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Macrophage-derived HMGB1 as a pain mediator in the early stage of acute pancreatitis in mice: targeting RAGE and CXCL12/CXCR4 axis

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Abstract Extracellular high mobility group box 1 (HMGB1) activates the receptor for advanced glycation end products (RAGE) or Toll-like receptor 4 (TLR4) and forms a heterocomplex with CXCL12 that strongly activates CXCR4, promoting inflammatory and pain signals. In the present study, we investigated the role of HMGB1 in pancreatic pain accompanying cerulein-induced acute pancreatitis in mice. Abdominal referred hyperalgesia accompanying acute pancreatitis occurred within 1 h after 6 hourly injections of cerulein. The anti-HMGB1 neutralizing antibody or recombinant human soluble thrombomodulin (rhsTM), known to inactivate HMGB1, abolished the cerulein-induced referred hyperalgesia, but not pancreatitis itself. Plasma or pancreatic HMGB1 levels did not change, but macrophage infiltration into the pancreas occurred 1 h after cerulein treatment. Minocycline, a macrophage/microglia inhibitor, ethyl pyruvate that inhibits HMGB1 release from macrophages, or liposomal clodronate that depletes macrophages prevented the referred hyperalgesia, but not pancreatitis. Antagonists of RAGE or CXCR4, but not TLR4, strongly suppressed the cerulein-induced referred hyperalgesia, but not pancreatitis. Upregulation of RAGE, CXCR4 and CXCL12, but not TLR4, were detected in the pancreas 1 h after cerulein treatment. Our data suggest that HMGB1 regionally secreted by macrophages mediates pancreatic pain by targeting RAGE and CXCL12/CXCR4 axis in the early stage of acute pancreatitis.

Keywords Macrophage · Pancreatic pain · HMGB1 · RAGE · CXCR4 · thrombomodulin

Introduction

Patients with pancreatic diseases, such as acute or chronic pancreatitis and pancreatic cancer, frequently experience pancreatic pain, which includes nociceptive, inflammatory and neuropathic components of pain (Ceyhan et al. 2009; Nechutova et al. 2014). The pathogenesis of pancreatic pain appears to involve various molecules including neurotrophic factors, neuropeptides (Ceyhan et al. 2008), proteinase-activated receptors (PARs) (Hoogerwerf et al. 2004; Kawabata et al. 2008; Terada et al. 2013), hydrogen sulfide (Fukushima et al. 2010; Nishimura et al. 2009; Terada et al. 2015), and ion channels, such as transient receptor potential (TRP) channels (Ceyhan et al. 2008; Schwartz et al. 2011; Terada et al. 2013) and T-type Ca^{2+} channels (Fukushima et al. 2010; Nishimura et al. 2009; Terada et al. 2015), etc. There is also evidence for a role of neuro-immune interactions in the development of pancreatitis and/or concomitant pancreatic pain (Ceyhan et al. 2008).

High mobility group box 1 (HMGB1), a nuclear protein, is one of damage-associated molecular patterns (DAMPs), and is passively released to the extracellular space from necrotic cells and actively secreted by certain cells such as macrophages (Malarkey and Churchill 2012; Yanai et al. 2012). Multiple signals including the JAK/STAT pathway and calcium/calmodulin-dependent protein kinases appear to regulate the cytoplasmic accumulation of HMGB1 and/or its packaging into secretory lysosomes (Lu et al. 2014; Zhang et al. 2011). Oxidative stress also mediates HMGB1 release via several distinct pathways (Yu et al. 2015). The extracellular HMGB1 activates the receptor for advanced glycation end products (RAGE) and Toll-like receptor 4 (TLR4), thereby facilitating inflammation (Malarkey and Churchill 2012; Yanai et al. 2012). HMGB1 also forms a heterocomplex with C-X-C motif chemokine ligand 12 (CXCL12), known as stromal cell-derived factor 1 (SDF1), at a 1:2 ratio and enhances the activation of C-X-C chemokine receptor 4 (CXCR4) by CXCL12 through the receptor dimerization (Schiraldi et al. 2012; Venereau et al. 2013). In rodents, HMGB1 participates in processing of inflammatory pain (Agalave et al. 2014; Tanaka et al. 2013; Yamasoba et al. 2016), and contributes to the pathogenesis of neuropathic pain (Feldman et al. 2012; Otoshi et al. 2011; Shibasaki et al. 2010; Zhang et al. 2015). Peripheral HMGB1 is also considered to mediate cystitis-related bladder pain in mice (Tanaka et al. 2014).

