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ORIGINAL ARTICLE

Improvements in endotoxemic syndromes using a disintegrin, rhodostomin, through integrin αvβ3-dependent pathway

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Summary. *Background and objectives:* Septic shock is a major cause of morbidity and mortality in intensive care units, but there is still no effective therapy for the patients. We evaluated the effects of rhodostomin (Rn), an Arg-Gly -Asp-containing snake venom disintegrin, on lipopolysaccharide (LPS)-activated phagocytes *in vitro* and LPS-induced endotoxemia *in vivo*. *Methods and results:* Rn inhibited adhesion, migration, cytokine production and mitogen-activated protein kinase (MAPK) activation of macrophage induced by LPS. Flow cytometric analysis revealed that Rn specifically blocked anti-αv mAb binding to RAW264.7. Besides inhibiting MAPK activation of THP-1, Rn bound to LPS-activated THP-1 and specifically blocked anti-αvβ3 mAb binding to THP-1. Binding assays proved that integrin αvβ3 was the binding site for rhodostomin on phagocytes. Rn reversed the enhancement of fibronectin and vitronectin on LPS-induced monocyte adhesion and cytokine release. Transfection of integrin αv siRNA also inhibited LPS-induced activation of monocyte, and Rn exerted no further inhibitory effect. Furthermore, Rn significantly decreased the production of tumor necrosis factor-α (TNF-a), interleukin (IL)-6, -1β and -10 and attenuated cardiovascular dysfunction, including blood pressure and heart pulse, and thrombocytopenia in LPS-induced endotoxemic mice. Rn also protected against tissue inflammation as evidenced by histological examination. *Conclusions:* Rn may interact with αvβ3 integrin of monocytes/macrophages leading to interfere with the activation of phagocytes triggered by LPS. These results suggest that the protective function of Rn in LPS-induced endotoxemia may be attributed to its anti-inflammation activities *in vivo*.

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Introduction

Lipopolysaccharide (LPS) is a molecule derived from the outer membrane of Gram-negative bacteria and is the best characterized bacterial antigen in immunology [1]. LPS interacts with monocytes, macrophages and other cells in the host circulation and leads to the release of inflammation cytokines, including tumor necrosis factor-α (TNF-α) and interleukin (IL)-1, IL-6, IL-8 and IL-12 [2]. When these proinflammation mediators are overproduced, they cause multi-organ dysfunction in sepsis, leading to shock and death in patients. It is well known that LPS is recognized by Toll-like receptor 4 (TLR4), resulting in activation of the mitogen-activated protein kinase (MAPK) signaling pathway and transcription factor, nuclear factor κB (NFκB) [1].

Another key receptor of phagocyte is integrins [3]. β2 integrins combine α subunits to form heterodimers on the leukocyte surface, which have essential roles in leukocyte trafficking and function [3]. It is also reported that αvβ3 modulates αLβ2-dependent transendothelial migration of monocyte [4]. In addition, vitronectin and αvβ3 contribute to the initiation of TLR2 responses to bacterial lipopeptides [5].

Recently, many disintegrins, a family of low-molecular weight peptides, have been reported to bind to integrins expressed on blood cells and other cells including vascular endothelial cells and some tumor cells [6,7]. Rhodostomin (Rn), a disintegrin purified from *Calloselasma rhodostoma* snake venom, consists of 71-amino-acid residues including an Arg-Gly-Asp (RGD) sequence at position 49–51 [8]. We previously reported that Rn inhibits platelet aggregation through integrin αIIbβ3 and exerts antiangiogenic activity through integrin αvβ3 [8,9]. In addition, Rn binds to neutrophils in a RGD-dependent manner to attenuate the adhesive activity and superoxide production of neutrophils [10]. However, the role of its anti-inflammation is still not well established. In the present study, we used Rn as a tool to examine the inhibitory effect of disintegrin on

LPS-induced endotoxemia in mice and to investigate the mechanism involved.

Methods

Materials

Rn was prepared by protein expression in *Pichia pastoris* as previously described [11]. LPS (*Escherichia coli*, serotype O127:B8) was from Sigma Chemicals Co. (St Louis, MO, USA). The murine mAb against αIIbβ3/αvβ3, 7E3, was kindly provided by Dr Barry S. Coller (Mount Sinai School of Medicine, NY, USA). Antibodies against mouse integrins, αv, β1 and β3, were from BD Biosciences (San Diego, CA, USA). Antibodies against human integrins, αv, β1, β2 and αvβ3, were from Chemicon (Temecula, CA, USA). Antibodies against human integrin β3, mouse TLR4 and human TLR4, pFAK, FAK and α-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The fluorescent dyes, BCECF-AM and fluorescein isothiocyanate (FITC), were from Molecular Probes (Leiden, the Netherlands). Other chemicals were from Sigma Chemicals Co.

Adhesion assay

RAW264.7 (mouse macrophages) and THP-1 (human monocytes) were cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 media, respectively. For adhesion assay, plates with 48 wells (Costar) were coated overnight with 100-μL matrices (collagen, fibrinogen, fibronectin, vitronectin) in 50 μg mL^{-1} or 1% (w/v) bovine serum albumin (BSA). Cells were labeled with BCECF-AM and activated with 1 μg mL^{-1} LPS or not, then incubated with various concentrations of Rn for 30 min at 37 °C. Control or the pretreated cells were applied to the plates and incubated for 120 min at 37 °C to allow adhesion. After washing, the plates were read with a CytoFluor 2300 fluorescence plate reader (Millipore, Billerica, MA, USA).

Migration assay

Migration assay of RAW264.7 macrophages was measured as described with modification [12]. In brief, Costar Transwells (5 μm pore size) were coated with 2% gelatin. RAW264.7 (5×10^5 cells) challenged with LPS (1 μg mL^{-1}) or not was incubated with different concentrations of Rn at 37 °C for 30 min, and then cells were seeded into the upper chamber. The lower chamber had only DMEM medium added. Cells that transmigrated on the membrane were counted. Results are expressed as a migration index, in which the number of control cells that migrated in the absence of LPS and Rn was set at one.

Cytokine production by LPS-activated phagocytes

After macrophages and monocytes were cultured with 0.1 μg mL^{-1} LPS alone or with varying concentrations of Rn or inhibitors (10 μM) for 24 h, media were collected by centrifugation. Cytokines were measured using an ELISA kit (Bender MedSystems, San Diego, CA, USA).

