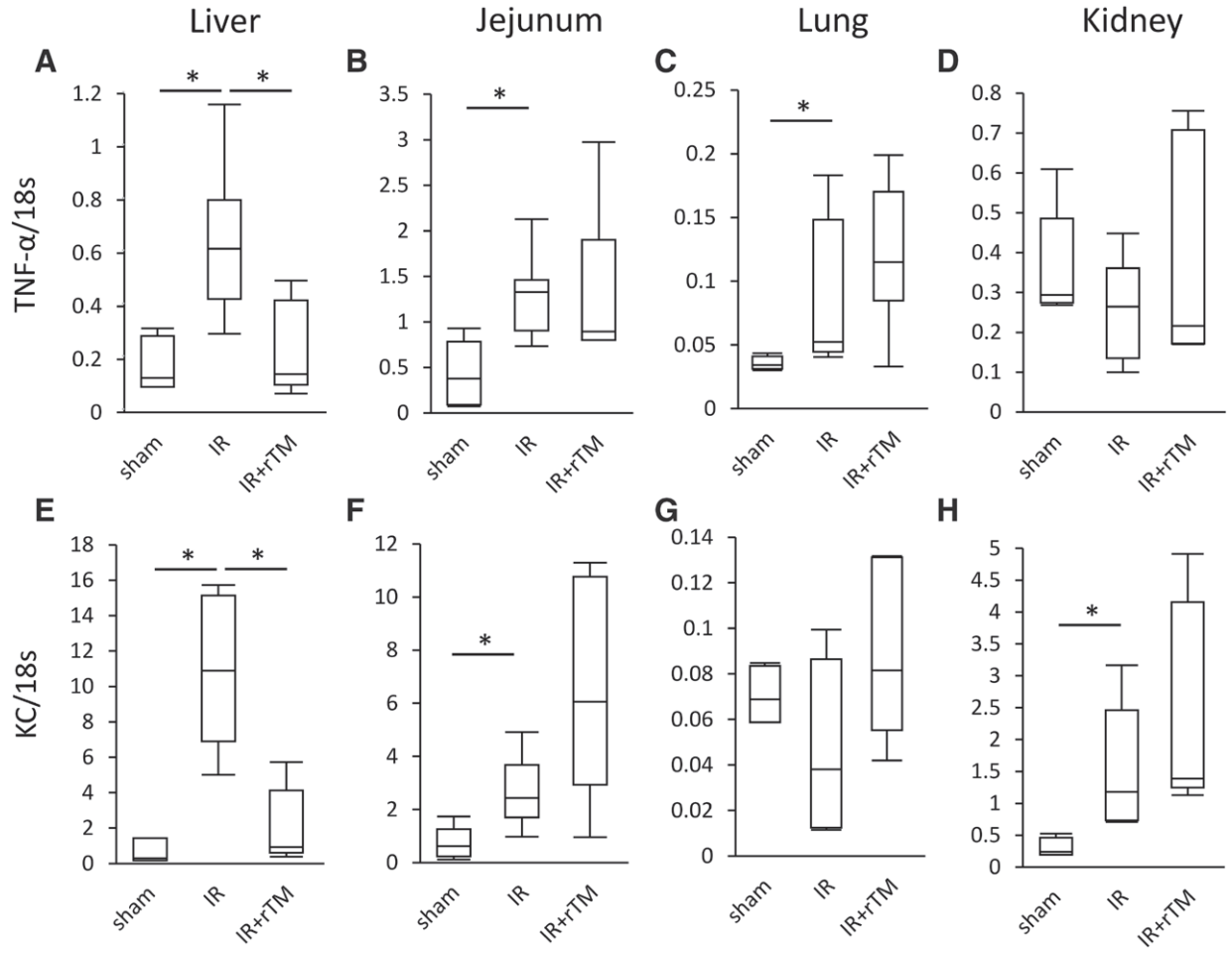

Fig. 4. Expression of inflammatory cytokines in mice subjected to intestinal ischemia–reperfusion. Intestinal ischemia–reperfusion (IR) model mice (30-min ischemia followed by 3 h of reperfusion) were treated with saline (IR group) or 10 mg/kg recombinant thrombomodulin (IR + rTM group). The expression of tumor necrosis factor-α (TNF-α) messenger RNA (mRNA) was evaluated in the liver (*A*; sham *vs.* IR group, $P = 0.006$; IR *vs.* IR + rTM group, $P = 0.004$), jejunum (*B*; sham *vs.* IR group, $P = 0.020$; IR *vs.* IR + rTM group, $P = 0.866$), lung (*C*; sham *vs.* IR group, $P = 0.048$; IR *vs.* IR + rTM group, $P = 0.587$), and kidney (*D*; sham *vs.* IR group, $P = 0.309$; IR *vs.* IR + rTM group, $P = 0.806$; n = 7 mice per group, n = 21 total). The expression of keratinocyte-derived chemokine (KC) mRNA was also evaluated in the liver (*E*; sham *vs.* IR group, $P = 0.004$; IR *vs.* IR + rTM group, $P = 0.002$), jejunum (*F*; sham *vs.* IR group, $P = 0.018$; IR *vs.* IR + rTM group, $P = 0.174$), lung (*G*; sham *vs.* IR group, $P = 0.644$; IR *vs.* IR + rTM group, $P = 0.309$), and kidney (*H*; sham *vs.* IR group, $P = 0.020$; IR *vs.* IR + rTM group, $P = 0.296$; n = 7 mice per group, n = 21 in total). The data are presented as whisker box plots wherein the middle line indicates the median; the upper and lower box lines indicate the third and first quartiles, respectively; and the whiskers extend to the highest and lowest values within 1.5 times the interquartile range from the box edges. *$P < 0.05$ *versus* respective control.

