

Suppressive action of resolvin D1 on the production and release of septic mediators in D-galactosamine-sensitized endotoxin shock mice

TAISUKE MURAKAMI¹, KAORI SUZUKI¹, HIROSHI TAMURA² and ISAO NAGAOKA¹

¹Department of Host Defense and Biochemical Research, Juntendo University, Graduate School of Medicine; ²Seikagaku Biobusiness Corporation, Tokyo, Japan

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Abstract. Endotoxin/septic shock is a severe condition induced during serious infections with Gram-negative bacteria. To evaluate the therapeutic potential of resolvin D1 (RvD1), a novel pro-resolving molecule, on endotoxin/septic shock, we investigated the effect of RvD1 on the extracellular release of high mobility group box-1 (HMGB1), the production of inflammatory cytokines, the accumulation of peritoneal cells and hepatocyte apoptosis *in vivo* using a D-galactosamine (GalN)-sensitized mouse endotoxin shock model. Serum HMGB1 levels were markedly elevated after challenge with lipopolysaccharide (LPS)/D-GalN, and RvD1 administration significantly reduced HMGB1 levels. Furthermore, the serum levels of inflammatory cytokines, such as TNF- α , IL-6, IL-10 and macrophage chemotactic protein (MCP)-1 were elevated in the endotoxin shock model. Importantly, RvD1 administration slightly reduced the TNF- α , IL-6 and IL-10 levels, and further lowered MCP-1 levels. Moreover, RvD1 administration affected the peritoneal cell accumulation and decreased the neutrophil population. Finally, LPS/D-GalN injection induced apoptosis in the liver (mostly of hepatocytes), and RvD1 administration reduced the apoptosis of hepatocytes. These observations suggest that RvD1 may be a therapeutic agent for sepsis/endotoxin shock by exerting suppressive action on the release and production of septic mediators (HMGB1 and inflammatory cytokines), the accumulation of peritoneal cells and hepatic apoptosis.

Introduction

Sepsis is caused by polymicrobial infections associated with severe systemic inflammatory response syndrome, which leads

to multiple organ failure, such as acute lung injury, renal and hepatic failure, and septic shock (1-3). Bacterial endotoxin lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and plays a pivotal role by stimulating mononuclear phagocytes (macrophages and monocytes) to secrete various inflammatory mediators, such as cytokines, reactive oxygen species, prostanooids/leukotrienes, proteases and NO (1,3). In addition, the high-mobility group box-1 (HMGB1), a highly conserved non-histone nuclear protein that binds with DNA, participates in gene transcription and functions as a cytokine in the extracellular milieu, up-regulates the expression of proinflammatory cytokines (e.g., TNF- α , IL-1 β and IL-8) in human mononuclear cells and neutrophils (4,5) and adhesion molecules, such as the intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells (6). Furthermore, HMGB1 is suggested to play a crucial role in endotoxin/septic shock as a late phase mediator. This is based on the findings that serum levels of HMGB1 increase in a late phase (8-32 h) after LPS exposure, and that administration of an anti-HMGB1 antibody attenuates endotoxin lethality in mice (7). Since these pro-inflammatory substances are overproduced and involved in the pathogenesis of septic shock, therapeutic strategies have targeted the blockade of these molecules; however, most of the strategies have been unsuccessful (8,9). Thus, the development of novel agents with therapeutic potential for sepsis/endotoxin shock is being explored.

The resolution phase of inflammation is now understood as not only a passive dilution of pro-inflammatory mediators, but also a highly regulated and active process via the production of various anti-inflammatory and pro-resolving mediators. Recently, novel ω -3 polyunsaturated fatty acid [e.g. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)]-derived mediators with potent anti-inflammatory and pro-resolving activities have been discovered and termed resolvins (10,11). E-series resolvins are derived from EPA, and D-series resolvins are from DHA. Resolvin D1 (RvD1, 7S,8R,17S-trihydroxy-DHA), a D-series resolvin, was originally identified in resolving inflammatory exudates and is produced by sequential oxygenation of DHA by 15-lipoxygenase (LOX) and 5-LOX *in vivo* (12). RvD1 exerts potent anti-inflammatory and pro-resolving actions on

Correspondence to: Dr Isao Nagaoka, Department of Host Defense and Biochemical Research, Juntendo University, Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
E-mail: nagaokai@juntendo.ac.jp