Western blotting of protein kinases

In the presence or absence of 1 μg mL^{-1} LPS, RAW and THP-1 were incubated with different concentration of Rn at 37 °C for 30 min. Aliquots of cell lysates were analyzed by Western blotting. For quantification of the Western analysis, the density of each band was quantified by ImageQuant software (GE Healthcare, Piscataway, NJ, USA).

Rn Binding assay

For detection of Rn binding on THP-1, Rn and BSA were conjugated with FITC according to the protocol described by Liu *et al.* [13]. After stimulating with 1 μg mL^{-1} LPS for 1 h at 37 °C, THP-1 cells were incubated with FITC-conjugated Rn or FITC-conjugated BSA at 4 °C for 30 min. Cells were analyzed by flowcytometry. Background binding (non-specific binding) was determined using FITC-conjugated BSA as the binding probe.

For analysis of Rn receptor, RAW and THP-1 cells were stimulated with 1 μg mL^{-1} LPS for 1 h at 37 °C and then incubated with 30 μg mL^{-1} Rn or not at 4 °C for 30 min. After extensive washes, cells were labeled with indicated mAbs at room temperature for 30 min. Cells were analyzed immediately by flowmetry.

Binding of integrins to immobilized Rn

Rn was applied to 15% SDS-PAGE and transferred to a poly(vinylidene difluoride) (PVDF) membrane. After blocking, the membrane was incubated with RAW264.11 or THP-1 cell lysate solution for 1 h at room temperature and was probed with anti-integrin mAbs as described.

Protein-based affinity pull-down

The affinity pull-down was performed with a specific kit (Pierce). To capture the prey proteins, Rn or BSA were biotinylated with Sulfo-NHS-LC-biotin, added to THP-1 or platelet cell lysate and incubated at 4 °C overnight. After 2-h incubation with streptavidin-Sepharose, the prey proteins were eluted. Dissociated proteins were separated under reducing conditions by SDS-PAGE, and detected by Western blotting.

RNA interference

THP-1 cells were transfected with small interfering RNA (siRNA) duplexes using RNAi-Mate Transfection Reagent (MDBio Inc., Taipei, Taiwan), according to the manufacturer's instruction. siRNA duplexes for integrin αv and negatively controlled non-targeting siRNA were from MDBio Inc. The siRNA sense sequences: human integrin αv, 5′-CACUCC-AAGAACAUGACUA (dTdT)-3′ and 5′-GACUGAGCU-

AAUCUUGAGA (dTdT)-3′; human integrin αM, 5′-UUGAGGAGCAGUUUGUUUCCAAGGG (dTdT)-3′; negative control, 5′-UUCUCCGAACGUGUCACGU (dTdT)-3′. After incubation for 48 h, cells were starved overnight.

Animals

Male ICR mice weighing 25–30 g were used in all studies. The animals were maintained on a 12-h light/dark cycle under controlled temperature (20 ± 1 °C) and humidity (55% ± 5%). Animals were given continuous access to food and water. The animal experimental protocols were approved by the Animal Care and Use Committee at College of Medicine, National Taiwan University.

LPS-induced endotoxemia

Mice were injected intraperitoneally (i.p.) with vehicle or LPS at a dose of 30 mg kg^{-1} body weight. After the i.p. injection, the mouse was intravenously (i.v.) injected with phosphate-buffered saline (PBS) (control, 50 μL) or 10 μg kg^{-1} Rn through the lateral tail vein.

Mice whole blood and serum collection

After mice were anesthetized by i.p. injection of sodium pentobarbital (50 mg kg^{-1}), whole blood was drawn by cardiac puncture and collected in citric acid-citrate dextrose (ACD; 9:1 blood vol/vol) at an indicated time (24 h after treatment with LPS). The number of platelets was counted using an automatic blood cell machine (KX-21N; Sysmex America Inc., Kobe, Japan). The plasma was separated by centrifugation at $1000 \times g$ for 10 min and the supernatant was collected. The concentration of cytokines, IL-6, TNF-α, IL-1β and IL-10 in the plasma was determined by an ELISA kit according to the manufacturer's instructions (Bender MedSystems).

Measurement of vital signs using the tail-cuff method

A thermostatically regulated, heating platform was used to maintain body temperature at 37 °C for 24 h after treatment with LPS. After the rectal temperature was measured during the experiment, conventional non-invasive blood pressure in conscious mice was measured using the tail-cuff method (BP-2000; Visitech Syatems, Inc., Apex, NC, USA), for the measurement of diastolic and systolic blood pressure as well as heart pulse.

Histological examination

Lung, liver and kidney segments were fixed in 10% v/v phosphate-buffered formalin for 48–72 h and then embedded in paraffin. Next, the samples were sectioned (5 μm) using a microtome, stained with hematoxylin and eosin (H&E), and examined with light microscopy at × 400 magnifications.

Statistical analysis

All values are presented as mean ± SEM. Analysis of three or more groups was assessed using one-way ANOVA and the Newman–Keuls multiple comparison test. Two groups were compared using the unpaired Student's *t*-test. The odds ratio (OR) with the confidence interval (CI) was calculated. *P*-values < 0.05 ($P < 0.05$) were considered as a significant difference.

Results

Rn blocks the activation of phagocyte

It is a prevalent condition in sepsis that leukocytes adhere to and transmigrate through the blood vessel wall to cause edema [2]. Rn concentration dependently inhibited the LPS-induced increase in macrophage, RAW264.7, attachment to fibronectin-coated wells and migration through gelatin-coated transwells (Fig. 1A,B). At 30 μg mL^{-1}, Rn almost completely inhibited cell migration.

Rn also concentration-dependently decreased LPS-induced IL-6 and TNF-α release in macrophage, RAW264.7 (Fig. 1C,D). Dexamethasone was used as a positive control. As both MAPK and NF-κB signal pathways are crucial in these cytokine production processes of macrophages [1], we also tested the effects of some specific inhibitors of these pathways, including SP600125 (JNK inhibitor), PD98059 (ERK inhibitor), SB203580 (p38 inhibitor) and PDTC (NF-κB inhibitor). As shown in Fig. 1C,D, all these inhibitors significantly reduced LPS-induced cytokines production.

In the meantime, we found that Rn concentration dependently reduced the LPS-induced phosphorylation of p38, JNK and ERK in RAW264.7 cells (Fig. 2A) and p38 and JNK in THP-1 cells (Fig. 2B).