ischemia–reperfusion increased vascular permeability in the intestine and all remote organs (fig. 5, E to H). However, mice treated with recombinant thrombomodulin exhibited a statistically significant reduction in vascular leakage only in the liver (fig. 5E). Organ samples could not be obtained from one mouse in the ischemia–reperfusion plus recombinant thrombomodulin group because of intraoperative death.

Intense Histone Accumulation in the Liver after Intestinal Ischemia–Reperfusion Injury Was Reduced by Recombinant Thrombomodulin Administration

Histone levels in the cytoplasm and extracellular spaces of tissues were evaluated by removing the nuclear fractions from homogenized organs harvested after intestinal ischemia–reperfusion. The increment of histones of jejunum, liver, lung, and kidney, which was calculated in comparison with histone contents from sham-operated mice, is shown in figure 6A. The jejunum exhibited the largest increase in histone accumulation among all assessed organs, whereas the liver exhibited the largest increase among the remote organs (fig. 6A). Treatment with recombinant thrombomodulin reduced histone accumulation in the liver but did not have a similar impact in the jejunum (fig. 6, B and C). Furthermore, recombinant thrombomodulin reduced the plasma histone levels after intestinal ischemia–reperfusion (fig. 6D). Similarly, the plasma levels of high morbidity group box-1, another damage-associated molecular pattern, were also increased by intestinal ischemia–reperfusion and reduced by recombinant thrombomodulin (fig. 6E).

Neutrophil Extracellular Trap Formation Contributed to Local and Distant Organ Injury during Intestinal Ischemia–Reperfusion

To determine whether the increases in histone accumulation in organ tissues were associated with neutrophil extracellular trap formation, we subjected cryosections of the jejunum, liver, lung, and kidney to immunofluorescence staining and confocal microscopy. In the ischemia–reperfusion–injured jejunum, Ly6G/C-positive cells (*i.e.*, neutrophils) colocalized with citrullinated histone H3 and DNA, indicating neutrophil extracellular trap formation (fig. 7A). Active neutrophil extracellular trap formation was also detected in the liver (fig. 7B), whereas few Ly6G/C-positive cells and little citrullinated histone H3 staining were observed in the lung and kidney after intestinal ischemia–reperfusion (Supplemental Digital Content 4, http://links.lww.com/ALN/C19; the figure shows representative images of immunofluorescence staining of the lung and kidney). A quantitative analysis of the percentages of citrullinated histone H3–positive and Ly6G/C-positive areas in each field of view revealed prominent increases in neutrophil extracellular trap formation in the jejunum and liver. Moreover, the amount of neutrophil extracellular traps showed similar distribution among the jejunum, liver, lung, and kidney to histone contents determined by enzyme-linked immunosorbent assay (fig. 7, C and D). The administration of recombinant thrombomodulin diminished neutrophil extracellular trap accumulation in the liver. However, neutrophil extracellular traps remained abundant in jejunal tissues from ischemia–reperfusion plus recombinant thrombomodulin mice (fig. 8A). A quantitative analysis of neutrophil extracellular traps revealed that recombinant thrombomodulin reduced the amount of neutrophil extracellular traps augmented by intestinal ischemia–reperfusion in the liver, but not in the jejunum (fig. 8B).

Discussion

Multiple-organ dysfunction syndrome, a leading cause of death in intensive care settings, increases the intensive care unit stays of critically ill patients and thus represents a major economic burden.[26,27] The prevention of multiple-organ dysfunction syndrome relies on the attenuation of distant organ injury consequent to a single-organ insult. In this study, we demonstrated that histones and neutrophil extracellular trap formation contribute to distant liver injury after an intestinal ischemic insult. We also demonstrated that recombinant thrombomodulin attenuated liver injury by abrogating the increases in histone accumulation and neutrophil extracellular trap formation, which may have led to improved survival in intestinal ischemia–reperfusion–injured mice.

Recent basic studies of intestinal ischemia–reperfusion models have demonstrated a critical role for the gut in the development of multiple-organ dysfunction. An intestinal ischemia–reperfusion injury exerts detrimental effects on various remote organs, including the liver, lung, and kidney.[3,4,25] These effects may be attributable to a mechanism involving platelet activation together with complements or the release of interleukin-17A from Paneth cells.[3,4] Nevertheless, the mechanisms of organ crosstalk between the ischemia–reperfusion–injured intestine and extraintestinal organs remain somewhat unclear. We observed marked increases in histone accumulation and neutrophil extracellular trap formation in the liver after intestinal ischemia–reperfusion, and these changes may contribute to additional liver injury and damage-associated molecular pattern propagation. Additionally, recombinant thrombomodulin clearly protected the liver from injury in this context. Because recombinant thrombomodulin binds to histones to neutralize their thrombogenic effect,[16] this agent may prevent subsequent liver injury by capturing histones and blocking neutrophil extracellular trap formation.

