ORIGINAL ARTICLE

AJT

The lectin-like domain of thrombomodulin is a drug candidate for both prophylaxis and treatment of liver ischemia and reperfusion injury in mice

Junya Kawasoe[1,3] | Yoichiro Uchida[1,3] | Tomoyuki Miyauchi[1,3] | Kentaro Kadono[1] | Hirofumi Hirao[1] | Kenichi Saga[1,3] | Takeshi Watanabe[2] | Shugo Ueda[3] | Hiroaki Terajima[1,3] | Shinji Uemoto[1]

[1]Division of Hepato-Biliary-Pancreatic Surgery and Transplantation, Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

[2]Division of Immunology, Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan

[3]Department of Gastroenterological Surgery and Oncology, The Tazuke Kofukai Medical Research Institute, Kitano Hospital, Osaka, Japan

Correspondence
Yoichiro Uchida
Email: uchiday@kuhp.kyoto-u.ac.jp

Funding information
The Tazuke Kofukai Medical Research Institute, Kitano Hospital; The Ministry of Education, Culture, Science and Sports, Japan, Grant/Award Number: 15K10041

Ischemia and reperfusion injury (IRI) can occur in any tissue or organ. With respect to liver transplantation, the liver grafts from donors by definition experience transient ischemia and subsequent blood reflow. IRI is a problem not only in organ transplantation but also in cases of thrombosis or circulatory disorders such as mesenteric ischemia, myocardial, or cerebral infarction. We have reported that recombinant human soluble thrombomodulin (rTM), which is currently used in Japan to treat disseminated intravascular coagulation (DIC), has a protective effect and suppresses liver IRI in mice. However, rTM may not be fully safe to use in humans because of its inherent anticoagulant activity. In the present study, we used a mouse liver IRI model to explore the possibility that the isolated lectin-like domain of rTM (rTMD1), which has no anticoagulant activity, could be effective as a therapeutic modality for IRI. Our results indicated that rTMD1 could suppress ischemia and reperfusion-induced liver damage in a dose-dependent manner without concern of associated hemorrhage. Surprisingly, rTMD1 suppressed the liver damage even after IR insult had occurred. Taken together, we conclude that rTMD1 may be a candidate drug for prevention of and therapy for human liver IRI without the possible risk of hemorrhage.

KEYWORDS
basic (laboratory) research / science, ischemia reperfusion injury (IRI), liver disease: immune / inflammatory, liver transplantation / hepatology

---

- - - - - - - - - -

1 | INTRODUCTION

Ischemia and reperfusion injury (IRI) results from the return of blood flow to a tissue or organ after an initial hypoxic insult.[1] IRI is particularly problematic in organ transplantation, as it results in damage to endothelium and immune cell activation. With respect to liver transplantation, IRI is the underlying problem in approximately 10% of early graft failures and it can lead to an even higher incidence of both acute and chronic rejection.[2] As such, overcoming IRI is a critical issue for the preservation of liver grafts and for successful liver transplantation. The production of reactive oxygen species (ROS), the activation of complement components such as the anaphylatoxins, and the release of various cytokines and biochemical mediators from immune cells, in response to microcirculatory pathology, are among the causative mechanisms underlying IRI.[3] It is well known that damage-associated molecular patterns (DAMPs) such as high-mobility group box 1 (HMGB-1) are released from macrophages and related inflammatory cells as well as parenchymal hepatocytes[4] at the onset of ischemia and reperfusion (IR), and that inflammatory responses triggered by HMGB-1 and other mediators result in severe damage to liver cells.[5,6] We have previously described that recombinant human soluble thrombomodulin (rTM) protects liver tissue from IRI via blocking of the damages induced by HMGB-1/Toll-like receptor 4 (TLR-4) interaction.[7] Thrombomodulin (TM) has been detected mainly on the surface of vascular endothelial cells and consists of a lectin-like amino-terminal domain (domain 1), followed by a hydrophobic segment, 6 contiguous epidermal growth factor (EGF)-like domains (domain 2), an O-glycosylated serine/threonine-rich domain (domain 3), a transmembrane segment, and a short cytoplasmic tail. TM binds to thrombin via interactions at domain 2, forming 1:1 complex via the 4th and 5th repeats of the EGF-like motif. The thrombomodulin-thrombin complex functions as an anticoagulant via interactions with protein C.[8-10] rTM includes domains 1, 2, and 3 but lacks the transmembrane segment and cytoplasmic tail. rTM has been utilized clinically in Japan for the treatment of disseminated intravascular coagulation (DIC) by an activity promoted by domain 2.[11] There are many reports that suggest that the administration of rTM might ameliorate IR-induced damage in various organs.[12-17] We have previously reported that rTM blocked HMGB-1 binding to TLR-4, which was followed by suppression of nuclear factor kappa B (NF-κB)–induced inflammation and diminished levels of IR-induced liver injury in mice.[7] Recent reports showed the anti-inflammatory effect of rTM relied mainly on the actions of domain 1 (rTMD1).[18-20] rTMD1 includes 154 amino acids and blocks TLR-4 signaling by binding to pathogen-associated molecular pattern molecules such as lipopolysaccharide (LPS).[20] rTMD1 was more effective than rTM for inhibition of the inflammatory response initiated by the interaction of LPS and TLR-4. It has also been reported that rTMD1 may bind to HMGB-1[18,20] and that the administration of rTMD1 alone promotes protection against IRI in lung[21] and heart[22] via the activated protein C (APC)–independent mechanisms. In the mouse lung IRI model, levels of IL-1β and granulocyte-monocytic colony-stimulating factor in bronchoalveolar lavage fluid obtained from *tmd1* gene–deleted (TMD1 KO) mice were significantly increased compared to those detected in the wild-type (WT).[21] Moreover, in the mouse myocardial IRI model, myocardial infarcts were

significantly larger in TMD1 KO mice compared to those sustained by their WT counterparts.[22] These reports suggest that the endogenous domain 1 of TM has a protective effect against IR-induced insult.

In the present study, we show that intravenous administration of rTMD1 suppresses liver IRI to the same extent as the entire rTM molecule and that rTMD1 was effective not only as prophylaxis but also as treatment after reperfusion was initiated. This is the first study that has shown clearly that rTMD1 has the capacity to suppress IR-induced liver damage even after reperfusion is in progress. These findings indicate that rTMD1 may be a candidate for further consideration as an important therapeutic agent for the prevention as well as therapy of IRI without the possible risk of hemorrhage.

2 | MATERIALS AND METHODS

2.1 | Animals

Male C57BL/6 mice (8 to 10 weeks old, weighing 20 to 25 g) were purchased from Shimizu Laboratory Supplies. *tlr-4* gene–deleted (TLR-4 KO) mice on a C57BL/6 genetic background were purchased from Oriental Bio Service (Kyoto, Japan). All animals were maintained in specific pathogen-free conditions and received humane care according to the Guide for Care and Use of Laboratory Animals. All experimental protocols were approved by the Animal Research Committee of The Tazuke Kofukai Medical Research Institute, Kitano Hospital.

2.2 | Reagents

rTM, rTMD1, and other rTM domain proteins (rTMD2, rTMD3) used in the present study were provided from Asahi Kasei Pharma. Each domain protein of rTM was produced in the *Pichia pastoris* expression system using the pPICZαA as a plasmid vector. Both 6 × His tag and c-Myc epitope were used for protein purification and detection. The purified proteins were examined by western blotting or Coomassie Brilliant Blue (CBB) staining after sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). The structures and western blotting patterns of rTM and rTMD1 are shown in Figure 1. Anti-TM antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Myc-Tag antibody was purchased from Cell Signaling Technology. CBB staining patterns of rTM and each rTM subdomain (rTMD1, rTMD2, and rTMD3) are shown in Figure S1.