Characterization of the interaction between Rn and macrophages/monocytes

We next analyzed the interaction between Rn and macrophage/monocyte. Stimulation with LPS on phagocytes could activate FAK and subsequently integrins to generate inside-out signaling for enhanced binding of its ligands [14]. In the flowcytometry assay, we found that the expression of integrins including αv, β1, β2, β3 and αvβ3 on monocytes and macrophages were significantly increased upon LPS stimulation (Table 1). Rn specifically reduced the binding of anti-αv mAb on RAW264.7, but not the binding of other anti-integrin mAbs, including anti-β1 and β3. In addition, Rn did not block the binding of the LPS receptor, TLR4, on macrophages (Fig. 3A and Table 1). Similarly, Rn also specifically blocked anti-αvβ3 mAb binding to LPS-activated THP-1, but not anti-β1, β2 and TLR4 mAbs binding (Fig. 3B and Table 1). Therefore, Rn inhibited anti-αv/αvβ3 mAbs binding to the activated monocytes and macrophages, but not to unstimulated cells (Fig. 3A,B and Table 1).

To elucidate the possible binding site(s) of Rn on monocyte, we incubated LPS-activated THP-1 cells with FITC-conjugated

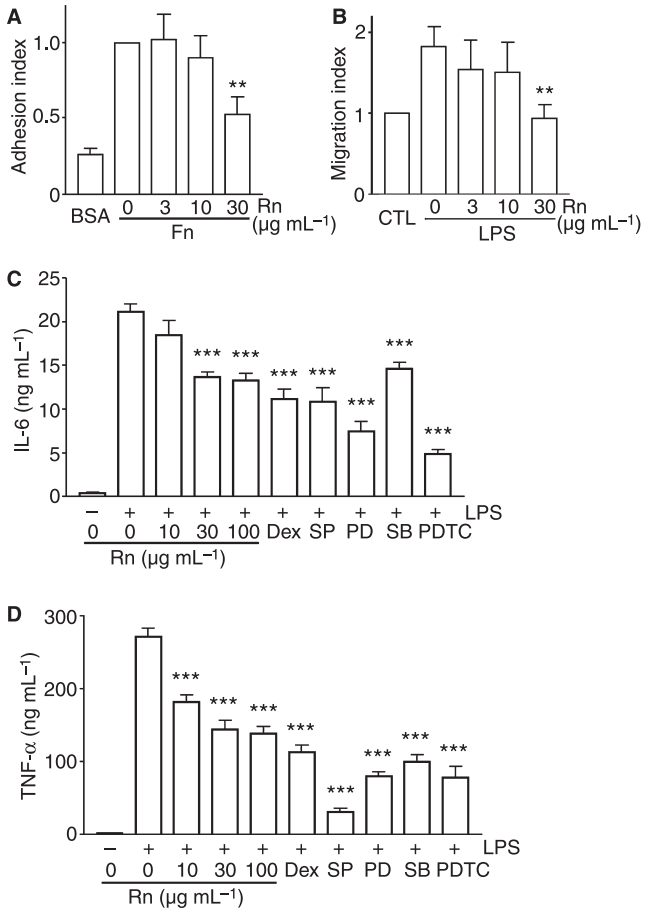

Fig. 1. The effect of rhodostomin (Rn) on lipopolysaccharide (LPS)-induced adhesion, migration and cytokine production of macrophage. (A) The adhesion index was calculated as the fold of adhered RAW264.7 cells normalized to that of the LPS-treated control group. (B) The migration index was calculated as the fold change of adhered RAW264.7 cells normalized to that of unstimulated RAW264.7 cells. CTL, non-activated control. (C, D) RAW264.7 was treated with Rn, dexamethason (Dex, 10 nM) and mitogen-activated protein kinase (MAPKs) inhibitors, including SP600125 (SP, 50 μM), PD98059 (PD, 50 μM) and SB203580 (SB, 50 μM), or NFκB inhibitor (PDTC, 50 μM), after activation with LPS or PBS alone (control, CTL). Concentrations of cytokines were measured by ELISA. Values are presented as mean ± SEM ($n = 3$), and $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with LPS-activated control groups.

Rn and found that the total fluorescence intensity of THP-1 cells increased in a concentration-dependent manner. After subtracting the non-specific binding probed by FITC-conjugated BSA, the specific binding of Rn reached a plateau at approximately 50 μg mL^{-1} (Fig. 3C). We used mAbs (20 μg mL^{-1}) to block the binding of FITC-Rn (30 μg mL^{-1}) to LPS-activated THP-1 cells, only anti-αvβ3 mAb, but not anti-β2, inhibited the binding of FITC-Rn (Fig. 3D).

The effect of Rn on integrin αvβ3 was further examined by binding assays. The immobilized Rn, which migrated as an 8-kDa protein, bound to integrin αv in RAW cell lysate, and integrin αvβ3 and β3 in THP-1 cell lysate, but not to integrin β1 and β2 (Fig. 3E). On the other hand, cell proteins bound to biotinylated Rn were isolated and eluted. After analysis with SDS-PAGE, a 105-kDa membrane protein integrin (β3) was specifically recognized by 7E3, a mAb recognizing integrin αvβ3 (THP-1)/αIIbβ3 (platelets) (Fig. 3F). These results indicate that Rn specifically binds to integrin αvβ3 on phagocytes, but not to integrin β1 or β2.

LPS-induced monocyte adhesion and cytokine release are integrin αv dependent

To evaluate the function of different matrices in LPS signaling, we seeded human monocyte THP-1 cells onto plates coated with different matrices and then added LPS. The response of monocytes to LPS activation, assessed as adhesion (Fig. 4A) and IL-6 release (Fig. 4B), was greater with cells adhered on fibronectin and vitronectin. However, Rn effectively reversed these enhancements of fibronectin and vitronectin to the basal level caused by other matrices (Fig. 4A,B).

Focal adhesion kinase (FAK) regulates integrin-mediated signaling and phosphorylation of tyrosine 397 of FAK is a marker of FAK activity [15,16]. As LPS incubation time was prolonged, FAK phosphorylation increased and reached maximum at 30 min (Fig. 4C). However, Rn concentration dependently inhibited FAK phosphorylation in response to LPS stimulation (Fig. 4C).