Why did we only observe a prominent augmentation of histones in the liver in this study? Two explanations appear relevant. First, extracellular histones and neutrophils primed to form neutrophil extracellular traps, which were released from the ischemia–reperfusion–injured intestine, may have become trapped initially in the liver sinusoids *via* the portal

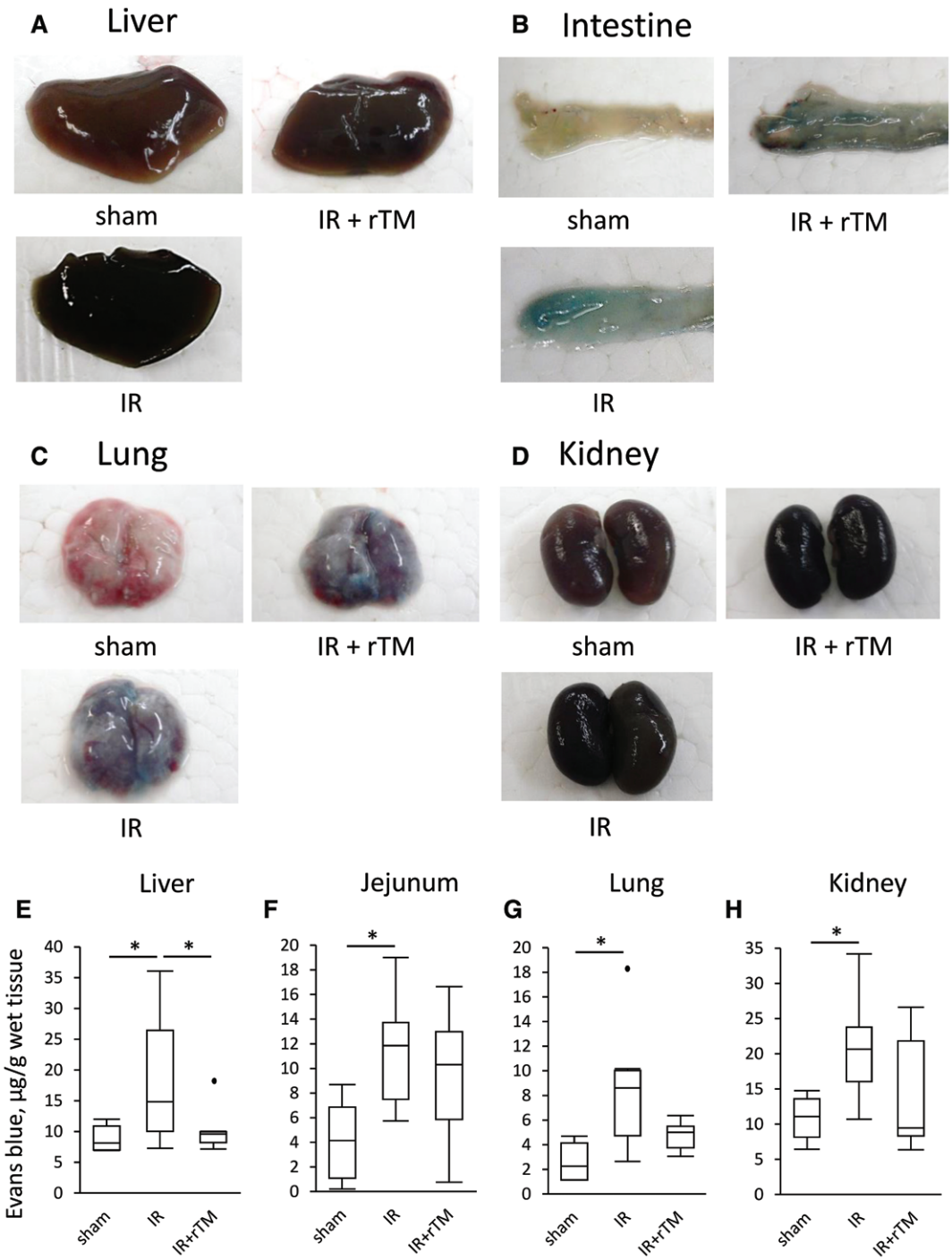

Fig. 5. Vascular permeability in mice subjected to intestinal ischemia–reperfusion. Intestinal ischemia–reperfusion (IR) model mice (30-min ischemia followed by 3 h of reperfusion) were treated with saline (IR group) or 10 mg/kg recombinant thrombomodulin (IR + rTM group). (*A to D*) Representative images of the liver, jejunum, lung, and kidney from the mice injected with Evans blue dye. Vascular leakage was evaluated in the liver (*E*; sham *vs.* IR group, $P = 0.039$; IR *vs.* IR + rTM group, $P = 0.035$), jejunum (*F*; sham *vs.* IR group, $P = 0.006$; IR *vs.* IR + rTM group, $P = 0.728$), lung (*G*; sham *vs.* IR group, $P = 0.044$; IR *vs.* IR + rTM group, $P = 0.157$), and kidney (*H*; sham *vs.* IR group, $P = 0.007$; IR *vs.* IR + rTM group, $P = 0.247$; n = 8 mice in the sham group, n = 10 in the IR group, and n = 9 in the IR + rTM group, n = 27 total). The data are exhibited as whisker box plots wherein the middle line indicates the median; the upper and lower box lines indicate the third and first quartiles, respectively; and the whiskers extend to the highest and lowest values within 1.5 times the interquartile range from the box edges. Outliers beyond the whiskers are indicated as *dots*. *$P < 0.05$ *versus* respective control.