2.3 | Liver IRI and treatment with rTM and rTM domain proteins

All mice were subjected to a nonlethal mouse model of segmental (70%) hepatic warm IR as described previously,[7,23,24] which is an ischemic insult for 60 minutes by interruption of the artery, portal venous supply, and bile duct to the left and middle liver lobes,
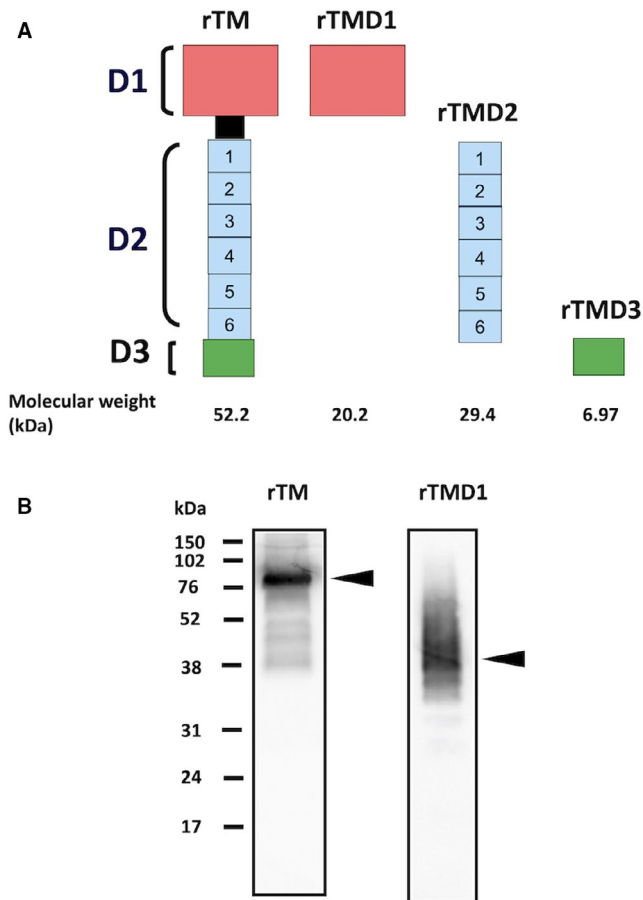
FIGURE 1 rTM and each rTM domain protein used in this study. A, The structures of rTM and each rTM domain protein are illustrated schematically. B, rTM and rTMD1 were detected by western blotting analysis using anti-TM antibody and anti-Myc-Tag antibody, respectively. rTM, recombinant human thrombomodulin; rTMD1, the domain 1 of recombinant thrombomodulin; TM, thrombomodulin [Colour figure can be viewed at wileyonlinelibrary.com]

followed by reperfusion for 6 hours. Thirty minutes before the ischemic insult, at the start of reperfusion, or 60 minutes after the start of reperfusion, mice were given an intravenous injection of each rTM domain protein dissolved in saline. Control mice were given saline only. Sham mice received the same procedure but without vascular occlusion.

2.4 | Hepatocyte function

Serum alanine aminotransferase (sALT) levels, used as a measure of liver injury, were determined by a standard spectrophotometric method with an automated clinical analyzer (JCABM9030, JEOL Ltd.).

2.5 | Histology

Liver paraffin sections (5 μm thick) were stained with hematoxylin and eosin (H&E). The severity of liver IRI (necrosis, sinusoidal

congestion, and centrilobular ballooning) was graded on a scale from 0 to 4 by an investigator who was blinded to the experimental conditions using a modified Suzuki's criteria.[25]

2.6 | Immunohistochemistry for detecting Ly-6G and CD11b

To detect Ly-6G and CD11b, antigen retrieval (Citrate, pH 6) was performed on paraffin-embedded sections of liver. After blocking, the sections were incubated in primary antibody overnight at 4°C. Then, biotinylated second antibody was applied. After incubation, immunoperoxidase (VECTASTAIN ABC Kit; Vector Labs) was applied to the sections and developed using 3,3′-diaminobenzidine (DAB). Ly-6G antibody was purchased from Tonbo Biosciences, and CD11b antibody was purchased from Abcam.

2.7 | Enzyme-linked immunosorbent assay (ELISA)

The serum HMGB-1 level was quantified with HMGB-1 ELISA Kit II (Shino-Test). Medium from peritoneal macrophage culture was analyzed for tumor necrosis factor (TNF)-α using an ELISA kit as per manufacturer's instructions (R&D Systems).
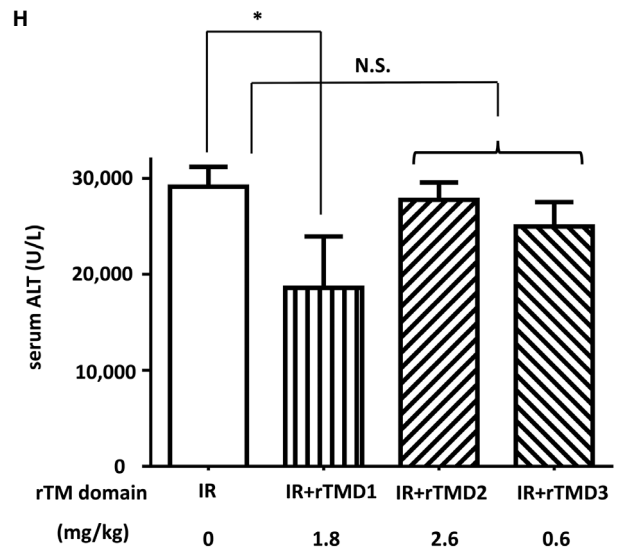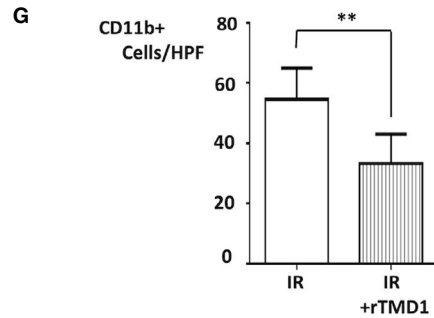
2.8 | Western blot assay

Western immunoblotting was performed using standard techniques as described previously.[24] Primary antibodies are shown as Supplementary data (Table S1).

2.9 | Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from liver tissue with RNeasy Mini Kit (QIAGEN). Complementary DNA was prepared using a PrimeScript RT Reagent Kit (Takara Bio). Quantitative polymerase chain reaction (qPCR) was performed using the StepOnePlus Real-Time PCR System (Life Technologies). Primers used to amplify specific gene fragments are listed in the legend in Figure 4. Target gene expression was calculated using the ratio to the housekeeping gene, β-actin.

2.10 | Apoptosis assay

Apoptosis in 5 μm liver paraffin sections was detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method using an in situ Apoptosis Detection Kit (Takara Bio) according to the manufacturer's instructions.

A

B

IR   IR +rTMD1(1.8mg/kg)   IR +rTMD1(18mg/kg)