We then used a combination of two siRNA duplexes targeting integrin αv to test its role in the LPS-activated THP-1. Transfection of integrin αv siRNA impaired endogenous αv protein expression as compared with αv expression in cells transfected with a negative control siRNA. However, transfection with negative control or αv siRNA did not change the expression of TLR4 (Fig. 4D), showing the specificity of the siRNA silencing of endogenous integrin αv.

The effect of transfection on cell viability was also examined using the MTT test, however, there was no significant difference observed for cells transfected with αv and negative control siRNA (data not shown). As shown in Fig. 4E–G, either transfection with integrin αv siRNA or treatment with Rn has similar and significant inhibitory effect on LPS-activated THP-1, and Rn has no further inhibitory effects on αv siRNA-transfected cells in terms of cell adhesion (Fig. 4E) and cytokine release (Fig. 4F,G), suggesting that integrin αv is involved in TLR4 signaling pathways leading to cell adhesion and cytokine release by activated THP-1.

We also used the αM siRNA transfected THP-1 cell to test the effect of Rn on LPS activation. Although αM siRNA transfection also significantly reduced LPS-induced cell adhesion and cytokine release, Rn still exhibited the inhibitory effects on αM siRNA-transfected cells (Fig. 4H,I). These results suggest that Rn interacts with receptors other than αM on THP-1 cells.

Rn reduced LPS-induced mortality

After i.p. challenge with LPS, a greater 24-h survival rate of Rn-treated animals ($n = 41$) was noted as compared with that of the untreated ones ($n = 45$) (90.24% vs. 71.11%; OR of

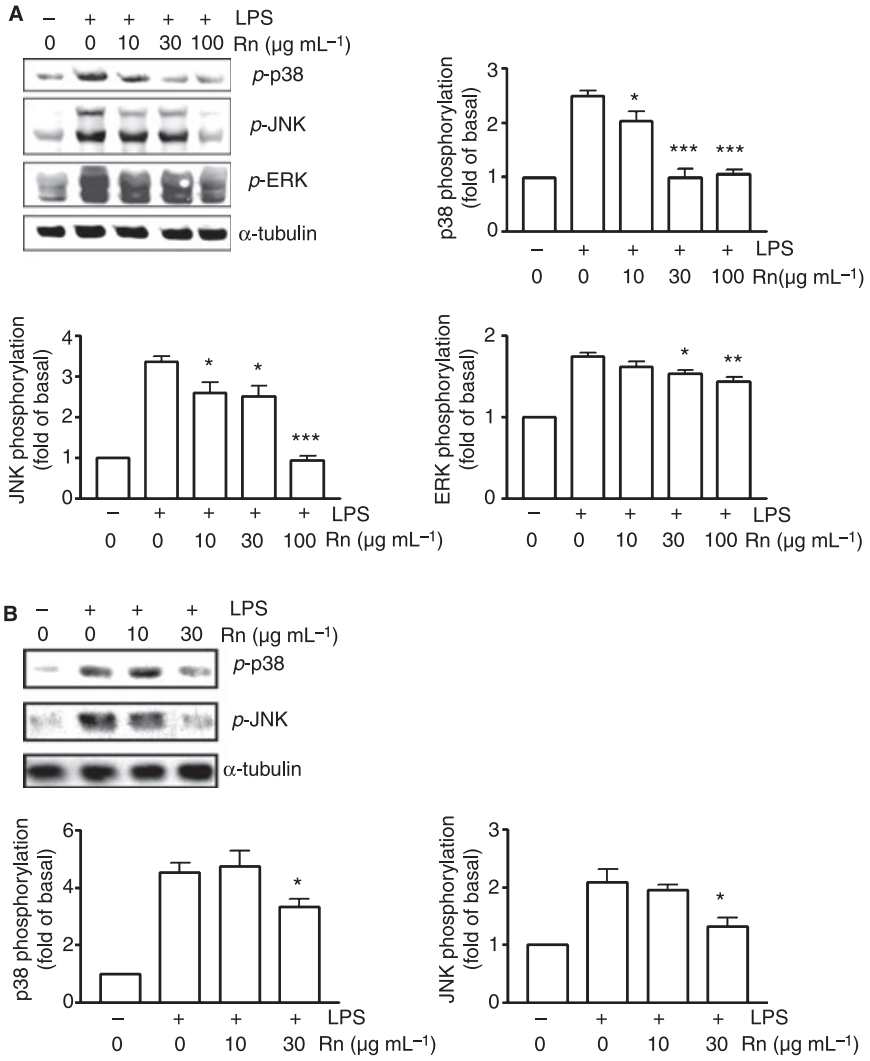

Fig. 2. The effect of rhodostomin (Rn) on lipopolysaccharide (LPS)-induced mitogen-activated protein kinase (MAPKs) activation in RAW264.7 and THP-1 Cells. LPS-activated RAW264.7 and THP-1 cells were incubated with different concentrations of Rn, and cell lysates were analyzed with Western blotting assay. (A) Immunoblotting analyzes of the level of phosphorylated p38, phosphorylated JNK, phosphorylated ERK and α-tubulin in RAW264.7 cells. (B) Immunoblotting analyzes of the level of phosphorylated p38, phosphorylated JNK and α-tubulin in THP-1 cells. Means ± SEM of three independent determinations of kinases activity were checked for statistical differences. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with LPS-activated control cells.

survival: 3.76, 95% CI: 1.16–12.03, $P < 0.05$). Moreover, we also monitored the vital signs of mice. After LPS challenge for 24 h, the body temperature and the systolic and diastolic pressure of mice were markedly lower (Fig. 5A,C,D), and all mice appeared fatigued and huddled together and lethargic. Tachycardia and bradycardia also occurred in some LPS-treated mice (Fig. 5B). In the Rn-therapeutic group, these vital signs, including blood pressure and body temperature, were significantly improved (Fig. 5A,C,D), and mice were much more energetic in appearance. Tachycardia and bradycardia symptoms were also normalized in Rn-treated mice (Fig. 5B).

Rn reverses LPS-induced thrombocytopenia

Bacterial infection can impair the hemostatic mechanisms including platelets, the coagulation system and the vessel wall. The development of thrombocytopenia usually indicates that the infection has become systemic [1,2]. The platelet count in LPS-challenged mouse blood was reduced from $1.10 \pm 0.02 \times 10^6/\mu L$ ($n = 16$) to $0.44 \pm 0.03 \times 10^6/\mu L$ ($n = 14$) after 24 h (Fig. 5E). However, Rn significantly increased the platelets number to $0.54 \pm 0.03 \times 10^6/\mu L$ ($P < 0.01$, $n = 16$) in septic mouse (Fig. 5E), indicating that Rn moderately reversed the LPS-induced thrombocytopenia.