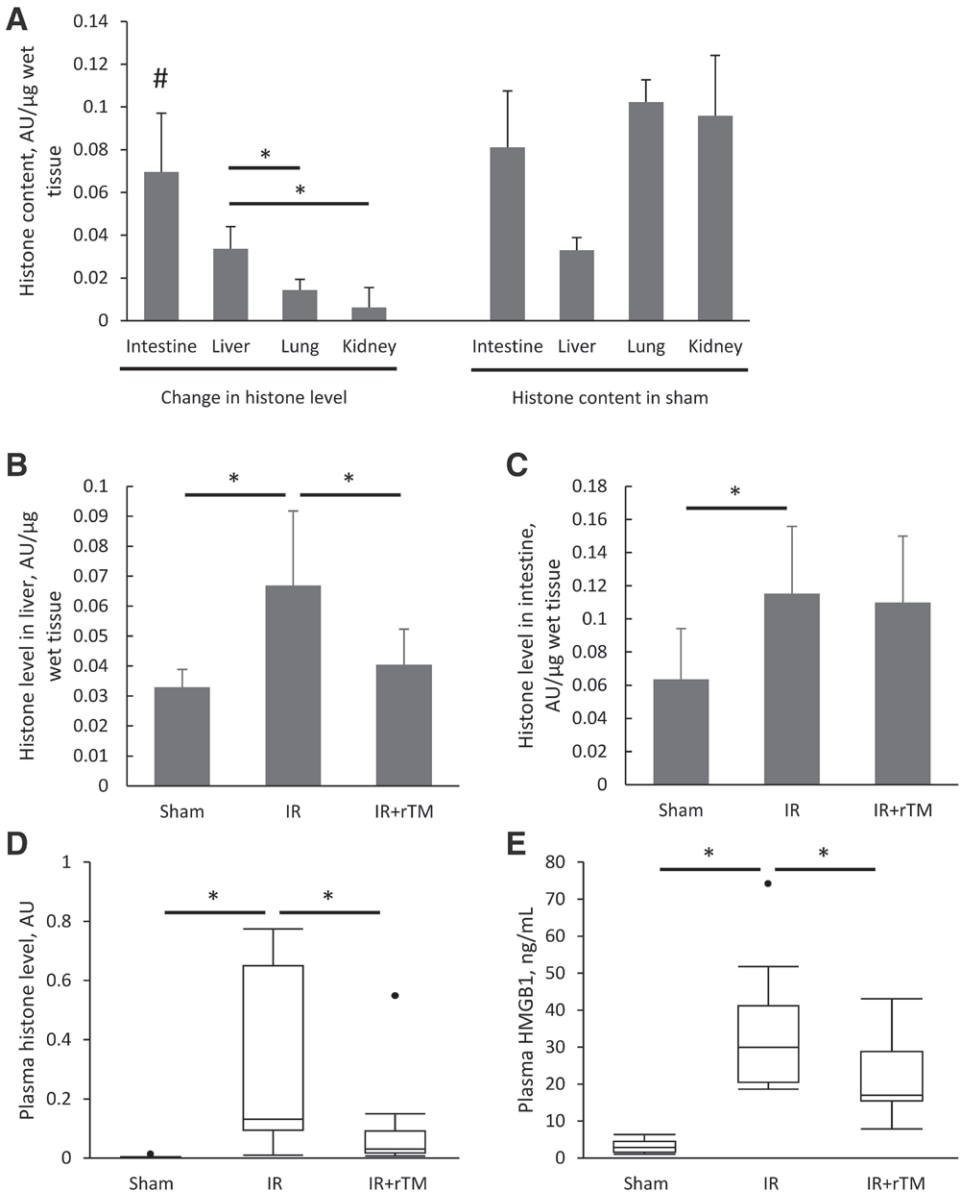

Fig. 6. Histone accumulation in the organs and plasma levels of high morbidity group box-1 after intestinal ischemia–reperfusion. (*A*) Intense histone accumulation was exhibited in the jejunum and liver harvested from mice subjected to 30-min ischemia followed by 3 h of reperfusion. Changes in the histone level in each organ were calculated by subtracting the average histone contents in sham-operated mice from histone measurements in the intestinal ischemia–reperfusion (IR)-injured mice (intestine *vs.* liver, $P = 0.011$; intestine *vs.* lung, $P = 0.003$; intestine *vs.* kidney, $P = 0.011$; liver *vs.* lung, $P = 0.008$; liver *vs.* kidney, $P = 0.011$; lung *vs.* kidney, $P = 0.241$; n = 7 mice per group, n = 28 total). Histone measurements in the sham-operated mice are also shown (n = 7 mice per group, n = 28 total). (*B* to *D*) The impact of 10 mg/kg recombinant thrombomodulin (rTM) on histone levels in the liver (*B*; sham *vs.* IR group, $P = 0.015$; IR *vs.* IR + rTM group, $P = 0.030$; n = 7 mice per group, n = 21 total), jejunum (*C*; sham *vs.* IR group, $P = 0.025$; IR *vs.* IR + rTM group, $P = 0.752$; n = 7 mice per group, n = 21 total), and plasma after intestinal IR (*D*; sham *vs.* IR group, $P = 0.001$; IR *vs.* IR + rTM group, $P = 0.024$; n = 7 mice per group, n = 21 total). (*E*) Levels of high morbidity group box-1 (HMGB1) in the plasma (sham *vs.* IR group, $P = 0.002$; IR *vs.* IR + rTM group, $P = 0.025$; n = 7 mice per group, n = 21 total). The histone and HMGB1 levels in the plasma are exhibited as whisker box plots wherein the middle line indicates the median; the upper and lower box lines indicate the third and first quartiles, respectively; and the whiskers extend to the highest and lowest values within 1.5 times the interquartile range from the box edges. Outliers beyond the whiskers are indicated as *dots*. #$P < 0.05$ *versus* all other groups; *$P < 0.05$ *versus* respective control. AU, absorbance unit.