Clinical studies indicate that serum HMGB1 levels increase in patients with severe acute pancreatitis within 72 h after the onset, and are correlated with the disease severity (Kang et al. 2014; Shen and Li 2015;

Yasuda et al. 2006). In experimental models for acute pancreatitis caused by several hourly injections of cerulein in rats or mice, serum and pancreatic tissue HMGB1 levels increase 6-12 h after the final dose of cerulein (Choi et al. 2014; Hagiwara et al. 2009), indicating the proinflammatory role of HMGB1 in the delayed stage of acute pancreatitis. However, it is still open to question whether extracellular HMGB1 participates in the early stage of pancreatitis and/or related pain. In the present study, we thus focused on the role of HMGB1 in processing of pancreatic pain signals accompanying cerulein-induced acute pancreatitis in mice, using the anti-HMGB1 neutralizing antibody and also recombinant human soluble thrombomodulin (rhsTM) that inactivates HMGB1 and inhibits HMGB1-mediated pain and inflammation (Conway 2012; Ito et al. 2008; Tanaka et al. 2013; Tanaka et al. 2014). Further, we also analyzed the origin and target molecules of HMGB1, which contribute to pancreatic pain. Here we provide evidence that macrophage-derived HMGB1 mediates pancreatic pain rather than inflammation mainly through activation of RAGE and CXCL12/CXCR4 signaling axis in the early stage of acute pancreatitis in mice.

Materials and Methods

Experimental animals

Male ddY mice (18-22 g, 4-5 weeks old) were purchased from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan). The animals were housed in a temperature (22-24ºC)-controlled room under a 12-h day/night cycle and had free access to food and water. All experimental protocols were approved by Kindai University's Committee for the Care and Use of Laboratory Animals and were in accordance with the Guiding Principles approved by The Japanese Pharmacological Society and with the *Guide for the care and Use of laboratory Animals* published by the US national Institutes of Health.

Major chemicals

Cerulein was purchased from Bachem (Bubendorf, Swizerland). The anti-HMGB1 neutralizing antibody and normal rat IgG (control) were made in house, the specificity of the antibody being described elsewhere

(Liu et al. 2007). Recombinant human soluble thrombomodulin (rhsTM) was provided by Asahi Kasei Pharma (Tokyo, Japan). Low molecular weight heparin (LH; molecular weight, 4500-6500; 79.5 U/mg), known to inhibit receptor for advanced glycation end products, was kindly provided by Fuso Pharmaceutical Industries, Ltd. (Osaka, Japan). Lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS) was obtained from InvivoGen (San Diego, CA, USA). AMD3100, ethyl pyruvate and minocycline were purchased from Sigma-Aldrich (St. Louis, MO, USA). FPS-ZM1 was from Calbiochem (San Diego, CA, USA). Liposomal clodronate (clophosome-A) and control liposomes were from FormuMax Scientific Inc. (Sunnyvale, CA, USA). Cerulein, LH, LPS-RS, AMD3100, ethyl pyruvate and minocycline were dissolved in saline. The anti-HMGB1 neutralizing antibody and control rat IgG were dissolved in 0.01 M phosphate-buffered saline (PBS), and rhsTM was in 0.002% Tween 80-containing saline. FPS-ZM1 was dissolved in DMSO and diluted with 10% Tween 80-containing saline (0.5% DMSO).

Creation of a mouse model for cerulein-evoked pancreatitis accompanied by referred allodynia/hyperalgesia in the upper abdomen

As described previously (Kawabata et al. 2006), pancreatitis in mice was created by six or twelve repeated intraperitoneal (i.p.) administrations of cerulein at 50 μg/kg at 1-h intervals. Referred hyperalgesia in the upper abdomen was evaluated 30 or 90 min after the final dose of cerulein. Immediately after the evaluation of referred hyperalgesia, blood samples were collected from the abdominal aorta in the mice under urethane (1.5 g/kg, i.p.) anesthesia, and the pancreas was excised and weighed. Plasma amylase activity was determined using an automatic analyzer (Dri-Chem 3500i) with its exclusive colorimetric assay kit (AMYL-P) (Fujifilm, Tokyo, Japan). The pancreatic tissue was fixed and stained with hematoxylin/eosin for histological observation.