Rn inhibits LPS-induced cytokine production in vivo

We used the LPS-induced endotoxemic animal model to investigate the effect of Rn on cytokine release *in vivo* (Fig. 6A). We found that the concentrations of cytokines were too low and undetectable in non-induction mice (i.p. injection with PBS, and data not shown). LPS caused elevated plasma IL-6

Table 1 The effect of lipopolysaccharide (LPS) and rhodostomin (Rn) on mean fluorescence intensity of phagocytes

mAb	LPS	Rn	RAW264.7	THP-1
IgG	–	–	10.32 ± 0.22	11.84 ± 0.51
	+	–	10.70 ± 0.54	11.70 ± 1.42
αvβ3	–	–	–	31.22 ± 1.56
		+		28.28 ± 1.38
	+	–	–	54.11 ± 2.66
		+		45.54 ± 4.07*
αv	–	–	28.22 ± 1.05	27.51 ± 2.56
		+	26.35 ± 1.18	23.03 ± 1.25
	+	–	32.02 ± 0.43	43.77 ± 1.49
		+	27.89 ± 0.88*	35.82 ± 1.05*
β1	–	–	65.16 ± 0.12	96.76 ± 2.46
		+	67.61 ± 4.75	104.30 ± 3.01
	+	–	78.33 ± 2.20	166.42 ± 1.38
		+	81.36 ± 2.77	160.26 ± 5.18
β2	–	–	–	188.82 ± 2.70
		+		195.87 ± 14.20
	+	–	–	233.13 ± 4.10
		+		242.52 ± 7.72
β3	–	–	36.98 ± 0.86	–
		+	35.70 ± 2.30	
	+	–	46.20 ± 1.18	–
		+	42.59 ± 1.39	
TLR4	–	–	44.31 ± 1.84	105.11 ± 2.71
		+	43.20 ± 2.28	100.22 ± 3.44
	+	–	52.92 ± 0.84	113.14 ± 3.56
		+	54.54 ± 2.47	115.84 ± 5.56

The Flowcytometry assay of the effect of Rn (30 μg mL^{-1}, +) on rest (–) and LPS-activated (+) RAW264.7 and THP-1. Data are expressed as the mean fluorescence intensity (Mean ± SEM, $n = 3$) and $^*P < 0.01$ compared between the presence and absence of Rn.

and TNF-α levels. When Rn (10 mg kg^{-1}) was i.v. administered immediately after LPS, LPS-induced IL-6 and TNF-α production were profoundly inhibited. Furthermore, Rn also significantly inhibited production of IL-1β and IL-10.

We also used mutant Rn (RGD/E) in the control experiment for specificity in the *in vivo* model; however, LPS-stimulated cytokine release was not significantly inhibited by RGE-Rn (data not shown). These results indicate that the RGD domain is important for the expression of anti-endotoxemic activities of Rn.

Protective effects of Rn on tissue injury in endotoxemia examined by histochemistry

Histological examination of lung and liver sections in LPS-induced septic mice revealed lung and liver injury in terms of infiltration of leukocytes in liver parenchyma (Fig. 6F) and the lung interstitium and alveoli, as well as thickening of the alveolar wall (Fig. 6C). The kidney was also damaged by LPS administration with evidence of glomerular hypercellularity (Fig. 6I). In contrast, the histologic appearance of the lung, liver and kidney sections in untreated animals was shown to be normal (Fig. 6B,E,H). The treatment of Rn significantly protected mice from liver, lung and kidney damage in LPS-induced endotoxemia, as shown by histological examination (Fig. 6D,G,J).

Discussion

In the present study, we found that Rn, an anti-platelet disintegrin, is useful in inhibiting the activation of LPS-stimulated phagocytes *in vitro* and alleviating the syndromes of LPS-induced extoxemia *in vivo*. The binding of Rn towards THP-1 monocyte was concentration dependent, and saturated at 50 μg mL^{-1} (6.82 μM) (Fig. 3C). It reveals that Rn specifically binds to monocytes in a receptor-mediated manner. It is known that disintegrins specifically bind to αvβ3 via the RGD sequence [5] and integrin αvβ3 staining is observed intensely in alveolar macrophages in inflamed lungs of sepsis patients [17]. In the present study, the flow cytometry assay showed that Rn specifically blocked the binding of anti-αv mAb to RAW264.7 and anti-αvβ3 mAb to THP-1 (Fig. 3A,B). The immobilized and pull-down assay also revealed that Rn selectively binds to αvβ3 in cell lysates of monocytes and macrophages (Fig. 3D,E). The silencing of integin αv inhibited LPS-induced THP-1 activation as effectively as Rn treatment (Fig. 4E–G). Disintegrin, jarastatin, is able to bind to αMβ2 integrin on neutrophil to inhibit migration, delay apoptosis and induce chemotaxis, and its activities are reversed by anti-αM antibody [18]. Flavoridin, a selective ligand of α5β1 and αvβ3, inhibits chemotaxis of neutrophil induced by fMLP [19] and has a mild pro-apoptotic effect on neutrophil [20]. Our group also reported that Rn blocks adhesion of neutrophils to immobilized fibrinogen, in parallel with decreasing the production of superoxide from adherent neutrophils. However, mAbs raised against the αM and β2 integrins did not inhibit the binding of Rn to activated neutrophils [10]. Although both the αMβ2 and αvβ3 integrins are possible targets of disintegrin on leukocyte, Rn specifically bound to the αvβ3 integrin on these phagocytes (Fig. 3). Taken together, the evidence indicates that integrin αvβ3 is the receptor of Rn responsible for antagonizing the stimulatory activities of phagocytes caused by LPS.