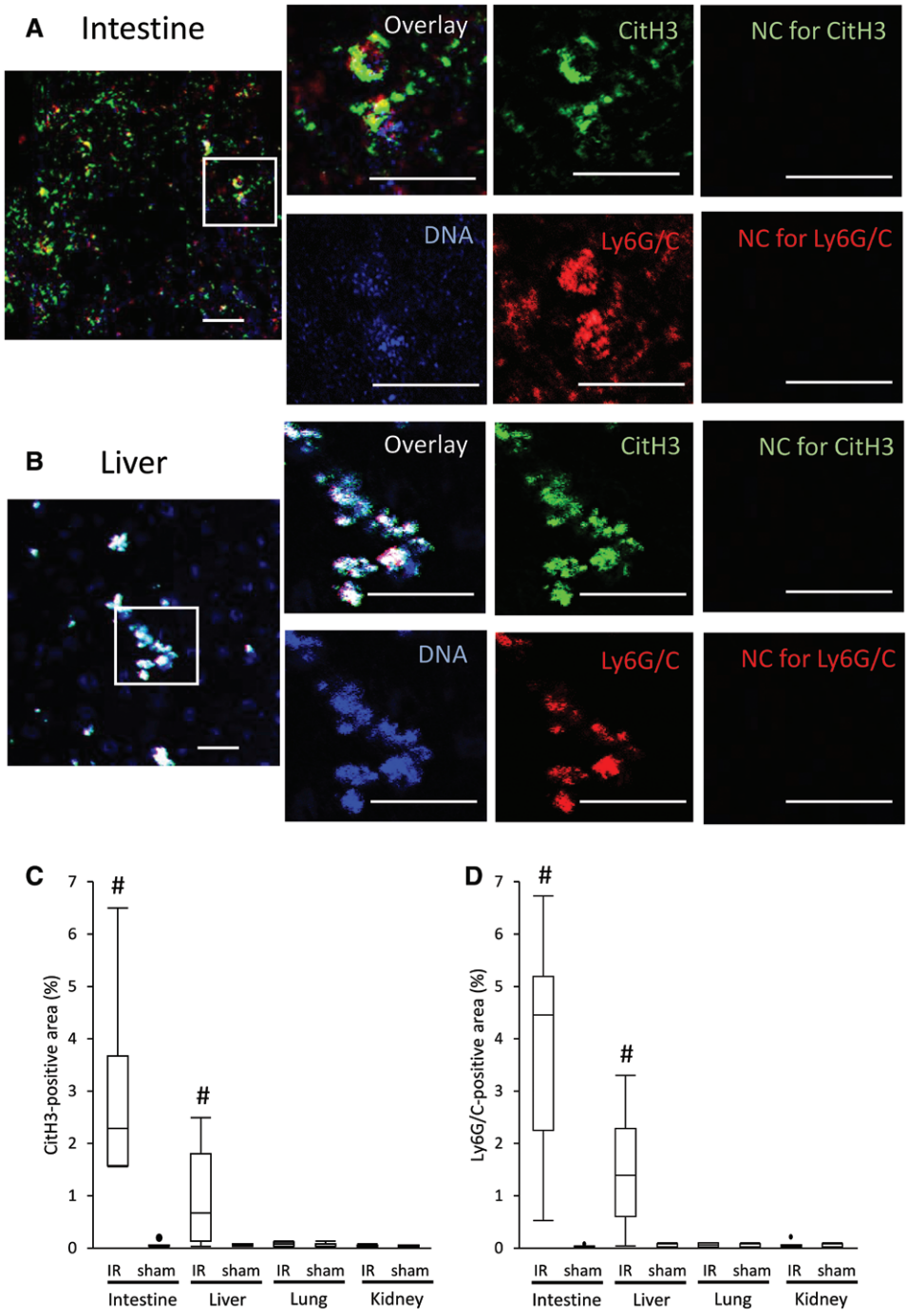

Fig. 7. Neutrophil extracellular trap formation in the intestine and liver after intestinal ischemia–reperfusion. Prominent neutrophil extracellular trap formation was detected in the jejunum (*A*) and liver (*B*) after intestinal ischemia–reperfusion (IR). Representative images of neutrophil extracellular traps are shown. Positive staining for citrullinated histone H3 (CitH3), Ly6G/C, and DNA are indicated in *green, red,* and *blue*, respectively. The *scale bar* indicates 30 μm. The colocalization of CitH3, Ly6G/C, and DNA indicates neutrophil extracellular trap formation. A sample not stained with primary antibodies for CitH3 and Ly6G/C is shown as a negative control (NC). (*C* and *D*) Areas of abundant CitH3-positivity (*C*; intestine, $P = 0.003$ *vs.* sham; liver, $P = 0.013$ *vs.* sham; lung, $P = 0.668$ *vs.* sham; kidney, $P = 0.519$ *vs.* sham; n = 7 mice per group, n = 56 total), and intense neutrophil infiltration (*D*; intestine, $P < 0.001$ *vs.* sham; liver, $P = 0.003$ *vs.* sham; lung, $P = 0.409$ *vs.* sham; kidney, $P = 1.000$ *vs.* sham; n = 7 mice per group, n = 56 total) were detected in the jejunum and liver. The data are exhibited as whisker box plots wherein the middle line indicates the median; the upper and lower box lines indicate the third and first quartiles, respectively; and the whiskers extend to the highest and lowest values within 1.5 times the interquartile range from the box edges. Outliers beyond the whiskers are indicated as *dots*. #$P < 0.05$ *versus* sham.