Determination of sensitivity to mechanical stimulation in the upper abdomen of mice

Each mouse was placed on raised wire-mesh floor under a clear transparent plastic box (23.5 x 16.6 x 12.4 cm), and acclimated to the experimental environment for 30 min. To determine referred hyperalgesia, the upper abdomen of each mouse was stimulated using four distinct von Frey filaments with strengths of 0.02,

0.07, 0.16 and 1.0 g in an ascending order of strength, as reported previously (Kawabata et al. 2006). The mechanical stimulation with each filament was applied five times at intervals of 5–10 s, and, after a 1 min resting period, another five times in the same manner, for a total of 10 times. Two successive applications of stimulation to the same point was avoided, taking into account ‘‘wind-up’’ effects or desensitization. Scoring of nociceptive behavior was defined as follows: score 0 = no response; score 1 = immediate escape or licking/scratching of the site stimulated by application of von Frey filaments; score 2 = strong retraction of the abdomen or jumping. The data are expressed as the total score of responses from 10 challenges with each filament.

Drug administration schedules

An anti-HMGB1 neutralizing antibody at 1 mg/kg and the control rat IgG at 0.1 or 1 mg/kg, rhsTM at 3 or 10 mg/kg, LH at 2.5 mg/kg, LPS-RS at 0.5 mg/kg or AMD3100 at 2.7 or 8 mg/kg were administered i.p. 30 min before the first dose or 5 min after the final dose of cerulein. FPS-ZM1 at 0.3 or 1 mg/kg was administered i.p. 30 min before the first dose of cerulein. Ethyl pyruvate at 80 mg/kg or minocycline at 30 mg/kg was administered i.p. 1 h before the first dose of cerulein. Liposomal clodronate at 1.05 mg/mouse or the control liposome was administered i.p. 24 h before the first dose of cerulein.

Determination of protein levels of HMGB1, RAGE, TLR4, CXCR4 and CXCL12

The blood samples and pancreatic tissue were obtained, as described above, 1 h after the final dose of cerulein in mice. The blood was also collected 18 h after i.p. administration of lipopolysaccharide (LPS) at 30 mg/kg in mice. The frozen tissue samples were homogenized in a RIPA buffer [PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] containing 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.15 U/ml aprotinin, and 1 mM sodium orthovanadate. After the addition of 2-mercaptoethanol and bromophenol blue, the supernatant was denatured at 95–100 °C for 5 min, and the proteins were separated by electrophoresis on a 12.5% or 7.5% SDS-polyacrylamide gel (Wako Pure Chemicals, Osaka, Japan) and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA). The membrane was blocked with a blocking solution containing 5% skim

milk, 137 mM NaCl, 0.1% Tween 20, and 20 mM Tris-HCl, pH 7.6. The membrane was washed and incubated overnight at 4°C with the affinity-purified anti-HMGB1 rabbit polyclonal antibody (1:10000 dilution) (SHINO-TEST Corporation, Kanagawa, Japan), the anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit polyclonal antibody (1:5000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the anti-RAGE rabbit polyclonal antibody (1:2000 dilution) (Abcam, Cambridge, UK), the anti-TLR4 rabbit polyclonal antibody (1:200 dilution) (Santa Cruz Biotechnology), the anti-CXCR4 rabbit polyclonal antibody (1:5000 dilution) (NOVUS BIOLOGICALS, Littleton, CO, USA), the anti-CXCL12 (SDF1) rabbit polyclonal antibody (1:3000 dilution) (Cell Signaling Technology). The membrane was washed again and incubated with a horseradish peroxidase-conjugated anti-rabbit IgG (1:5000 dilution) (Cell Signaling Technology) or anti-mouse IgG (1:3000 dilution) (Cell Signaling Technology). Immunolabeled proteins were visualized with an enhanced chemiluminescence detection reagent (Nacalai Tesque, Kyoto, Japan) and detected by Image Quant 400 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The detected bands were quantified using a densitometric software (Image J downloaded from http://imagej.nih.gov/ij/download.html). Plasma HMGB1 levels were determined spectrophotometrically using a commercial enzyme-linked immunosorbent assay (ELISA) kit (SHINO-TEST Corporation), according to the manufacturer's instructions.