C

D

Sham   Sham+rTMD1

IR   IR+rTMD1

E

Ly6G+ Cells/HPF

F

Sham   Sham+rTMD1

IR   IR+rTMD1

G

CD11b+ Cells/HPF

H

rTM domain	0	1.8	2.6	0.6
(mg/kg)	IR	IR+rTMD1	IR+rTMD2	IR+rTMD3
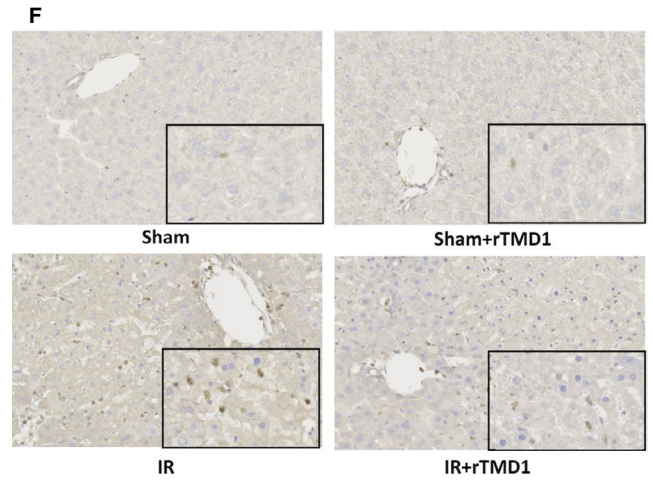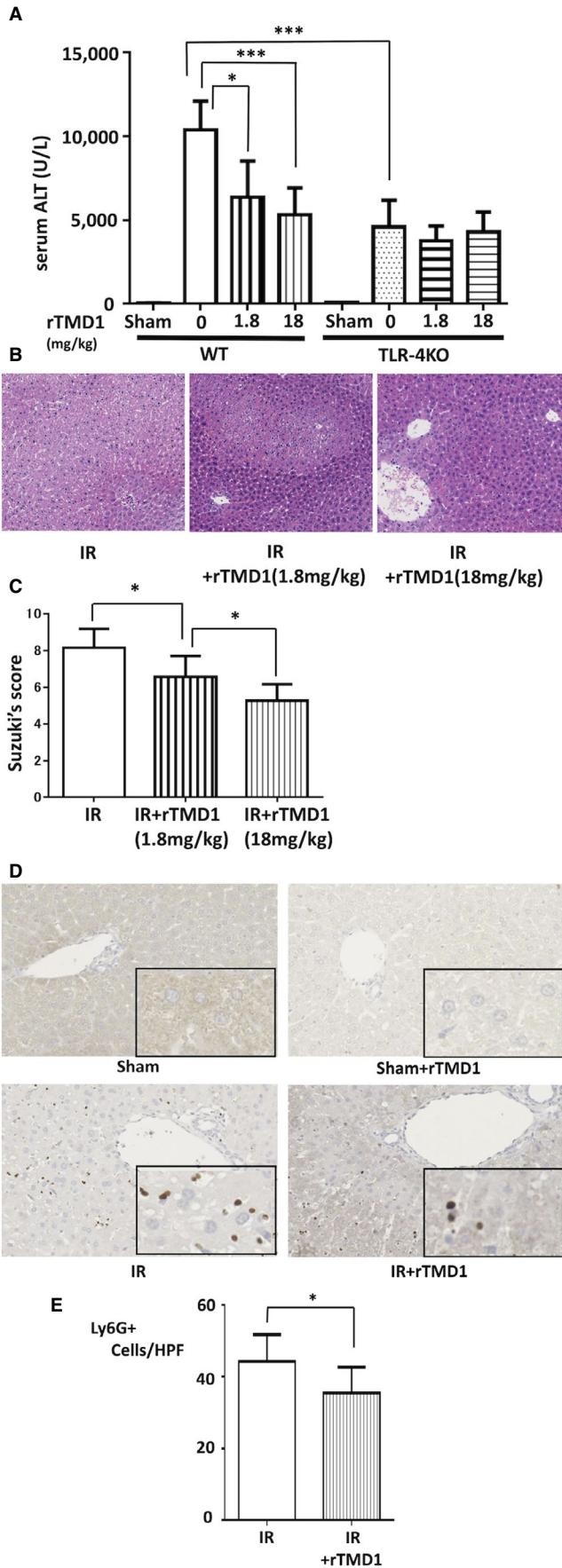
	Average Molecular Weight (Da)	Dosage for mice (mg/kg)				
		1	2	3	5	10
rTM	52171	1	2	3	5	10
rTMD1	20191	0.36	0.72	1.09	1.81	3.62
rTMD2	29392	0.53	1.05	1.58	2.64	5.27
rTMD3	6973	0.13	0.25	0.38	0.63	1.25

2.11 | Cell cultures

Thioglycollate-elicited peritoneal macrophages, which were collected using a method as we described previously,[7] were plated at 2.0×10^5 cells/well in a volume of 0.5 mL/well into 24-well cell culture plates and incubated in a humidified atmosphere of 5% CO_2 and 95% air. After 3 hours, wells were washed 3 times with phosphate-buffered saline to remove nonadherent cells, which were then incubated with or without bovine HMGB-1 (Chondrex, Redmond, WA) (1 μg/mL) and various concentrations of rTM domain proteins (n = 3 in each group). Forty-eight hours after treatment, supernatants were collected and stored at −80℃ until measurement of TNF-α by ELISA.

2.12 | Statistical analysis

All data are presented as the means ± the standard deviation. Differences between experimental groups were analyzed using one-way analysis of variance of Student's *t* test for unpaired data. All differences were considered statistically significant at the *P*-value of less than .05.

3 | RESULTS

3.1 | Pretreatment with rTMD1, but not with rTMD2 or D3, improved IR-triggered hepatocellular damage in a dose-dependent manner

Our previous study revealed that the administration of rTM attenuated liver damage in response to IR in a TLR-4 dependent manner.[7] The present study clearly indicates that a single dose of rTMD1 suppressed the mouse partial warm liver IRI to the same extent as the entire rTM molecule. rTMD1 (at 1.8 or 18 mg/kg) was administered intravenously at 30 minutes prior to the ischemic insult. Levels of sALT were measured at 6 hours after reperfusion and used as an index of liver damage resulting from IR. Pretreatment with rTMD1 resulted in significantly diminished levels of sALT to approximately 34% of the value observed in mice
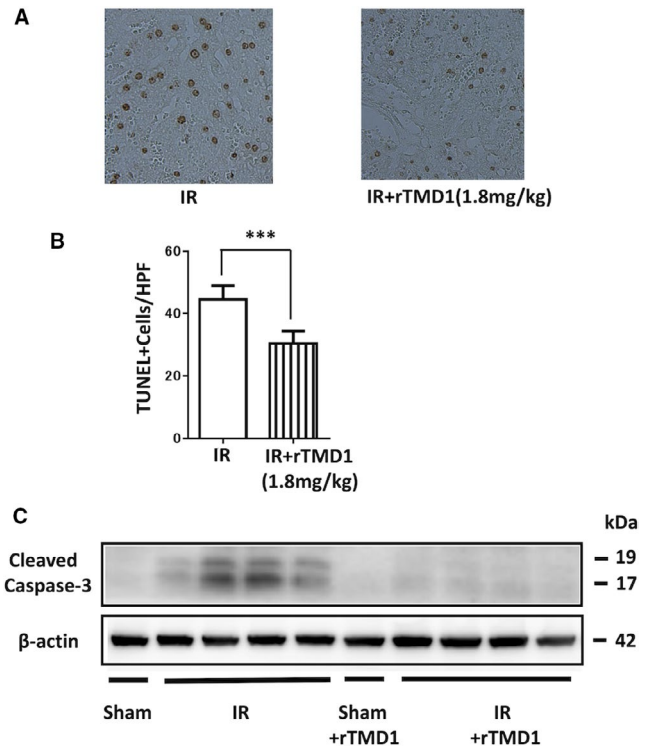
FIGURE 3 The assessment of apoptosis in hepatocytes after liver IR. A, Representative TUNEL-assisted detection of hepatic apoptosis in liver tissues after IR (magnification ×400). B, Quantification of hepatic apoptosis (n = 6 mice/group; ***P < .001). C, Western blot–assisted expression of cleaved caspase-3 at 6 h of reperfusion after 60 min of ischemia. β-actin was used as internal control. IR, ischemia and reperfusion; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling

without rTMD1 pretreatment (Figure 2A). High doses of rTMD1 (18 mg/kg) reduced the sALT levels more effectively than low doses (1.8 mg/kg). By contrast, in TLR-4 KO mice, sALT levels were lower than in the WT, and did not respond further to the administration of rTMD1. These results indicated that rTMD1 suppressed the liver injury resulting from activation of TLR-4-mediated pathways. Among the WT mice subjected to IR and not treated with rTMD1, the livers displayed pathological changes including