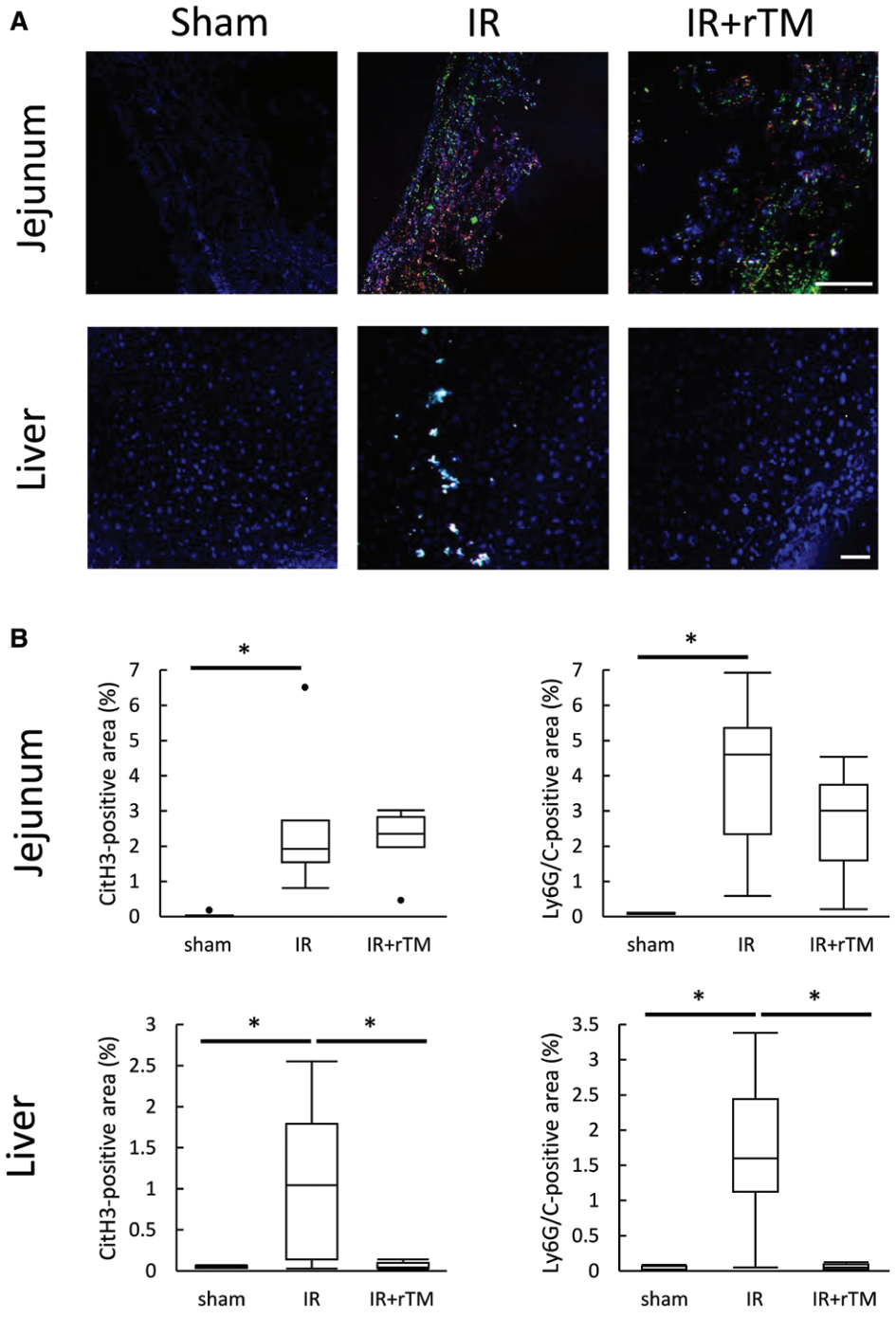

Fig. 8. Impact of recombinant thrombomodulin on neutrophil extracellular trap formation after intestinal ischemia–reperfusion (IR). (*A*) Representative images of neutrophil extracellular traps in the jejunum and liver. Positive staining for citrullinated histone H3 (CitH3), Ly6G/C, and DNA are indicated in *green*, *red*, and *blue*, respectively. Treatment with recombinant thrombomodulin (rTM) reduced neutrophil extracellular trap formation in the liver but not in the jejunum. The *scale bar* indicates 50 μm. (*B*) CitH3-positive (sham *vs.* IR group, $P = 0.022$; IR *vs.* IR + rTM group, $P = 0.029$; n = 7 mice group, n = 21 total) and Ly6G/C-positive areas (sham *vs.* IR group, $P = 0.012$; IR *vs.* IR + rTM group, $P = 0.007$; n = 7 mice per group, n = 21 total) in the liver and CitH3-positive (sham *vs.* IR group, $P = 0.006$; IR *vs.* IR + rTM group, $P = 0.801$; n = 7 mice per group, n = 21 total) and Ly6G/C-positive areas (sham *vs.* IR group, $P = 0.009$; IR *vs.* IR + rTM group, $P = 0.286$; n = 7 mice per group, n = 21 total) in the jejunum. The data are exhibited as whisker box plots wherein the middle line indicates the median; the upper and lower box lines indicate the third and first quartiles, respectively; and the whiskers extend to the highest and lowest values within 1.5 times the interquartile range from the box edges. Outliers beyond the whiskers are indicated as *dots*. *$P < 0.05$ *versus* respective control.

vein. Consistent with this speculation, an assessment of distant organs from a model of ischemic acute kidney injury detected neutrophil extracellular trap formation mainly in the lung, which is anatomically the first organ to encounter histones and neutrophils from injured kidneys.[11] Second, the distribution pattern of histones in remote organs may reflect the enriched reticuloendothelial system in the liver, which contains a diverse population of immunologically active cells.[28,29] Recent basic studies of methicillin-resistant *Staphylococcus aureus* bacteremia models reported that this bacterial strain accumulated most intensely in the liver, leading to severe liver injury *via* active neutrophil extracellular trap production.[9,30]