To mount effective responses against pathogens for the initiation of innate immune responses, TLRs act together with other microbe-recognition molecules [1,5]. In the present results, the ligation of integrin αvβ3 in peripheral blood monocytes resulted in the promotion of LPS induction of adhesion and cytokine release; however, silencing and blocking of integrin αv resulted in attenuating the enhanced cell adhesion and cytokine release by LPS (Fig. 4). Integrins αvβ3 and α4β1 were reported to serve as receptors and mediate pro-inflammatory action [21]. In addition, it is also indicated that the inhibition of αv-associated integrins might have potent anti-inflammatory effects in acute kidney allograft rejection [22]. Previous reports indicated that several Gram-negative bacterial pathogens interact with integrin αvβ3 to assist invasion directly or via extracellular matrix (ECM)-binding proteins, including *Borrelia burgdorferi*, *Neisseria meningitides*, *Neisseria gonorrhea* and *Porphyromonas gingivalis* [23]. On the basis of our data, we propose that integrin αvβ3 might act as an integral part in facilitating the TLR4 responses to pathogenic bacteria.

It is reported that the αvβ3 integrin appears to transduce an inhibitory signal for the down-regulation of LPS-induced

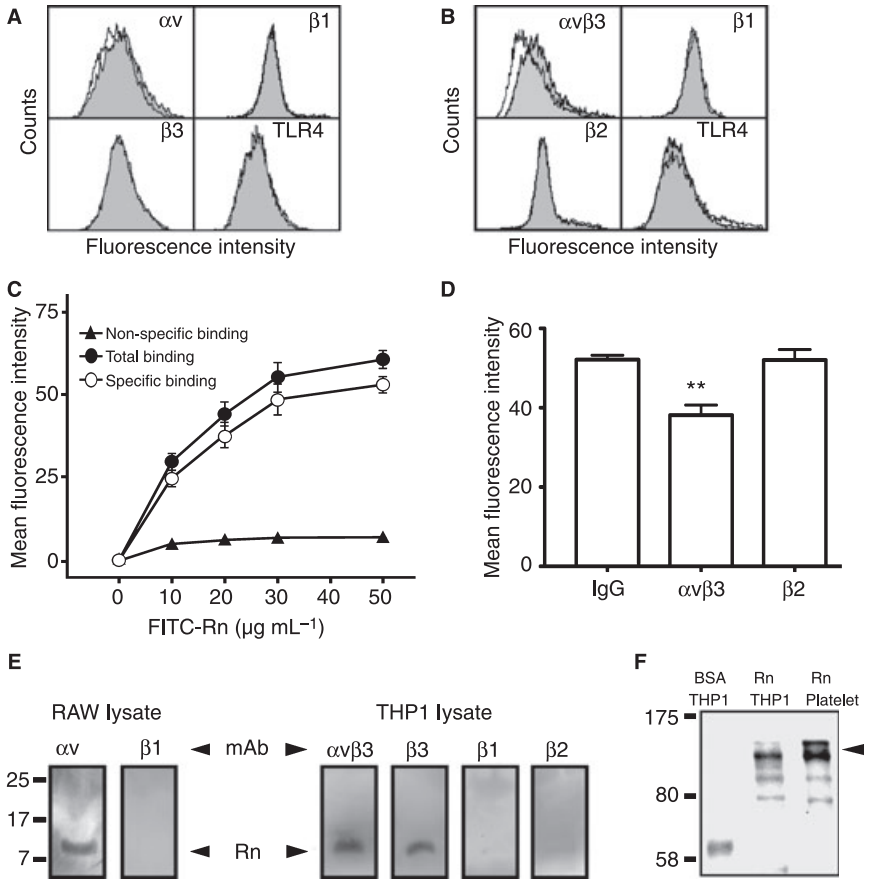

Fig. 3. The binding of rhodostomin (Rn) to RAW264.7 macrophage and THP-1 monocyte. (A) Lipopolysaccharide (LPS)-activated RAW264.7 cells were incubated with Rn (30 μg mL^{-1}, blank area) or not (gray area) before probing with anti-αv, anti-β3, anti-β1 or anti-TLR4 mAbs, respectively. (B) LPS-activated THP-1 cells were incubated with Rn (30 μg mL^{-1}, blank area) or not (gray area) before probing with anti-αvβ3, anti-β1, anti-β2 or anti-TLR4 mAbs, respectively. (C) LPS-activated THP-1 cells were incubated with increasing concentration of FITC-Rn for the fluorescence intensity measurement. Total binding (black circle) and non-specific binding (triangle) were determined by FITC-Rn and FITC-BSA, respectively. Specific binding (blank circle) was calculated by subtracting the non-specific binding from total binding. Data are the mean ± SEM (n = 3). (D) LPS-activated THP-1 cells were incubated with FITC-Rn (30 μg mL^{-1}) in the presence of different mAbs (20 μg mL^{-1}; control IgG, anti-αvβ3 and anti-β2) for the fluorescence intensity measurement. Data are the mean ± SEM (n = 3). $^{**}P < 0.01$, compared with control IgG group. (E) Rhodostomin (Rn, 20 μg) was applied to 20% SDS-PAGE and transferred to a poly(vinylidene difluoride) (PVDF) membrane. The membrane was incubated with cell lysate of RAW264.7 or THP-1 and followed by blotting with mAbs as indicated. (F) THP-1 or platelet proteins eluted from biotinylated Rn or bovine serum albumin (BSA) and streptavidin-Sepharose beads were blotted by an anti-β3 monoclonal antibody (7E3).

NO synthesis [24]. Besides activating MAPK pathways, LPS can also activate integrin αvβ3 signaling, e.g. phosphorylations of integrin β3, FAK and Pyk2 (FAK family members) [25]. In the LPS-activated phagocytes, we found that Rn not only inhibited phosphorylation of MAPK (Fig. 2A,B), but also phosphorylation of FAK (Fig. 4C). Either silencing or blocking of integrin αvβ3 (Rn treatment) attenuated LPS-induced cytokine release in monocytes but with no synergistic effect in monocyte. In addition, Rn apparently had no direct effect on TLR4 binding as probed by TLR4 mAb (Fig. 3A,B). It is suggested that Rn might inhibit MAPKs phosphorylation through blocking LPS-activated integrin signaling. However, the cross-talk between TLR4 and the integrin αvβ3 signaling pathways is still under investigation. It has been reported that RGDS with high specificity for αv integrins attenuates the inflammatory cascade through the MAP kinase leading to the NF-κB pathway during LPS-induced development of acute lung injury [26]. Stimulation with LPS on phagocytes could activate FAK and integrins to generate inside-out signaling for enhanced binding of its ligands [14]. Integrin-dependent adhesion and/or clustering mediate outside-in signaling to phosphorylate the tyrosine of FAK/Src, and then activate the Ras/MEK/ERK cascade [27], therefore, LPS signaling activated the integrin signal that amplifies ERK and JNK activations in macrophages [28]. However, anti-αv or anti-β3 mAb inhibited LPS-induced c-Src and FAK activation [24] and Rn also suppressed LPS-induced FAK phosphorylation in monocytes (Fig. 4C). Collectively these findings suggest that the specific inhibition of integrin signaling by Rn results in suppression of proinflammatory mediator productions through blockade of the FAK-dependent MAPK pathways.