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various cell types. For example, RvD1 was found to suppress the transendothelial migration of human neutrophils *in vitro* (12) and neutrophil infiltration *in vivo* in a murine peritonitis model (12), as well as the release of pro-inflammatory cytokines from LPS-stimulated mouse macrophages *in vitro* (13,14). Based on these potential pro-resolving activities of RvD1, it is reasonable to speculate that RvD1 may attenuate the pathological condition of sepsis/endotoxin shock. In this study, we evaluated the effect of RvD1 on the extracellular release of HMGB1, the production of inflammatory cytokines, the accumulation of peritoneal cells and hepatocyte apoptosis *in vivo* using a D-galactosamine (D-GalN)-sensitized mouse endotoxin shock model.

Materials and methods

Reagents. LPS (from *E. coli* serotype O111:B4) and D-(+)-galactosamine hydrochloride (D-GalN) were purchased from Sigma Chemicals (St. Louis, MO, USA). RvD1 was obtained from Cayman Chemical (Ann Arbor, MI, USA). A mouse inflammation kit (BD™ Cytometric Bead Array system) was purchased from BD Bioscience (San Jose, CA, USA).

D-GalN-sensitized endotoxin shock model. A D-GalN-sensitized mouse model (15), which is highly susceptible to LPS, was utilized to assess the potential of RvD1 to suppress an inflammatory reaction *in vivo*. Male C57BL/6 mice aged 7–9 weeks (Sankyo Laboratories, Tokyo, Japan) were intraperitoneally injected with 400 μ l of D-GalN (18 mg, dissolved in saline) or D-GalN + LPS (25 ng) without or with RvD1 (0.1 or 1 μ g). Thereafter, mice were anesthetized by intraperitoneal injection of pentobarbital, and blood was collected by cardiac puncture for the assays of the cytokines (1 h after the challenge) and HMGB1 (5 h after the challenge). All animal procedures were approved by the Ethics Committee of Juntendo University, Graduate School of Medicine and performed according to the institutional guidelines.

Quantification of serum cytokine levels. Serum levels of TNF- α , IL-6, IL-10, IL-12p70, MCP-1 and IFN- γ were quantified with the mouse inflammation kit. Sera were prepared from blood by centrifugation at 1000 g for 10 min and diluted 10-fold with an assay diluent. Then, 50- μ l aliquots were assayed for cytokines according to the manufacturer's instructions. The system includes six fluorescently distinguishable capture beads coated with antibodies against six analytes (TNF- α , IL-6, IL-10, IL-12p70, MCP-1 and IFN- γ) and detects these cytokines by ELISA on microbeads. The detection limits were 7.3 pg/ml for TNF- α , 5 pg/ml for IL-6, 17.5 pg/ml for IL-10, 10.7 pg/ml for IL-12p70, 52.7 pg/ml for MCP-1 and 2.5 pg/ml for IFN- γ .

Quantification of serum HMGB1. Serum HMGB1 levels were quantitated with 10- μ l aliquots of sera using an HMGB1 ELISA kit II (Shino-Test Corp., Kanagawa, Japan) according to the manufacturer's instructions. The detection limit of HMGB1 was 1 ng/ml.

Peritoneal exudate cell counting. Mice were euthanized by ether anesthesia 5 h after receiving the peritoneal LPS injection

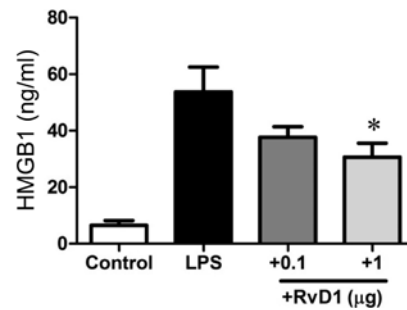


Figure 1. Effect of RvD1 on the levels of serum HMGB1 in D-GalN-sensitized endotoxin shock mice. Mice were intraperitoneally injected with (D-GalN) 18 mg/mouse and (LPS) 25 ng/mouse without or with administration of RvD1 (0.1 or 1 μ g/mouse; +RvD1). Mice injected with D-GalN alone were used as controls. Blood was collected 5 h after the challenge, and the serum level of HMGB1 was quantitated by ELISA. Data are the mean \pm SEM of 11 to 13 mice in each group. Values were compared: without and with RvD1 administration (LPS vs. +RvD1). * P <0.05.

tion and D-GalN, and peritoneal exudate cells were recovered by washing with 5 ml ice-cold PBS. Cells were stained with Turk's solution for counting the total cell number. Alternatively, cells were cytocentrifuged (Cytospin 4; ThermoShandon, Cheshire, UK) and stained with May-Grünwald-Giemsa solution (Muto Pure Chemicals, Tokyo, Japan) for differential cell counts (neutrophils and mononuclear cells); at least 200 cells per slide on duplicate cytopsins were examined under a light microscope.