FIGURE 2 Pretreatment with rTMD1 ameliorates liver IRI in mice in a dose-dependent manner by inhibiting TLR-4-mediated signaling. A, The hepatocellular damage was measured by the serum level of ALT. High dose (18 mg/kg) or low dose (1.8 mg/kg) of rTMD1 was administered intravenously to WT or TLR-4 KO mice 30 min before the ischemia maneuver. IR-treated mice were given saline only. Sham-operated mice underwent the same procedure but without vascular occlusion (n = 8 mice/group; *P < .05, ***P < .001). B, Representative liver histology (hematoxylin and eosin staining) after IR insult in WT mice (magnification ×400). C, Suzuki's histological grading in each group (n = 8 mice/group; *P < .05). D, Representative immunohistochemistry of Ly-6G (dark brown spots) at 6 h of reperfusion (magnification ×400; in box, ×800). E, Quantitation of hepatic Ly-6G accumulation (n = 8 mice/group; *P < .05). F, Representative immunohistochemistry of CD11b (dark brown spots) at 6 h of reperfusion (magnification ×400; in box, ×800). G, Quantitation of hepatic CD11b accumulation (n = 8 mice/group; **P < .01). H, Each rTM domain protein was administered 30 min before initiating liver ischemia, and sALT was examined 6 h after reperfusion was initiated (n = 3-4 mice/group; *P < .05). The dosage of each rTM domain protein was calculated by its average molecular weight (rTMD1, 20.2kDa; rTMD2, 29.4kDa; rTMD3, and 6.97kDa, respectively). ALT, alanine aminotransferase; IR, ischemia and reperfusion; IRI, ischemia and reperfusion injury; rTMD1, the domain 1 of thrombomodulin; sALT, serum alanine aminotransferase; TLR-4, Toll- like receptor 4; TLR-4 KO, *tlr-4* gene–deleted; WT, wild-type

lobular edema, congestion, ballooning, and prominent hepatocellular necrosis. By contrast, the livers from rTMD1-pretreated mice subjected to IR revealed significantly less pathology as shown in Figure 2B. Suzuki's score was also significantly lower in rTMD1-pretreated mice. This protection against hepatocellular pathology was even more evident in response to increasing amounts of rTMD1 (Figure 2C). The numbers of neutrophils (Ly-6G^{+}) and macrophages (CD11b^{+}) infiltrating into liver tissues after IR were significantly lesser in rTMD1-pretreated mice (Figure 2D-G). We further determined whether other domains of rTM might have a

similar impact on liver IRI. As shown in Figure 2H, administration of either rTMD2 or rTMD3 to WT mice resulted in no suppression of IR-induced liver damage. Because it has been reported that IRI is associated with hepatocyte apoptosis,[26-28] we examined this issue in mice receiving rTMD1 pretreatment. The numbers of TUNEL-positive cells induced by IR were significantly diminished in rTMD1-pretreated WT mice compared with mice without rTMD1 pretreatment (Figure 3A,B). Moreover, the elevated levels of cleaved caspase-3 in liver cells in response to IR were reduced by the administration of rTMD1 (Figure 3C and Figure S2).
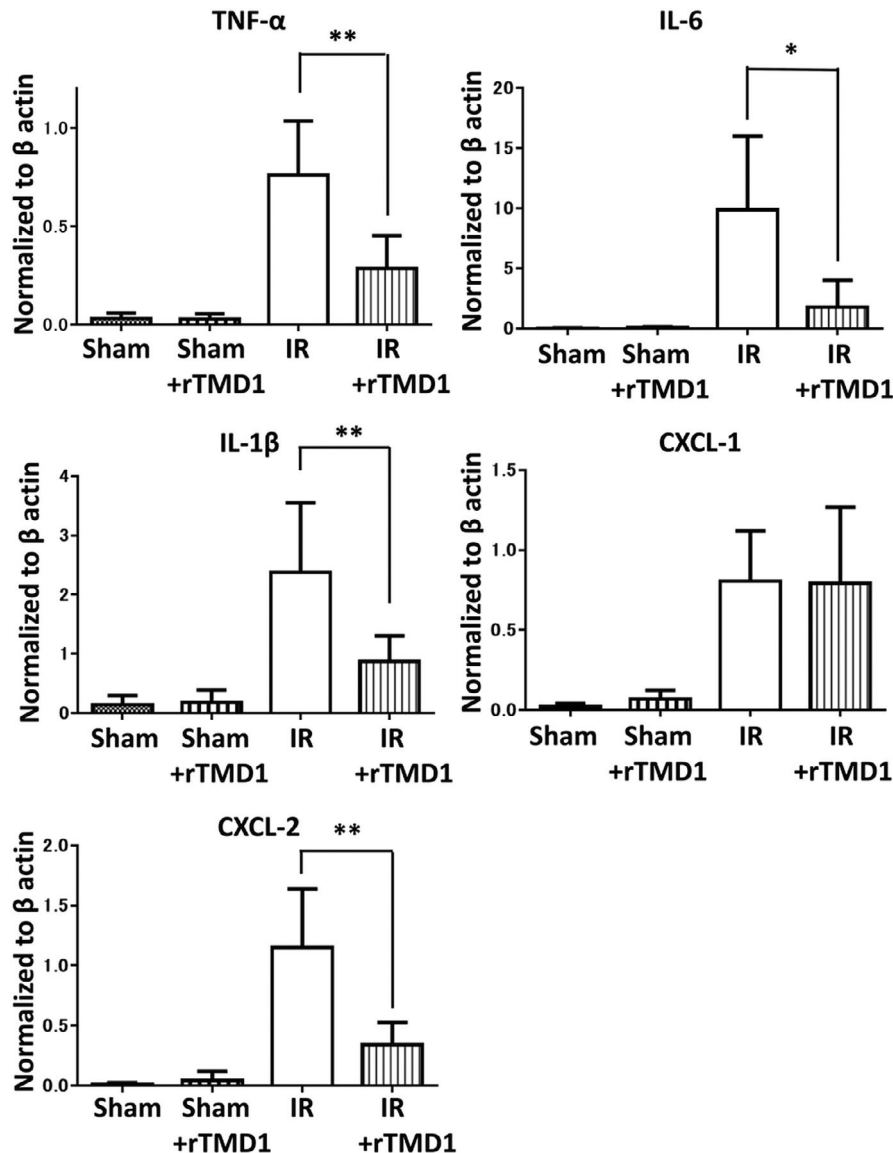

FIGURE 4 rTMD1 pretreatment suppressed pro-inflammatory cytokines and chemokine. Quantitative reverse transcription-polymerase chain reaction detection of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, CXCL-1, and CXCL-2) at 6 h of reperfusion. rTMD1 was administered before the initiation of ischemia. Data were normalized to β-actin gene expression (n = 6 mice/group; *P < .05, **P < .01). The primer couples used to amplify specific gene fragments are as follows. IL-6: (f) ACCAGAGGAAATTTTCAATAGGC, (r) TGATGCACTTGCAGAAAACA; TNF-α: (f) AGGGTCTGGGCCATAGAACT, (r) CCACCACGCTCTTCTGTCTAC; IL-1β: (f) CAGGTCGCTCAGGGTCACA, (r) CAGAGGCAAGGAGGAAACACA; CXCL-1: (f) GCTTGAAGGTGTTGCCCTCAG, (r) AAGCCTCGCGACCATTCTTG; CXCL-2: (f) TCCAGGTCAGTTAGCCTTGC, (r) CGGTCAAAAAGTTTGCCTTG; f, forward; r, reverse. rTMD1, the domain 1 of thrombomodulin; TNF-α, tumor necrosis factor-α

3.2 | Pretreatment with rTMD1 suppressed the production of pro-inflammatory cytokines and chemokines in the liver after IR

Inflammatory cytokines play a critical role in the development of liver IRI.[29,30] Moreover, CXC chemokines, which are neutrophil chemoattractants, are crucial contributors to the pathophysiology of liver IRI in mice.[30,31] Pretreatment with rTMD1 resulted in significant decrease in the expression of pro-inflammatory cytokines and CXC chemokine induced by IR in the liver including TNF-α, IL-6, IL-1β, and CXCL-2, but interestingly, not CXCL-1 (Figure 4). The reduction in inflammatory mediators likely facilitates the suppression of hepatic inflammation.

3.3 | rTMD1 pretreatment suppressed the level of serum HMGB-1 and the expression of TLR-4 in liver tissues after IR

Our previous study revealed that rTM suppresses IR-induced liver damage by modulating the HMGB-1/TLR-4 pathway. To clarify whether the anti-inflammatory effect of rTM relies mainly on rTMD1, we examined the levels of HMGB-1 in serum and the expression of TLR-4 in liver tissues after IR. The levels of serum HMGB-1 increased significantly 1 hour after reperfusion, and rTMD1 markedly reduced the levels of serum HMGB-1 6 hours after reperfusion (Figure 5A).

Moreover, the increased expression of TLR-4 in liver tissues after reperfusion was significantly suppressed by rTMD1 pretreatment (Figure 5B).