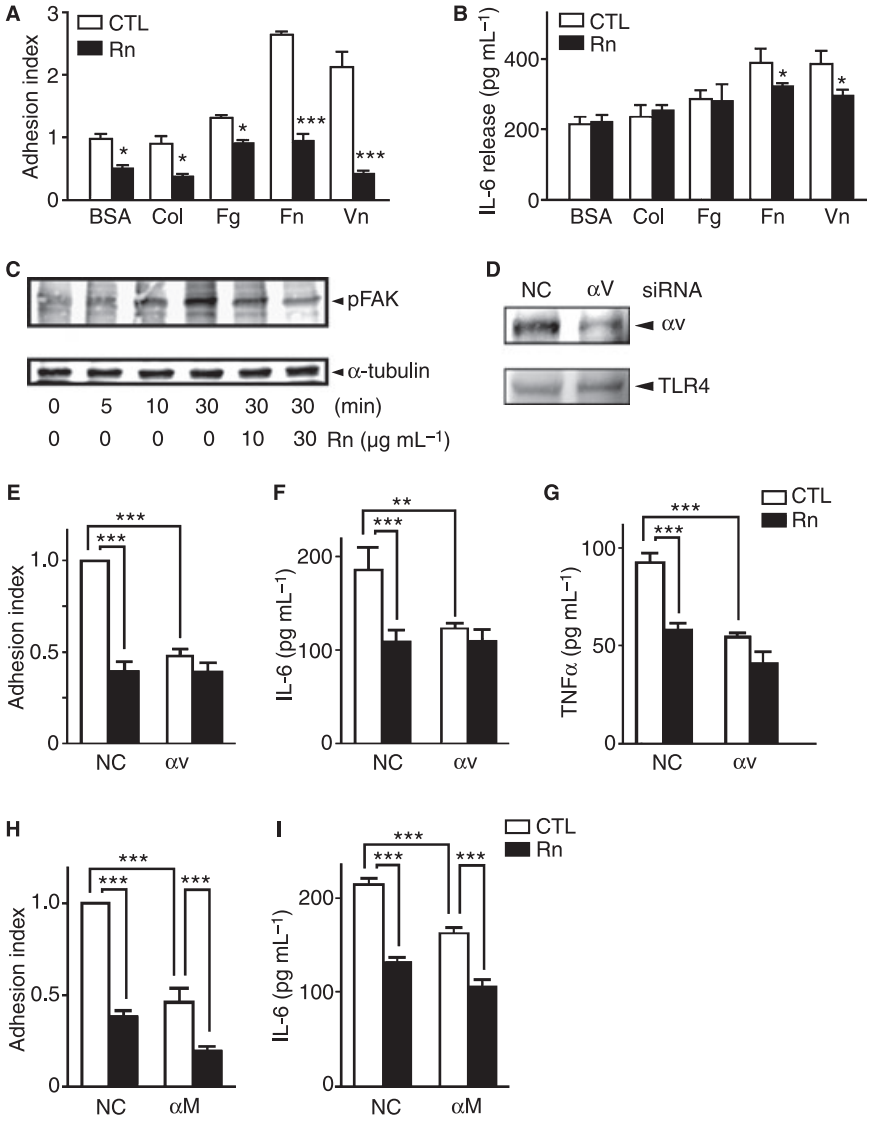

Fig. 4. Rhodostomin (Rn) inhibits the adhesion and interleukin (IL)-6 release of the lipopolysaccharide (LPS)-stimulated THP-1 through blockade of integrin αvβ3 instead of αMβ3. (A) Adhesion and (B) IL-6 production by LPS-stimulated THP-1 cells seeded on plates coated with albumin (BSA), collagen (Col), fibrinogen (Fg), fibronectin (Fn) and vitronectin (Vn), in the absence (CTL) or presence of Rn (Rn). Means ± SEM of three separate experiments were checked for statistical differences. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, compared with LPS-activated control cells. (C) LPS-activated THP-1 cells were incubated with different concentrations of Rn for an indicated time interval and cell lysates were analyzed with Western blotting assay. Immunoblotting analysis of the level of phosphorylated FAK and α-tubulin was carried out. (D–I) THP1 cells were transfected with integrin αv (αv), integrin αM (αM) or negative control (NC) siRNA for 48 h followed by different assays. (D) Western blotting for integrin αv and TLR4. (E and H) The adhesion index was calculated as the fold of adhered cells normalized to that of the LPS-treated control group. (F and I) IL-6 and (G) TNF-α release measured with the ELISA kit. Blank bar, untreated control (CTL). Black bar, incubated with Rn (Rn). Results are expressed as the mean ± SEM ($n = 3$) and $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with the indicated groups.

Thrombocytopenia occurs early in the course of septicemia and may alert the clinician to the possibility of septicemia [1,2]. Triflavin is another RGD-containing disintegrin, which was reported to prevent thrombocytopenia [29]. We found that Rn also alleviated thrombocytopenia induced by LPS *in vivo*. Rn markedly inhibits platelet aggregation and blocks the adhesion of platelets to subendothelial matrices, resulting in decreased platelet activation and thrombus formation. This may increase the blood flow and improve organ failure in the sepsis-challenged host. Coagulation and inflammation may proceed in a self-propagating feedback loop to amplify each other and to induce a lethal disseminated intravascular coagulation (DIC) [30]. We also injected heparin (180 U kg^{-1}) i.v. after LPS, and observed a less favored effect, as compared with Rn, with a moderate reduction of cytokine release (data not shown). Therefore, the significantly protective effect of Rn on the syndromes and cytokine production in endotxemic mice may be synergistically caused by both of its intrinsic antiplatelet and