Evaluation of hepatocyte apoptosis. Mice were euthanized by ether anesthesia 5 h after the peritoneal LPS injection and D-GalN administration, and the liver was then resected, trimmed and fixed with 4% paraformaldehyde for 12 h at 4°C, followed by immersion in a series of PBS/sucrose solution until reaching the sucrose concentration of 30%. The tissue was embedded in optimal cutting temperature (OCT) embedding medium (Sakura Finetechnical, Tokyo, Japan), by freezing in liquid nitrogen and sectioned at 50 μ m with a cryostat (CM3051 S, Leica, Wetzlar, Germany). Sections were stained with a TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) reagent for detecting apoptosis (*In Situ* Cell Death Detection kit, Fluorescein, Roche Diagnostics, Penzberg, Germany) and mounted with an aqueous medium fluoromount (Diagnostic Biosystems, Pleasanton, CA, USA). Fluorescent images were captured with a microscope system Axioplan 2 (Carl Zeiss, Jena, Germany). The number of TUNEL⁺ cells was counted in 2 high power fields/mouse (x200 magnification) and averaged. Sections were also stained with May-Grünwald-Giemsa; we confirmed that the inflammatory cells, such as neutrophils and mononuclear cells, were not infiltrated into the injured liver tissues 5 h after the injection of LPS/D-GalN (data not shown).

Statistical analysis. Data represent the mean \pm standard error of the mean (SEM). Statistical significance was determined by one-way ANOVA analysis (Graphpad PRISM, La Jolla, CA, USA). A P -value <0.05 was considered to be significant.

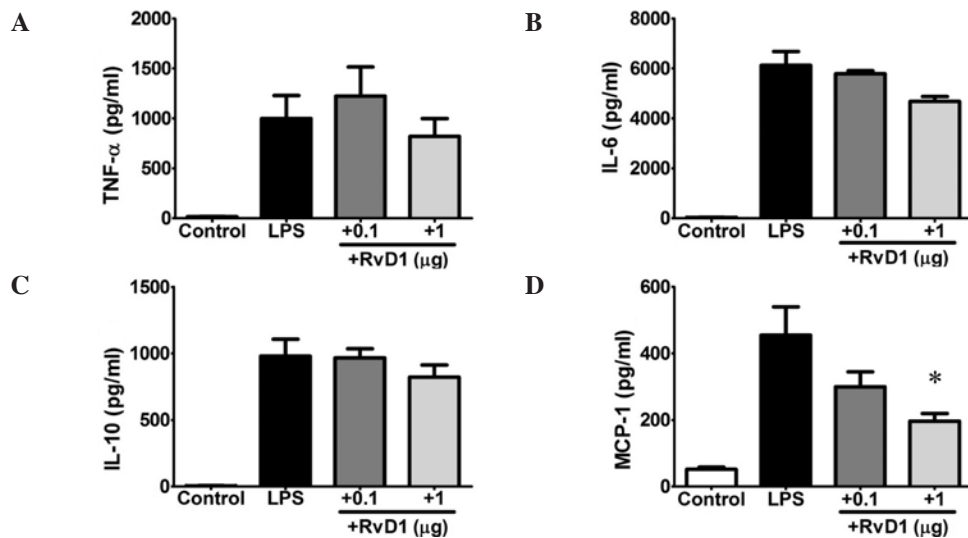


Figure 2. Effect of RvD1 on the levels of serum cytokines in D-GalN-sensitized endotoxin shock mice. Mice were intraperitoneally injected with D-GalN (18 mg/mouse) and LPS (25 ng/mouse) without or with RvD1 (0.1 or 1 μg/mouse; +RvD1). Mice injected with D-GalN alone were used as the controls. Blood was collected 1 h after the challenge, and the serum levels of TNF-α (A), IL-6 (B), IL-10 (C) and MCP-1 (D) were quantitated by cytometric bead array system. Data are the mean ± SEM of 5 to 7 mice in each group. Values were compared: without and with RvD1 administration (LPS vs. +RvD1). *P<0.05.