3.4 | rTMD1, but not rTMD2 or D3, is an important domain of rTM for the suppression of TNF-α production induced by HMGB-1 in macrophages

HMGB-1 has been characterized as a major factor underlying liver IRI via its capacity to activate macrophages, which results in the production of various pro-inflammatory cytokines.[32] To clarify
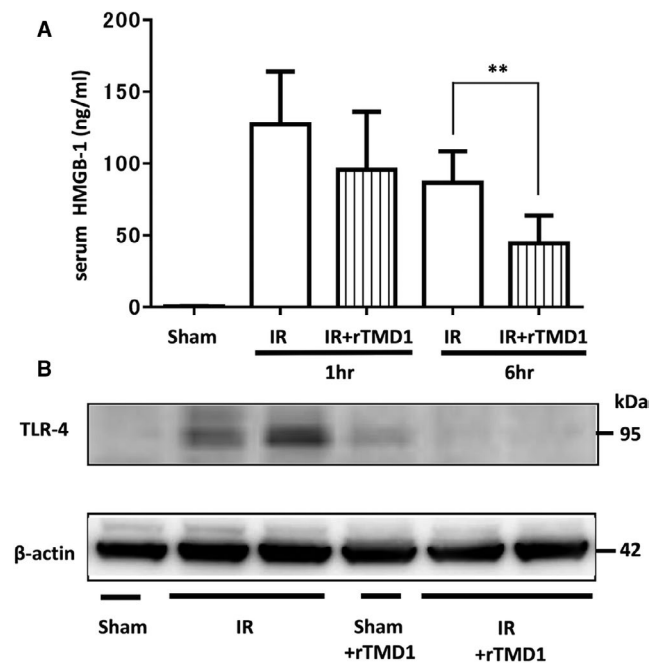
FIGURE 5 The levels of serum HMGB-1 and expression of TLR-4. A, The levels of serum HMGB-1 at 1 and 6 h after reperfusion in IR-treated and rTMD1-pretreated mice (n = 8 mice/group; **P < .01). B, The expression of TLR-4 in liver tissues at 6 h after reperfusion in IR-treated and rTMD1-pretreated mice. HMGB-1, high-mobility group box 1 protein; IR, ischemia and reperfusion; rTMD1, the domain 1 of thrombomodulin; TLR-4, Toll-like receptor 4
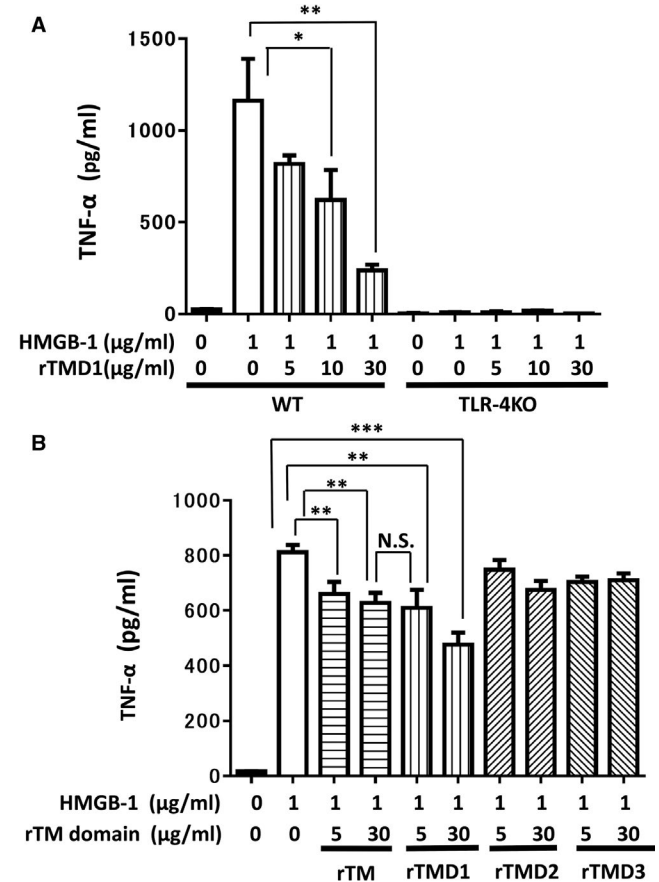
FIGURE 6 rTMD1 suppressed HMGB-1-mediated activation of peritoneal macrophages. A, Peritoneal macrophages from WT or TLR-4 KO mice were cultured with HMGB-1 (1 μg/mL) alone or with the mixture of HMGB-1 (1 μg/mL) and rTMD1 (5, 10, and 30 μg/mL, respectively) for 48 h. TNF-α release was measured by ELISA (n = 3 samples/group; *P < .05, **P < .01). B, Peritoneal macrophages from WT mice were cultured with HMGB-1 (1 μg/mL) alone or with the mixture of HMGB-1 (1 μg/mL), and each rTM domain protein (rTM, rTMD1, rTMD2, rTMD3; 5 and 30 μg/mL, respectively) for 48 h. TNF-α release was measured by ELISA (n = 3 samples/group; **P < .01, ***P < .001). ELISA, enzyme-linked immunosorbent assay; HMGB-1, high-mobility group box 1 protein; rTM, recombinant human thrombomodulin; rTMD1, the domain 1 of thrombomodulin; TLR-4 KO, tlr-4 gene–deleted; TNF-α, tumor necrosis factor-α; WT, wild-type

how rTMD1 affects HMGB-1-induced production of pro-inflammatory cytokines, we explored the responses of peritoneal macrophages treated with HMGB-1 ex vivo. Peritoneal macrophages from WT mice were cultured in RPMI-1640 medium containing bovine HMGB-1 (1 μg/mL) or medium alone. After 48 hours with HMGB-1, we observed significantly increased levels of TNF-α in supernatants from macrophage culture (Figure 6A, the first and second column). After 48 hours culture of peritoneal macrophages pretreated with either 5, 10, or 30 μg/mL of rTMD1, premixed with 1 μg/mL of HMGB-1, we observed a clear reduction in TNF-α levels, which responded to rTMD1 in a dose-dependent manner (Figure 6A, the third to fifth column). TNF-α production was not observed in peritoneal macrophages isolated from TLR-4 KO mice upon stimulation with HMGB-1 (right side of Figure 6A). Taken together, these results indicate that rTMD1 alone, without the other domains of the entire rTM molecule, was capable of profound

inhibition of TNF-α production induced by HMGB-1. Likewise, we note here that HMGB-1-induced inflammation in peritoneal macrophages is entirely dependent on the TLR-4-mediated signaling. We then evaluated TNF-α production induced by HMGB-1 in the presence of rTM or one of the rTM domain proteins (rTMD1, rTMD2, and rTMD3). Five or 30 μg/mL of each rTM protein (rTM, rTMD1, rTMD2, or rTMD3, respectively) was first preincubated with HMGB-1 protein (1 μg/mL), and then mixtures were added to the macrophage cultures. Of interest, preincubation of HMGB-1 with the lower amounts of rTMD1 (5 μg/mL) resulted in suppression of TNF-α production to the same degree as observed in response to the higher amounts of rTM (30 μg/mL) (Figure 6B). Moreover, preincubation of the higher amounts of rTMD1 (30 μg/
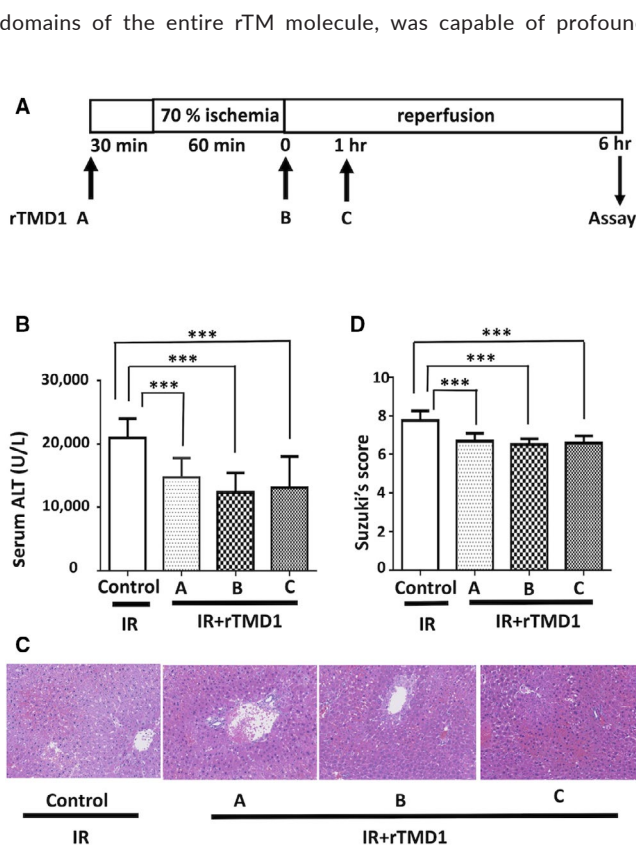