Immunohistochemical analysis of macrophage migration in the pancreas of mice with cerulein-induced pancreatitis

Under anesthesia with i.p. urethane at 1.5 g/kg, the mice were transcardially perfused with 4% paraformaldehyde 1 h after the final dose of cerulein. The pancreas was isolated and embedded in paraffin. The paraffin-embedded pancreas was sectioned at 5 μm. After deparaffinization, the section was washed with Tris-buffered saline (TBS, pH 7.6) for 5 min and incubated with antigen retrieval solution (DAKO, Glostrup, Denmark) at 121°C for 15 min. After keeping at room temperature for 20 min, the section was washed with ultrapure water for 5 min. After 3 times washing with 0.15 M NaCl and 0.05% Tween 20 containing 0.1 M Tris-HCl buffer (TNT, pH 7.5) for 3 min ×3, the section was immersed in 0.03% methanol for 30 min. After washing with TNT for 3 min, the sections were incubated with the anti-F4/80 rat monoclonal antibody (1:500; Bio Rad, Hercules, CA) or normal rat IgG (Santa Cruz Biotechnology) for

overnight at 4°C. After 5 times washing with TNT for 3 min, the sections were incubated with Histofine simple stain mouse MAX-PO (rat) (Nichirei Biosciences, Tokyo Japan). After 5 times washing with TNT for 3 min, the sections Tyramide signal amplification (TSA) system (PerkinElmer, Waltham, MS, USA) was used for the detection of positive signals. After 5 times washing with TNT for 3 min, the nucleus are stained with 4',6-diamidino-2-phenylindole (DAPI, blue, 1:2000, Sigma Aldrich) and sections were visualized using a OLYMPUS FLUOVIEW FV10i. The number of F4/80 positive cells were counted in five microscopic fields for each mouse pancreas.

Flow cytometry

The mouse was killed by cervical dislocation 1 h after the final administration of cerulein. The spleen was isolated and homogenized in cold Hank's balanced salt solution (HBSS) in a volume of 4 mL using a sterile syringe bottom in plastic dish. After filtering through a 70 μm cell strainer (Corning, Corning, NY, USA.), the sample was isolated with Pasteur pipette and collected in 15 mL tube. HBSS in 2 mL was added in plastic dish to wash and the sample was collected again. After centrifuging at 300 g for 5 min at 4°C, Tris-NH_4Cl (pH:7.65, Tris:NH_4Cl 1:9 solution) was added in the pellet and incubated at 37°C for 2 min to remove the erythrocyte. After counting the cell number, 0.2% bovine serum albumin (BSA) and 0.1% NaN_3 containing PBS was added to prepare the cell solution in a volume of 1 mL (2 x 10^7 cell). The cell solution in a volume of 100 μL (2 x 10^6 cell) was incubated with FITC-F4/80 (1:100, diluted with 1% BSA and 0.1% NaN_3 containing PBS, Biolegend, Inc., San Diego, CA, USA) and PE-CD11b (1:200, diluted with 1% BSA and 0.1% NaN_3 containing PBS, Biolegend, Inc.) in a volume of 50 μL on ice for 30 min in the dark. After staining, cells were washed twice with 0.2% BSA and 0.1% NaN_3 containing PBS. Data were determined with a BD LSR II (BD Biosciences, ranklin Lakes, NJ, USA) and analyzed using FlOWJO (TOMY DIGITAL BIOLOGY CO., LTD., Tokyo, Japan).

Statistics analysis

Data are represented as means ± S.E.M. Statistical significance for parametric data was analyzed by Student's t-test for comparison between two groups and by analysis of variance followed by Tukey's test

for multiple comparisons. For nonparametric analysis, Wilcoxon t-test was used for comparison between two groups, and Kruskal-Wallis H test followed by a least significant difference-type test was employed for multiple comparisons. Significance was set at a level of $P < 0.05$.