Results

Effects of resolvin D1 on the release and production of septic mediators in a D-GalN-sensitized endotoxin shock model. To determine whether RvD1 modulates the levels of septic mediators in LPS/D-GalN mice, we examined the effect of RvD1 on the release and production of septic mediators in sera. We first investigated the effect of RvD1 on the serum level of HMGB1, a non-histone nuclear protein that is extracellularly released from dying cells and functions as a late-phase mediator in endotoxin/septic shock (7,16,17). As presented in Fig. 1, the HMGB1 level was strikingly elevated from 6.5 ± 1.7 to 53.7 ± 8.8 ng/ml 5 h after LPS administration ($P < 0.001$). Notably, the administration of 1 μg of RvD1 significantly reduced the HMGB1 level to 30.6 ± 5.0 ng/ml ($P < 0.05$). We then examined the effect of RvD1 on the serum levels of inflammatory cytokines. As presented in Fig. 2, TNF-α, IL-6, IL-10 and MCP-1 apparently increased 1 h after the LPS injection ($P < 0.001$). Notably, RvD1 administration slightly reduced the levels of TNF-α, IL-6 and IL-10 and significantly suppressed the level of MCP-1 at 1 μg ($P < 0.05$).

Effect of resolvin D1 on the number of peritoneal exudate cells. RvD1 was found to suppress the transendothelial migration of neutrophils *in vitro* and peritoneal infiltration of neutrophils *in vivo* in a murine zymosan-induced peritonitis model (12). Thus, we examined the effect of RvD1 on the number of peritoneal exudate cells in the LPS/D-GalN mice. As presented in Fig. 3A, the total peritoneal cell number was marginally increased at 5 h after the LPS injection, which was due to the accumulation of neutrophils (Fig. 3B) and the concomitant decrease in mononuclear cells (mostly macrophages) in the peritoneal cavity (Fig. 3C). Notably, the RvD1 administration slightly decreased the total peritoneal cell number and neutrophil population, while it increased the mononuclear cell population, although these changes were not statistically significant.

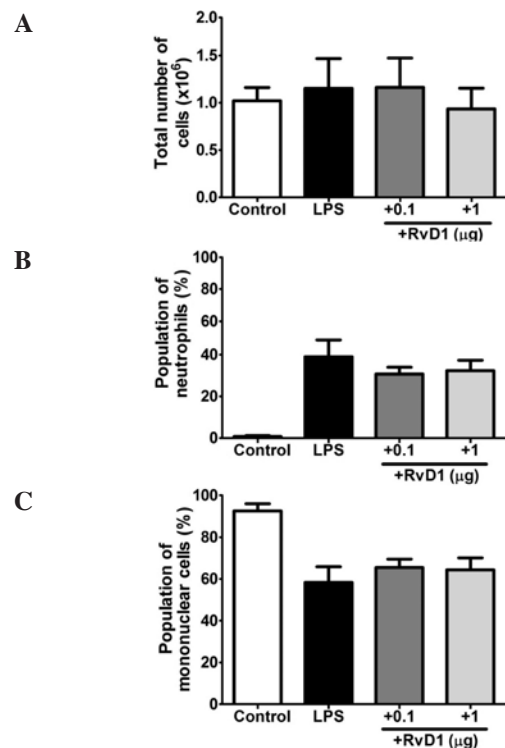


Figure 3. Effect of RvD1 on the number of peritoneal exudate cells in D-GalN-sensitized endotoxin shock mice. Mice were intraperitoneally injected with D-GalN (18 mg/mouse) and LPS (25 ng/mouse) without or with administration of RvD1 (0.1 or 1 μg/mouse; +RvD1). Mice injected with D-GalN alone were used as controls. Peritoneal exudate cells were collected 5 h after the challenge. Cells were stained with Turk's solution for counting the total cell number (A). Alternatively, cells were cytocentrifuged and stained with May-Grünwald-Giemsa for assessing the differential cell counts, neutrophils (B) and mononuclear cells (C). Data are the mean ± SEM of 6 to 9 mice in each group.