FIGURE 7 rTMD1 ameliorated IR-induced liver damage even after IRI had occurred. A, WT mice were divided into 4 groups: Control group mice were treated with saline instead of rTMD1 before initiating liver ischemia; Group A, rTMD1 was administered 30 min before initiating liver ischemia; Group B, rTMD1 was administered immediately after initiation of reperfusion; Group C, rTMD1 was administered 1 h after reperfusion was initiated. B, sALT in Group A, B, C, and Control group (n = 8 mice/group; ***P < .001). C, Representative liver histology (hematoxylin and eosin staining) after IR insult in each group (magnification ×400). D, Suzuki's histological grading in each group (n = 8 mice/group; ***P < .001). IR, ischemia and reperfusion; IRI, ischemia and reperfusion injury; rTMD1, the domain 1 of thrombomodulin; sALT, serum alanine aminotransferase; WT, wild-type
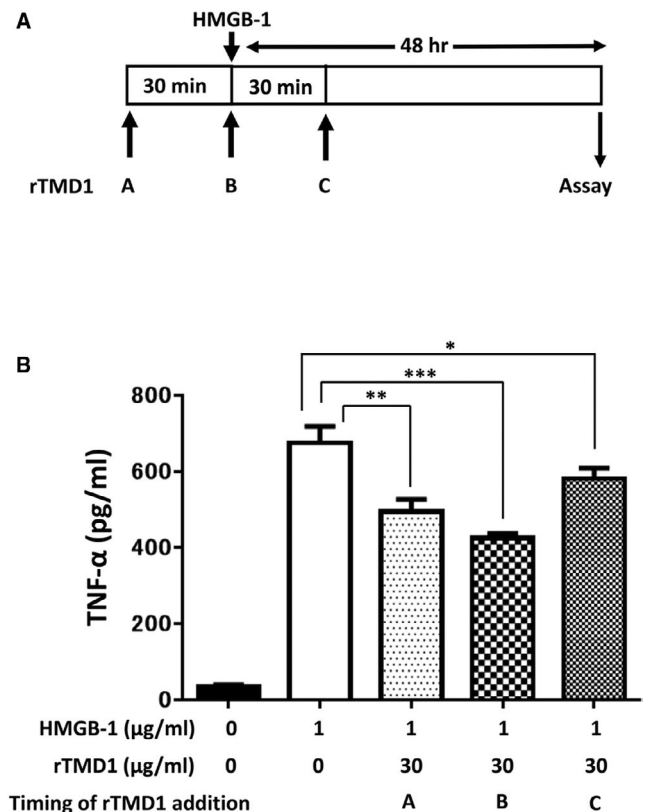

FIGURE 8 rTMD1 can suppress TNF-α production even after the addition of HMGB-1. A, Peritoneal macrophages from WT mice were divided into 5 groups. Nonstimulated group, macrophages were cultured with culture fluid alone; Control group, macrophages were cultured with HMGB-1 (1 μg/mL); Group A, rTMD1 (30 μg/mL) was added to the macrophages 30 min prior to the addition of HMGB-1 (1 μg/mL); Group B, rTMD1 (30 μg/mL) and HMGB-1 (1 μg/mL) were first mixed in a tube and incubated for 30 min, and then mixtures were added together to macrophages; Group C, rTMD1 (30 μg/mL) was added to the macrophages 30 min after the addition of HMGB-1 (1 μg/mL). All cells were cultured for 48 h. B, TNF-α release was measured by ELISA (n = 3 samples/group; *P < .05, **P < .01, ***P < .001). ELISA, enzyme-linked immunosorbent assay; HMGB-1, high-mobility group box 1 protein; rTMD1, the domain 1 of thrombomodulin; TNF-α, tumor necrosis factor-α ; WT, wild-type

mL) with HMGB-1 resulted in significantly stronger suppression of TNF-α production. Taking into account the molecular weight of rTM and rTMD1 (rTM at 52.2 kDa and rTMD1 at 20.2 kDa), rTMD1 appears to be equally effective to rTM on a molar basis at suppressing the production of TNF-α. Two other subdomain proteins of rTM (rTMD2 and rTMD3) had no apparent suppressive activity against HMGB-1-induced TNF-α production from macrophages (Figure 6B).

3.5 | rTMD1 can suppress IR-induced liver damage even after the IR insult has started

To investigate the timing of rTMD1 administration, rTMD1 was injected into mice at different time points during the course of liver IR (Figure 7A). As we presented in Figure 5A, serum HMGB-1 levels were at high values as early as 1 hour after the initiation of reperfusion in mice subjected to IR. The rTMD1-treated mice were divided into 3 groups: Group A, rTMD1 was administered 30 minutes before initiating liver ischemia; Group B, rTMD1 was administered immediately after initiation of reperfusion; Group C, rTMD1 was administered 1 hour after reperfusion was initiated. Mice in the Control group received liver IR but were administered saline instead of rTMD1 30 minutes before initiating liver ischemia. Surprisingly, rTMD1 was effective in suppressing acute liver damage in all 3 groups compared with Control group (Figure 7B). Histological changes in liver improved to an equivalent degree (Figure 7C) and Suzuki's score was also lowered in all 3 groups (Figure 7D). These results indicate that rTMD1 administration both before and, interestingly, even after reperfusion showed the suppression of liver damage to the same extent.

3.6 | rTMD1 can suppress the TNF-α production from macrophages even after the stimulation with HMGB-1

Finally, we explored the impact of rTMD1 administered at different addition times to HMGB-1-stimulated peritoneal macrophage cultures (Figure 8A). Group A, rTMD1 (30 μg/mL) was added to the macrophages 30 minutes prior to the addition of HMGB-1 (1 μg/mL); Group B, rTMD1 (30 μg/mL) and HMGB-1 (1 μg/mL) were first mixed in a tube and incubated for 30 minutes and then mixtures were added together to macrophages; and Group C, rTMD1 (30 μg/mL) was added to the macrophages 30 minutes after the addition of HMGB-1 (1 μg/mL). All cells were cultured for 48 hours. As shown in Figure 8B, addition of rTMD1 at any of the 3 time points resulted in significant suppression of TNF-α production by the peritoneal macrophages, compared to the control without addition of rTMD1. These results indicate that rTMD1 is equally effective against an HMGB-1-mediated inflammatory response, even after an initial interaction between HMGB-1 and TLR-4 has taken place.

4 | DISCUSSION

rTM, an established therapeutic drug for DIC in Japan, has strong anticoagulant activity that is associated with protein C activation and inhibition of thrombin production.[33] Our previous report revealed that rTM suppressed the liver IRI in mice by inhibiting the HMGB-1/TLR-4 signaling pathway and the ensuing inflammatory cascade.[7] However, it was uncertain whether the anticoagulant activity might preclude the use of rTM as a therapeutic option in human liver IRI. As reported in the Phase III Clinical Study on rTM, among the patients with DIC, 43.1% (50/116) of the patients experienced adverse bleeding-related events within 7 days of the start of the infusion.[34] By contrast, rTMD1, which is a single subunit of that includes the lectin-like domain only and has no anticoagulant activity, is under consideration as a novel anti-inflammatory drug.[35] We have documented previously that a single dose of rTMD1 suppressed liver damage to the same extent as an entire molecule of rTM in the warm liver IRI model in mouse.[7] Likewise, we have shown in the present study that administration of even a high dose of rTMD1 (18 mg/kg, 10 times higher than usual dose) prior to inducing ischemia suppressed liver damage more effectively than the usual dose (1.8 mg/kg) without inducing any hemorrhage (Figure 2A). Thus rTMD1 was equally effective as rTM and reduced hepatocellular injury to 35%-45% of that observed in the absence of treatment in WT mice. Whole molecule of rTM as well as rTMD1 suppressed HMGB-1-induced macrophage activation in a TLR-4-dependent manner (Figure 6A). On the other hand, rTMD2 or rTMD3 had no impact on pathology in mouse liver IRI (Figure 2H), indicating that suppressive effect of rTM on liver IRI by inhibiting the HMGB-1/TLR-4 pathway depends entirely on rTMD1, not on other rTM domains.

rTMD1 reduced the number of apoptotic cells in the liver that develop in response to IR insult (Figure 3A,B). Moreover, the expression of cleaved caspase-3 in liver tissues after IR was reduced in rTMD1-pretreated mice, whereas the expression of B cell lymphoma-extra large (Bcl-xL) and phospho-Akt (p-Akt) was increased (Figure 3C and Figure S2). During the liver IR insult, hepatocytes generate ROS, which triggers the recruitment of immune cells and promotes cellular apoptosis in liver tissues.[23,36] Earlier studies revealed that rTMD1 suppressed the production of ROS induced by LPS stimulation[20]; as such, rTMD1 might suppress IR-induced liver cell apoptosis also via inhibition of ROS generation and the suppression of the caspase-3 cleavage by blocking the TLR-4-mediated signaling pathway.