During intestinal ischemia–reperfusion, necroptosis of the ischemic intestine may trigger the release of damage-associated molecular patterns and induce neutrophil extracellular trap formation. A recent report suggests that necroptosis inhibitors such as necrostatin-1 may potently intervene in this process.[31] Second, extracellular histones and neutrophil extracellular trap-forming neutrophils may be trapped in the liver and thus cause acute liver injury. In the liver, strongly positively charged extracellular histones would have direct cytotoxic effects on the surrounding hepatocytes.[32] Neutrophil extracellular trap-derived neutrophil elastase also has devastating proteolytic effects on bystander cells in the liver.[9] The causal relationship of histones and neutrophil extracellular trap formation with liver injury is supported by previous studies demonstrating the abilities of anti-histone antibodies and peptidyl arginine deiminase-4 inhibitor to attenuate liver injury in hepatic ischemia–reperfusion models.[5,10] Our study showed that recombinant thrombomodulin administration blocked the accumulation of histones and neutrophil extracellular traps in the liver and attenuated subsequent injury. Whereas our results imply that postintestinal ischemia–reperfusion survival could be improved by blocking histones and neutrophil extracellular traps, some reports suggested that the elimination of neutrophil extracellular trap formation increased mortality in sepsis models by decreasing immunoprotection against various pathogens.[33,34] Further studies are warranted to evaluate the impacts of damage-associated molecular pattern and neutrophil extracellular trap blockade on clinically important outcomes in patients with multiple-organ dysfunction syndrome.

Our study revealed increased expression of inflammatory cytokines in the jejunum and liver, corresponding to the abundant histone accumulation and neutrophil extracellular trap formation observed after intestinal ischemia–reperfusion. Histone blockade by the injection of recombinant thrombomodulin substantially suppressed the proinflammatory response. These results suggest that histones activate neutrophils through pattern recognition receptors, including Toll-like receptors, to increase the production of inflammatory cytokines, consistent with previous findings from both hepatic and renal ischemia–reperfusion models.[5,11] Highly positively charged histones have been shown to damage endothelial cells and cause increased vascular permeability.[35] In this study, increased vascular permeability was observed in all organs after intestinal ischemia–reperfusion, regardless of the distribution of histones. However, recombinant thrombomodulin treatment attenuated vascular leakage only in the liver, indicating a partial contribution of histones to increased vascular permeability in the intestinal ischemia–reperfusion model.