Effect of resolvin D1 on hepatocyte apoptosis. Finally, to examine the effect of RvD1 on organ failure in endotoxin/septic shock, we evaluated the extent of liver injury in LPS/D-

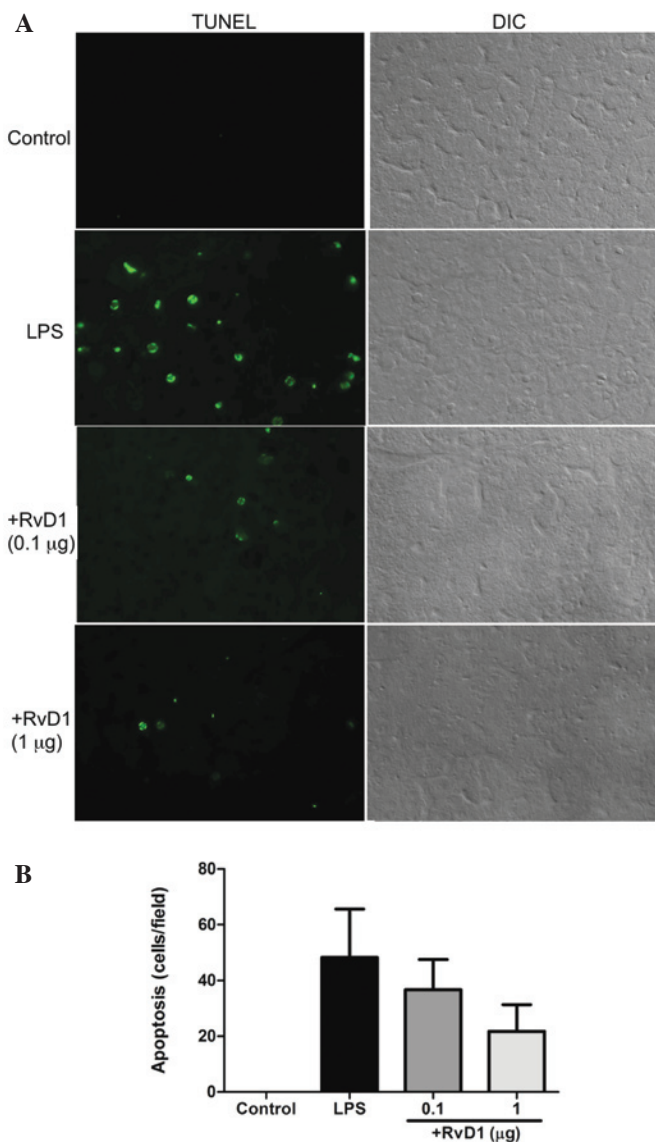


Figure 4. Effect of RvD1 on the apoptosis of hepatocytes in the liver of D-GalN-sensitized endotoxin shock mice. Mice were intraperitoneally injected with D-GalN (18 mg/mouse) and LPS (25 ng/mouse) without or with RvD1 (0.1 or 1 µg/mouse; +RvD1). Mice injected with D-GalN alone were used as controls. After the challenge (5 h), liver sections were stained with a TUNEL reagent (apoptotic cells; green). (A) Left panels present the images of TUNEL staining; right panels present the images of differential interference contrast (DIC) microscopy. All images are representative of 3 to 6 samples. (B) The number of TUNEL⁺ cells was counted in 2 high power fields (x200 magnification)/mouse and averaged. Data are the mean \pm SEM of 3 to 6 mice.

GalN mice (18). As revealed in Fig. 4A, TUNEL⁺ apoptotic cells (mostly hepatocytes) were apparently increased in the liver of the LPS/D-GalN mice, compared to the control mice injected with D-GalN alone. To note, RvD1 administration dose-dependently reduced the apoptosis of parenchymal cells (hepatocytes) in the liver (Fig. 4B).

Discussion

Endotoxin/septic shock is a severe condition that is induced during serious infections with Gram-negative bacteria (1-3). Although it is characterized by systemic inflammatory

responses of the host to invading microorganisms, most anti-inflammatory therapeutic strategies have failed to improve the prognosis of patients (8,9). Thus, the development of novel agents with therapeutic potential for sepsis/endotoxin shock has been explored. In this study, to evaluate the therapeutic potential of RvD1, a pro-resolving molecule, on endotoxin/septic shock, we investigated the effect of RvD1 on the extra-cellular release of HMGB1, the production of inflammatory cytokines, the accumulation of peritoneal cells and hepatocyte apoptosis *in vivo* using a D-GalN-sensitized mouse endotoxin shock model.