In liver IRI, inflammatory cytokines such as IL-1β, TNF-α, IL-6, and chemokines such as CXCL-1 and CXCL-2 play important roles in promoting liver damage.[29-31] The expression of many of these cytokines and chemokines was significantly diminished in rTMD1-pretreated mice. However, rTMD1 pretreatment suppressed the production of CXCL-2 but had no impact on CXCL-1 (Figure 4). In immunostaining assays, both of the infiltration of Ly-6G^{+} neutrophils and CD11b^{+} macrophages/monocytes were significantly reduced in liver tissues in rTMD1-pretreated mice (Figure 2D-G). It has been reported that CXCL-1 produced by endothelial cells and pericytes in

a TNF-stimulated environment may regulate neutrophil recruitment, whereas CXCL-2 is produced by neutrophils and macrophages, and regulates mainly accumulation of macrophages.[37] Lentsch et al[38] reported in a mouse IRI model that MIP-2 (CXCL-2) messenger RNA was induced within 3 hours after reperfusion and increased to a greater extent in the ischemic lobe even after 9 hours of reperfusion. In contrast, KC (CXCL-1) mRNA expression was not increased after 3 hours of reperfusion but increased 9 hours after, suggesting that CXCL-2 rather than CXCL-1 may be mainly involved in the recruitment of neutrophils to the ischemic liver lobe at early stage of IRI. Fan and Malik[39] have suggested that TLR-4 signaling augments chemokine (CXCL-2)–induced neutrophil migration by modulating cell surface expression of chemokine receptors. These reports indicate that CXCL-2 rather than CXCL-1 dominantly plays a role in neutrophil accumulation into liver during IRI.

HMGB-1 is known to be released from hepatocytes damaged by ischemic condition and serum level of HMGB-1 increases during IR.[4] The released HMGB-1 causes liver injury through the induction of inflammatory responses in macrophages via TLR-4. As shown in Figure 5A, serum levels of HMGB-1 peaked right after the start of reperfusion and gradually decreased. rTMD1 pretreatment significantly decreased the serum HMGB-1 levels especially 6 hours after reperfusion, indicating that administration of rTMD1 lowered the upregulated serum HMGB-1 level. The expression of TLR-4 in liver tissues was also lowered in rTMD1-pretreated mice (Figure 5B). Nace GW et al reported that TLR-4 is expressed not only on macrophages and dendritic cells but also on hepatocytes in liver, and that the release of HMGB-1 from hepatocytes strictly relies mostly on the TLR-4 expressed on hepatocytes.[4] It has been suggested that portal vein occlusion during liver IR results in congestion of the intestinal wall, leading to the release of gut-derived molecules, including endotoxin such as LPS, into the bloodstream.[40] Such gut-derived molecules may trigger the release of HMGB-1 from hepatocytes via TLR-4. On the basis of our in vitro assay, rTMD1 could strongly suppress the interaction between extracellular HMGB-1 and TLR-4 on macrophages, and also may interfere the interaction of HMGB-1 with TLR-4 on hepatocytes. Thus, administration of rTMD1 possibly suppress the release of HMGB-1 from hepatocytes and expression of TLR-4 by inhibiting the putative endotoxin-TLR-4 interaction.

In previous reports, rTM and other treatments were generally administered before the onset of ischemia.[7,12-14,17,23,40-44] The IR insult was also more profound in genetically modified mice lacking the N-terminal lectin-like domain of TM.[21,22] These reports suggested that preconditioning with rTM or rTMD1 before ischemia appears to be required for the suppression of liver IRI. However, as we have shown clearly in Figure 7, administration of rTMD1 at a time point up to 1 hour after the start of reperfusion resulted in suppression of IRI-related pathology. To mimic the in vivo scenario, rTMD1 was added in vitro to macrophage culture at 30 minutes after the addition of HMGB-1 as shown in Figure 8A. Addition of rTMD1 resulted in a significant decrease in TNF-α production even after the addition of HMGB-1. As the level of serum HMGB-1 reached its peak 1 hour after initiation of reperfusion (Figure 5A), the administration

of rTMD1 at 1 hour after the reperfusion might result in blockade of HMGB-1/TLR-4 signaling. In the present study, we showed that administration of rTMD1 even after reperfusion exhibits an efficient suppressive effect in the mouse liver IRI model. Of interest, a very recent publication reported that intraperitoneal administration of propofol, an intravenous anesthetic with antioxidant property, immediately after reperfusion also reduced the extent of pathology that results from liver IRI.[45] However, in this report, the "postconditioning" strategy was in fact similar to that used for Group B (but not Group C) of the present study as shown in Figure 7A. To date, there have been no other reports suggesting the utility of any drug or method that could suppress the extent of IR-induced liver damage once reperfusion has been initiated.

This is the first study that provides documentation of the impact of rTMD1 on liver IRI even after the onset of injury. Thinking more broadly, rTMD1 may be effective for the post hoc treatment of inflammatory disorders related to extracellular HMGB-1 such as mesenteric ischemia or cerebral or myocardial infarction. It is desirable to conduct human clinical trials on rTMD1 as a therapeutic drug for inflammatory disease in the future. In conclusion, we demonstrate here that rTMD1 may be effective both as prophylactic well as therapeutic indications in acute liver IRI without the possible risk of bleeding.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Yoichiro Uchida https://orcid.org/0000-0003-2266-7722
Takeshi Watanabe https://orcid.org/0000-0001-7850-4178

REFERENCES

1. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *New Engl J Med.* 1985;312(3):159-163.
2. Farmer DG, Amersi F, Kupiec-Weglinski J, Busuttil RW. Current status of ischemia and reperfusion injury in the liver. *Transplant Rev.* 2000;14(2):106-126.
3. Collard CD, Gelman S. Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. *Anesthesiology.* 2001;94(6):1133-1138.

4. Nace GW, Huang H, Klune JR, et al. Cellular-specific role of toll-like receptor 4 in hepatic ischemia-reperfusion injury in mice. *Hepatology (Baltimore, MD)*. 2013;58(1):374-387.

5. van Golen RF, Reiniers MJ, Marsman G, et al. The damage-associated molecular pattern HMGB1 is released early after clinical hepatic ischemia/reperfusion. *Biochim Biophys Acta*. 2019;1865(6):1192-1200.

6. Scheuermann U, Zhu M, Song M, et al. Damage-associated molecular patterns induce inflammatory injury during machine preservation of the liver: potential targets to enhance a promising technology. *Liver Transplant*. 2019;25(4):610-626.

7. Kadono K, Uchida Y, Hirao H, et al. Thrombomodulin attenuates inflammatory damage due to liver ischemia and reperfusion injury in mice in toll-like receptor 4-dependent manner. *Am J Transplant*. 2017;17(1):69-80.

8. Fuentes-Prior P, Iwanaga Y, Huber R, et al. Structural basis for the anticoagulant activity of the thrombin-thrombomodulin complex. *Nature*. 2000;404(6777):518-525.

9. Ikezoe T. Thrombomodulin/activated protein C system in septic disseminated intravascular coagulation. *J Intensive Care*. 2015;3(1):1.