Results

Pretreatment with the anti-HMGB1 neutralizing antibody or rhsTM prevents the development of referred hyperalgesia accompanying cerulein-induced acute pancreatitis in mice

As reported previously (Nishimura et al. 2009), six repeated i.p. administration of cerulein at 50 μg/kg caused referred hyperalgesia in the upper abdomen (Fig. 1A) accompanying acute pancreatitis, characterized by increased pancreatic weight, an indicator of edema (Fig. 1B), and plasma amylase activity (Fig. 1C), and by histological observations, such as acinar cell vacuoles and inflammatory cell infiltration, in the pancreatic tissue (Fig. 1D). The anti-HMGB1 neutralizing antibody, preadministered i.p. at 1 mg/kg, completely prevented the referred hyperalgesia (Fig. 1A), but not pancreatitis-related inflammatory symptoms (Fig. 1B, C, D), in the mice treated with cerulein. Similarly, i.p. preadministration of rhsTM at 3-10 mg/kg, known to inactivate HMGB1 (Ito et al. 2008; Tanaka et al. 2013; Tanaka et al. 2014), significantly reduced the referred hyperalgesia (Fig. 1A), without affecting pancreatitis itself (Fig. 1B, C, D).

Posttreatment with the anti-HMGB1 neutralizing antibody or rhsTM relieves the referred hyperalgesia in mice with cerulein-induced acute pancreatitis

We next tested if inactivation of HMGB1 after the development of acute pancreatitis suppresses the pancreatitis-related hyperalgesia. The anti-HMGB1 antibody at 1 mg/kg, administered 5 min after the final dose of cerulein, completely relieved the referred hyperalgesia accompanying pancreatitis (Fig. 2A). Similarly, the referred hyperalgesia accompanying cerulein-induced pancreatitis disappeared 90 min, but not 30 min, after i.p. administration of rhsTM at 10 mg/kg following the final dose of cerulein (Fig. 2B). It

is to be noted that neither anti-HMGB1 antibody nor rhsTM affected the established pancreatitis-related inflammatory symptoms such as the increased pancreatic weight and plasma amylase activity (data not shown).

Plasma and pancreatic tissue levels of HMGB1 in the early stage of cerulein-induced acute pancreatitis in mice

Although LPS treatment greatly increased plasma HMGB1 levels in 18 h, we could not detect significant increase in plasma or pancreatic tissue HMGB1 levels after 6 repeated doses of cerulein (Fig. 3A, B), in spite of the present evidence for the pronociceptive role of endogenous HMGB1 in the early stage of cerulein-induced acute pancreatitis (see Figs. 1 and 2). These findings indicate that HMGB1 is neither upregulated in the pancreas nor greatly washed into the blood stream at this time point. It is noteworthy that HMGB1 levels in the pancreas, but not plasma, significantly increased after 12 repeated doses of cerulein (Fig. 3A, B)

Involvement of macrophage-derived HMGB1 in the early stage of pancreatitis-related hyperalgesia in cerulein-treated mice

Considering the evidence that macrophages actively secrete HMGB1 (Malarkey and Churchill 2012; Yanai et al. 2012), we hypothesized that macrophages might infiltrate into the pancreatic tissue in the early stage of cerulein-induced acute pancreatitis and locally secrete HMGB1, leading to pancreatic pain. To address this question, we tested the possible involvement of macrophages in the early stage of pancretitis-related pain in mice. Minocycline, an inhibitor of macrophage and microglia activation, at 30 mg/kg and ethyl pyruvate, known to inhibit HMGB1 release from macrophages (Entezari et al. 2014; Ulloa et al. 2002), at 80 mg/kg, preadministered i.p., prevented the referred hyperalgesia accompanying acute pancreatitis (Fig. 4A). Surprisingly, neither minocycline nor ethyl pyruvate attenuated the increased pancreatic tissue weight or plasma amylase activity (Fig. 4B, C). To further confirm the role of macrophages in pancreatitis-related pain, we depleted macrophages using liposomal clodronate (Pinto et al. 1991; Saeki et al. 2012). Liposomal clodronate, administered i.p. at 1.05 mg/mouse, dramatically decreased the number of splenic macrophages

expressing F4/80 and CD11b in 24 h (Fig. 4D, E). Treatment with liposomal clodronate abolished the pancreatitis-related referred hyperalgesia (Fig. 4F), and tended to reduce the increased pancreatic weight and plasma amylase activity in mice (Fig. 4G, H). The immunohistochemical analysis indicates that the number of F4/80-positive cells (arrows) in the pancreatic tissue remarkably increased 1 h after 6 doses of cerulein in mice (Fig. 5A, B).