Evaluations of lung and kidney tissues revealed moderately elevated levels of histones, vascular permeability, and cytokine expression. After intestinal ischemia–reperfusion, the histones and activated neutrophils first accumulated in the liver would next reach the lung *via* the inferior vena cava and, subsequently, the kidney. Additionally, previous studies have reported platelet aggregation in the lung and increased Paneth cell–derived interleukin-17A in the kidney after intestinal ischemia–reperfusion, and these immune effectors may promote inflammatory responses and vascular leakage.[3,4] The factors described above would likely affect lung and renal injury after intestinal ischemia–reperfusion.

Recent animal studies revealed that high morbidity group box-1 levels begin to increase within 1 h after ischemia–reperfusion injury, in contrast to the role of this factor as a late mediator in sepsis.[36,37] Our study similarly observed an increased plasma high morbidity group box-1 level after 3 h of reperfusion. The high morbidity group box-1 level decreased after recombinant thrombomodulin treatment, in accordance with a previous report describing the anti-inflammatory function of thrombomodulin by binding to high morbidity group box-1 *via* its lectin-like domain.[15] As shown in a previous study of acute kidney injury models, high morbidity group box-1 might play a pivotal role in the occurrence of distant organ injury after intestinal ischemia–reperfusion by enhancing the inflammatory response and neutrophil extracellular trap formation.[21]

Interestingly, recombinant thrombomodulin administration was ineffective in the jejunum, which exhibited the highest level of histone accumulation among the assessed organs. This result implies some potential causes of the distinct effects of recombinant thrombomodulin in the intestine and liver. First, the level of histones in the ischemia–reperfusion–injured intestine may exceed the neutralization capacity of recombinant thrombomodulin. Second, other immune effectors such as superoxide, which is not a target of recombinant thrombomodulin, contribute to local intestinal injury after an ischemia–reperfusion insult.[38,39] Third, intestinal ischemia–reperfusion causes a loss of paracellular barrier integrity in the intestine, which enables the penetration of pathogen-associated molecular patterns that cannot be eliminated by recombinant thrombomodulin.[40] These patterns may act synergistically with damage-associated molecular patterns to activate both innate and adaptive immunity in the intestine. Fourth, intestinal ischemia may induce intestinal necroptosis by inhibiting caspase 8, which

would allow assembly of the necroptosome and the release of cell death–associated molecular patterns.[41] This process cannot be prevented by recombinant thrombomodulin. Finally, intraperitoneally injected recombinant thrombomodulin may initially accumulate in the liver *via* the portal vein, leading to the specific attenuation of injury in the liver.[42]

Several limitations of our study should be acknowledged. First, our results do not directly demonstrate a causal relationship of histone accumulation and neutrophil extracellular trap formation with local/remote organ dysfunction because recombinant thrombomodulin is expected to have several different targets. Further investigation is needed to determine whether recombinant thrombomodulin–mediated histone blockade reduces the severity of organ injury, using preparations such as recombinant thrombomodulin with preblocked histone binding sites. Second, some experimental evidence suggests that the oxygen and carbon dioxide tension may affect inflammation in both local and distant organs after intestinal ischemia–reperfusion.[43,44] These factors were not examined in this study. Uncontrolled oxygenation and diffusion of carbon dioxide and insufficient resuscitation under inflammatory conditions could be a major problem in translation of our results into a clinical context. Third, the difference in the numbers of parameters included in the organ injury scoring systems might have led to inconsistencies in the sensitivity of tissue damage detection among the organs. Fourth, the Cell Death Detection ELISA^{plus} could not specifically detect histones derived from neutrophil extracellular traps. However, we conducted an immunofluorescence assay to detect neutrophil extracellular traps and quantified neutrophil extracellular trap formation to confirm the enzyme-linked immunosorbent assay results. Fifth, our results may not be generalizable to females. We studied only male mice because they do not have estrous cycle that may affect pharmacokinetics of study drugs and inflammatory response.[45,46]

In conclusion, this study demonstrated that histone accumulation and neutrophil extracellular trap formation might mediate distant organ damage after intestinal ischemia–reperfusion. The administration of recombinant thrombomodulin ameliorated remote liver injury by blocking histones and neutrophil extracellular trap production, thereby contributing to decreased mortality after intestinal ischemia–reperfusion. Histones and neutrophil extracellular traps are potential therapeutic targets in critically ill patients with multiple-organ dysfunction syndrome. Therefore, drugs that target these mediators, including recombinant thrombomodulin, are expected to improve the outcomes of such patients.

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Competing Interests

Asahi Kasei Pharma (Tokyo, Japan) generously provided recombinant thrombomodulin but did not contribute to the study design, data analysis, or preparation of the manuscript. The authors declare no competing interests.

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