10. Stearns-Kurosawa DJ, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. *Proc Natl Acad Sci USA*. 1996;93(19):10212-10216.

11. Mori S, Ai T, Sera T, Ochiai K, Otomo Y. Human Soluble Recombinant Thrombomodulin, ART-123, resolved early phase coagulopathies, but did not significantly alter the 28 day outcome in the treatment of DIC associated with infectious systemic inflammatory response syndromes. *J Clin Med*. 2019;8(10):1553.

12. Kashiwadate T, Miyagi S, Hara Y, et al. Recombinant human soluble thrombomodulin (ART-123) prevents warm ischemia-reperfusion injury in liver grafts from non-heart-beating donors. *Transpl Proc*. 2012;44(2):369-372.

13. Kashiwadate T, Miyagi S, Hara Y, et al. Soluble thrombomodulin ameliorates ischemia-reperfusion injury of liver grafts by modulating the proinflammatory role of high-mobility group box 1. *Tohoku J Exp Med*. 2016;239(4):315-323.

14. Kimura K, Yoshizumi T, Inokuchi S, et al. Potential effect of recombinant thrombomodulin on ischemia-reperfusion liver injury in rats. *Hepatol Res*. 2018;48(5):391-396.

15. Loghmani H, Conway EM. Exploring traditional and nontraditional roles for thrombomodulin. *Blood*. 2018;132(2):148-158.

16. Sharfuddin AA, Sandoval RM, Berg DT, et al. Soluble thrombomodulin protects ischemic kidneys. *J Am Soc Nephrol: JASN*. 2009;20(3):524-534.

17. Tanemura A, Kuriyama N, Azumi Y, et al. Thrombomodulin administration attenuates ischemia-reperfusion injury of the remnant liver after 70% hepatectomy in rats: simulated model of small-for-size graft in living donor liver transplantation. *Transpl Proc*. 2014;46(4):1107-1111.

18. Abeyama K, Stern DM, Ito Y, et al. The N-terminal domain of thrombomodulin sequesters high-mobility group-B1 protein, a novel antiinflammatory mechanism. *J Clin Investig*. 2005;115(5):1267-1274.

19. Lattenist L, Teske G, Claessen N, Florquin S, Conway EM, Roelofs JJ. The lectin like domain of thrombomodulin is involved in the defence against pyelonephritis. *Thromb Res*. 2015;136(6):1325-1331.

20. Shi C-S, Shi G-Y, Hsiao H-M, et al. Lectin-like domain of thrombomodulin binds to its specific ligand Lewis Y antigen and neutralizes lipopolysaccharide-induced inflammatory response. *Blood*. 2008;112(9):3661-3670.

21. Geudens N, Van de Wouwer M, Vanaudenaerde BM, et al. The lectin-like domain of thrombomodulin protects against ischaemia-reperfusion lung injury. *Eur Respiratory J*. 2008;32(4):862-870.

22. Herzog C, Lorenz A, Gillmann H-J, et al. Thrombomodulin's lectin-like domain reduces myocardial damage by interfering with HMGB1-mediated TLR2 signalling. *Cardiovasc Res*. 2014;101(3):400-410.

23. Miyauchi T, Uchida Y, Kadono K, et al. Preventive effect of antioxidative nutrient-rich enteral diet against liver ischemia and reperfusion injury. *JPEN J Parenter Enteral Nutr*. 2019;43(1):133-144.

24. Miyauchi T, Uchida Y, Kadono K, et al. Up-regulation of FOXO1 and reduced inflammation by beta-hydroxybutyric acid are essential diet restriction benefits against liver injury. *Proc Natl Acad Sci USA*. 2019;116(27):13533-13542.

25. Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, Cejalvo D. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. *Transplantation*. 1993;55:1265-1272.

26. Rudiger HA, Clavien PA. Tumor necrosis factor alpha, but not Fas, mediates hepatocellular apoptosis in the murine ischemic liver. *Gastroenterology*. 2002;122(1):202-210.

27. Kohli V, Madden JF, Bentley RC, Clavien PA. Calpain mediates ischemic injury of the liver through modulation of apoptosis and necrosis. *Gastroenterology*. 1999;116(1):168-178.

28. Kohli V, Selzner M, Madden JF, Bentley RC, Clavien PA. Endothelial cell and hepatocyte deaths occur by apoptosis after ischemia-reperfusion injury in the rat liver. *Transplantation*. 1999;67(8):1099-1105.

29. Colletti LM, Kunkel SL, Walz A, et al. The role of cytokine networks in the local liver injury following hepatic ischemia/reperfusion in the rat. *Hepatology (Baltimore, MD)*. 1996;23(3):506-514.

30. Palomino DC, Marti LC. Chemokines and immunity. *Einstein (Sao Paulo, Brazil)*. 2015;13(3):469-473.

31. Lentsch AB, Yoshidome H, Cheadle WG, Miller FN, Edwards MJ. Chemokine involvement in hepatic ischemia/reperfusion injury in mice: roles for macrophage inflammatory protein-2 and KC. *Hepatology (Baltimore, MD)*. 1998;27(4):1172-1177.

32. Tsung A, Sahai R, Tanaka H, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J Exp Med*. 2005;201(7):1135-1143.

33. Conway EM. Thrombomodulin and its role in inflammation. *Semin Immunopathol*. 2012;34(1):107-125.

34. Aoki N, Matsuda T, Saito H, et al. A comparative double-blind randomized trial of activated protein C and unfractionated heparin in the treatment of disseminated intravascular coagulation. *Int J Hematol*. 2002;75(5):540-547.

35. Li YH, Kuo CH, Shi GY, Wu HL. The role of thrombomodulin lectin-like domain in inflammation. *J Biomed Sci*. 2012;19:34.

36. Quesnelle KM, Bystrom PV, Toledo-Pereyra LH. Molecular responses to ischemia and reperfusion in the liver. *Arch Toxicol*. 2015;89(5):651-657.

37. Girbl T, Lenn T, Perez L, et al. Distinct compartmentalization of the chemokines CXCL1 and CXCL2 and the atypical receptor ACKR1 determine discrete stages of neutrophil diapedesis. *Immunity*. 2018;49(6):1062-1076.e1066.

38. Lentsch AB, Yoshidome H, Cheadle WG, Miller FN, Edwards MJ. Chemokine involvement in hepatic ischemia/reperfusion injury in mice: roles for macrophage inflammatory protein-2 and Kupffer cells. *Hepatology (Baltimore, MD)*. 1998;27(2):507-512.

39. Fan J, Malik AB. Toll-like receptor-4 (TLR4) signaling augments chemokine-induced neutrophil migration by modulating cell surface expression of chemokine receptors. *Nat Med*. 2003;9(3):315-321.

40. Uchida Y, Freitas MC, Zhao D, Busuttil RW, Kupiec-Weglinski JW. The protective function of neutrophil elastase inhibitor in liver ischemia/reperfusion injury. *Transplantation*. 2010;89(9):1050-1056.

41. Uchida Y, Ke B, Freitas MCS, et al. The emerging role of T cell immunoglobulin mucin-1 in the mechanism of liver ischemia and reperfusion injury in the mouse. *Hepatology (Baltimore, MD)*. 2010;51(4):1363-1372.

42. Hirao H, Uchida Y, Kadono K, et al. The protective function of galectin-9 in liver ischemia and reperfusion injury in mice. *Liver Transplant*. 2015;21(7):969-981.

43. Uchida Y, Freitas MC, Zhao D, Busuttil RW, Kupiec-Weglinski JW. The inhibition of neutrophil elastase ameliorates mouse liver damage due to ischemia and reperfusion. *Liver Transplant*. 2009;15(8):939-947.

44. Uchida Y, Ke B, Freitas MCS, et al. T-cell immunoglobulin mucin-3 determines severity of liver ischemia/reperfusion injury in mice in a TLR4-dependent manner. *Gastroenterology*. 2010;139(6):2195-2206.

45. Ge M, Chen H, Zhu Q, et al. Propofol post-conditioning alleviates hepatic ischaemia reperfusion injury via BRG1-mediated Nrf2/HO-1 transcriptional activation in human and mice. *J Cell Mol Med*. 2017;21(12):3693-3704.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.