HMGB1, a 30-kDa non-histone nuclear protein, has been reported to play a crucial role in endotoxin/septic shock by functioning as a late phase mediator (16,19,20). In this context, serum HMGB1 levels are highly elevated in septic patients who succumb to the disease (non-survivors) compared to patients with non-lethal infection (survivors); moreover, the administration of an anti-HMGB1 antibody was found to attenuate endotoxin lethality in mice (7). Thus, HMGB1 is recognized as a potential therapeutic target for the treatment of endotoxin/septic shock (20). In this study, serum HMGB1 levels were markedly elevated 5 h after the LPS-challenge in LPS/D-GalN mice. Notably, RvD1 significantly reduced the HMGB1 level.

Inflammatory cytokines such as TNF- α , IL-6, IL-10 and MCP-1 are produced and released by mononuclear phagocytes (macrophages) shortly after exposure to LPS, and play a pivotal role in the pathogenesis of lethal systemic inflammation in endotoxin/septic shock (1). TNF- α and IL-6 activate neutrophils, lymphocytes and vascular endothelial cells, up-regulate cellular adhesion molecules and induce the production of lipid mediators, nitric oxide and reactive oxygen species, whereas IL-10 negatively regulates these responses. In addition, MCP-1, as a chemokine, activates inflammatory cells (particularly macrophages) to migrate into tissues. Notably, RvD1 has been found to suppress the production of inflammatory cytokines from LPS-stimulated macrophages (13,14). Moreover, RvD1 apparently down-regulates MCP-1 and IL-8 production from LPS-stimulated human aortic endothelial cells (14). The present study revealed that the serum levels of inflammatory cytokines (particularly TNF- α , IL-6, IL-10 and MCP-1) were elevated in our endotoxin shock model. Importantly, the RvD1 administration slightly reduced the TNF- α , IL-6 and IL-10 levels and further lowered the MCP-1 level.

Since RvD1 was found to suppress the peritoneal infiltration of neutrophils in a murine zymosan-induced peritonitis model (12), we investigated the effect of RvD1 on the peritoneal cell count in LPS/D-GalN mice. RvD1 administration slightly affected the peritoneal cell accumulation; it decreased the neutrophil population, but increased the mononuclear cell population.

It has been demonstrated that the death of LPS/D-GalN mice is mainly a result of injury to the liver, but not to other organs, such as the lung, as D-GalN is specifically hepatotoxic (15). Thus, we evaluated the effect of RvD1 on hepatic apoptosis in the LPS/D-GalN mice. As previously reported (18), the LPS/D-GalN injection induced apoptosis in the liver, and TUNEL⁺ cells were mostly hepatocytes. Notably, RvD1 administration reduced the apoptosis of hepatocytes.

The present study revealed the suppressive action of RvD1 on the release of HMGB1, the production of inflammatory cytokines, the accumulation of peritoneal neutrophils and hepatic apoptosis, and suggests that RvD1 may be a therapeutic agent for sepsis/endotoxin shock, although the molecular mechanisms for the suppressive actions remain to be elucidated. RvE1, a member of the resolvins family, has been demonstrated to markedly suppress leukocyte infiltration/migration and cytokine production by attenuating NF- κ B signaling via the action on specific receptors, such as ChemR23 and BLT (leukotriene B₄ receptor 1) (21,22). Notably, RvD1 has recently been suggested to exert pro-resolving actions on neutrophils and macrophages via the candidate receptors ALX (lipoxin A₄ receptor) and GPR32 (G-protein-coupled receptor 32) (23). Moreover, it is known that the activation of NF- κ B is involved in the extracellular release of HMGB1 and apoptotic cell death (24,25). Based on these observations, it may be speculated that RvD1, an analogue of RvE1, also exerts pro-resolving activities by attenuating NF- κ B signaling, thereby suppressing inflammatory cytokine production and peritoneal cell infiltration, as well as HMGB1 release and hepatocyte apoptosis via the action on specific receptors, such as ALX and GPR32. Nevertheless, the precise mechanisms for the suppressive actions of RvD1 on LPS/D-GalN mice observed in this study should be clarified in the future.

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