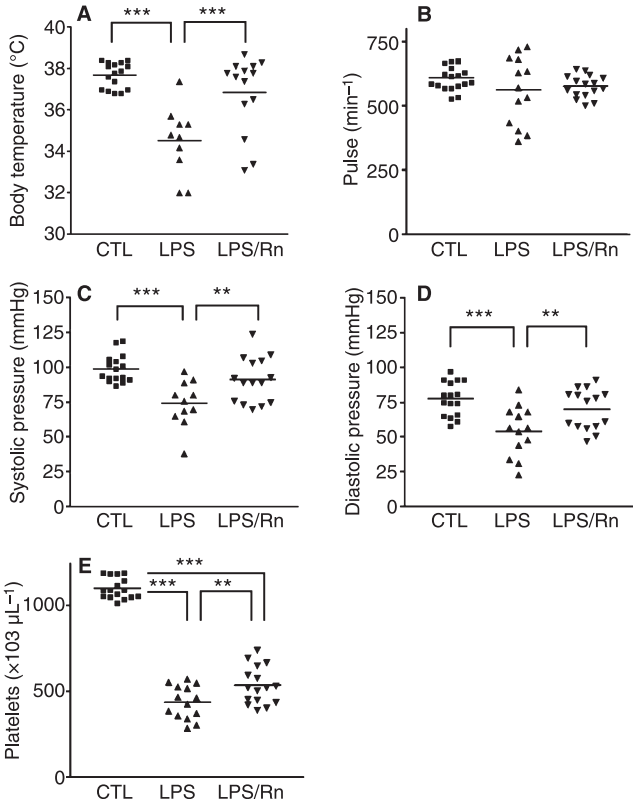

Fig. 5. The effects of rhodostomin (Rn) on cardiovascular dysfunction induced by lipopolysaccharide (LPS) administration *in vivo*. The rectal temperature (A), heart pulse (B) and sysytolic (C) and diastolic blood pressure (D) of mice were measured. Platelet numbers (E) of euthanized mice were counted. CTL, untreated control. LPS, LPS only. LPS/Rn, Rn administration after LPS. Bars represent the mean of each group (n = 11–16), and $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the indicated groups.

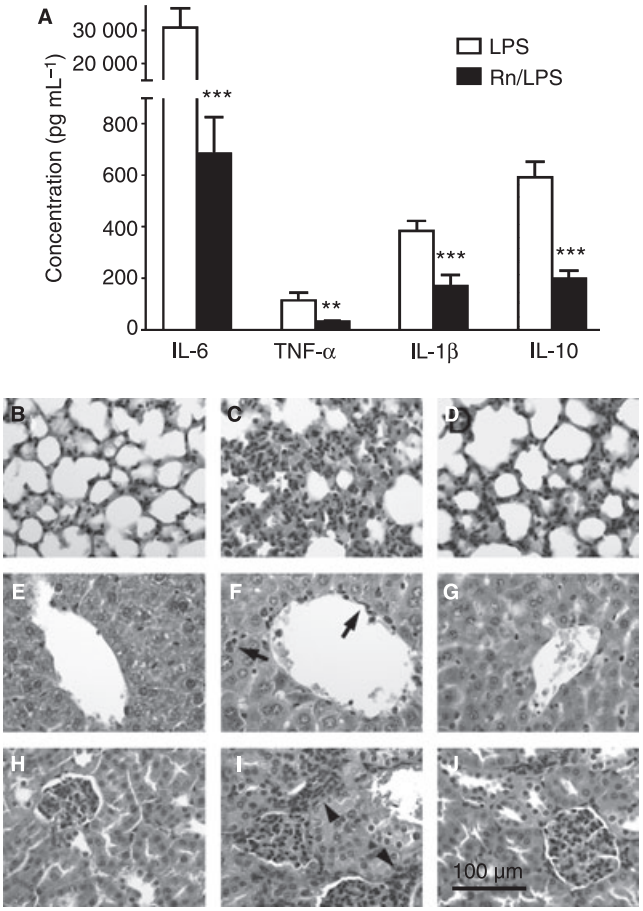

Fig. 6. The protective effects of rhodostomin (Rn) on cytokine production and tissue inflammation induced by lipopolysaccharide (LPS) administration *in vivo*. (A) Mice were anesthetized and blood was collected for serum isolation, and concentration of cytokines was measured by ELISA. Values are presented as mean ± SEM (n = 10), and $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with LPS-treated control group. (B–J) Morphological changes in the mouse lung (B–D), liver (E–G) and kidney (H–J) sections [hematoxylin and eosin (H&E) stain, all panels are × 400 with the same scale bar in panel J]. (B, E and H) Control mice, (C, F and I) LPS-treated mice, (D, G and J) LPS-treated mice with Rn. Adherent and infiltrated leukocytes in liver (arrow) and glomerular hypercellularity in kidney (arrowhead) were indicated.

anti-inflammation activities. By inhibiting the formation of thrombin and the release of proinflammatory cytokines, recombinant human-activated protein C has been approved by FDA as adjunctive therapy in septic patients with a high risk of death [31]. In contrast to a long list of other immunomodulators developed for sepsis, anti-thrombotic therapy might be a good and effective strategy. Sepsis and septic shock are a serious epidemiological problem worldwide, and they are a leading cause of death without suitable effective monotherapy. The syndromes involving inflammation, hypercoagulation and multi-organ dysfunction are profound and complicated in sepsis/endotoxemia. In the present study, Rn was chosen as a good candidate for an anti-endodotoxemic agent with a high potential, because it possesses dual antiplatelet and anti-inflammatory activities, although some effects of Rn were modest. APC and Rn are both anticoagulant and anti-inflammatory. With some further optimal modification of this disintegrin and some more animal experiments, disintegrins might be developed as one of the candidates for sepsis. However, drug treatment before the induction of sepsis usually does not reflect the clinical practice. we performed the post-treatment study, in which Rn was given 1 h after LPS administration. One hours after the LPS injection, the mice became endotoxemic, and were lethargic and had diarrhea. It was shown that LPS-induced thrombocytopenia was partially reversed and the LPS-induced production of IL-6 in plasma was also reduced by Rn-treatment (data not shown). These data indicate that Rn is still effective even after the syndromes of endotoxemia appeared in mice.

In conclusion, we demonstrated that Rn markedly reduced cytokine production, blocked phagocytes activity and reversed thrombocytopenia, and thus alleviating the syndromes of LPS-induced endotoxemia. We also found that integrin αvβ3 on phagocytes not only increases adhesion but also facilitates the innate inflammation initiated by TLR4 ligation. However, further studies are required to investigate how Rn interrupts the LPS-induced signaling pathway.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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