Analysis of possible targets for HMGB1 involved in the early stage of pancreatitis-related pain in cerulein-treated mice

To clarify the target molecules for HMGB1 responsible for pancreatic pain, we tested the effects of FPS-ZM1, a RAGE antagonist, low-molecular weight heparin (LH), known to block RAGE (Liu et al. 2009; Myint et al. 2006), LPS-RS, a TLR4 antagonist, or AMD3100, a CXCR4 antagonist. The blockade of RAGE by pretreatment with i.p. FPS-ZM1 at 1 mg/kg or LH at 2.5 mg/kg completely prevented cerulein-induced referred hyperalgesia accompanying acute pancreatitis (Fig. 6A), but did not significantly affect the increased pancreatic weight or plasma amylase activity in mice (Fig. 6B, C). Blocking TLR4 with i.p. LPS-RS at 0.5 mg/kg, did not show significant effects (Fig. 6A, B, C). The CXCR4 antagonism by i.p. preadministration of AMD3100 at 8 mg/kg abolished the referred hyperalgesia, but not increased pancreatic weight or plasma amylase activity, caused by cerulein treatment in mice (Fig. 6A, B). We next tested the role of RAGE, TLR4 and CXCR4 in the maintenance of pancreatic pain and the therapeutic usefulness of their blockers, which were administered i.p. after the final dose of cerulein. Blocking CXCR4 with AMD3100, strongly counteracted the referred hyperalgesia accompanying cerulein-induced acute mild pancreatitis (Fig. 6D). Blocking RAGE with LH and TLR4 with LPS-RS revealed some and no suppressive effects, respectively, on the referred hyperalgesia (Fig. 6D). Interestingly, RAGE and CXCR4, but not TLR4, in the pancreatic tissue were dramatically upregulated 1 h after the final dose of cerulein (Fig. 7A, B, C). In addition, we also detected cerulein-induced increase in protein levels of pancreatic CXCL12 (Fig. 7D) that forms a heterocomplex with HMGB1 and causes accelerated activation of CXCR4 (Schiraldi et al. 2012; Venereau et al. 2013).

Discussion

Our results clearly indicate that macrophage-derived HMGB1 mediates pancreatic pain, but not inflammation, in the early stage of cerulein-induced acute pancreatitis in mice. Our data also suggest that RAGE and CXCL12/CXCR4 axis are upregulated in the early stage of acute pancreatitis, and contribute to both development and maintenance of the HMGB1-dependent pancreatic pain. Collectively, we propose that macrophages infiltrate into the pancreatic tissue in the early stage of cerulein-induced acute pancreatitis and locally secrete a small amount of HMGB1, which in turn causes pancreatic pain via activation of the upregulated RAGE and CXCL12/CXCR4 axis (Fig. 8).

The data from experiments employing the anti-HMGB1 antibody and rhsTM capable of inactivating HMGB1 (Conway 2012; Ito et al. 2008) (see Figs. 1 and 2) demonstrate the pronociceptive role of endogenous HMGB1 in the early stage of acute pancreatitis, namely within 1 h after 6 repeated doses of cerulein. Nevertheless, plasma and pancreatic tissue HMGB1 levels still remained unchanged at the same time point in the present study (see Fig. 3). It is noteworthy that HMGB1 levels in the pancreas significantly increased after 12 repeated doses of cerulein, in agreement with the previous evidence that HMGB1 contributes to the severe pancreatitis or the delayed aggravation of acute pancreatitis (Hagiwara et al. 2009). It is of interest that macrophage infiltration into the pancreatic tissue and the upregulation of RAGE, CXCR4 and CXCL12 in the pancreas were already detectable within 1 h after the final dose of cerulein (see Figs. 5 and 7). We thus consider that HMGB1 secreted regionally by infiltrated macrophages in the pancreas triggers or promotes pancreatic pain by activating the upregulated RAGE and CXCL12/CXCR4 axis in the early stage of pancreatitis. The macrophage-derived HMGB1, once washed into the blood stream, might be inactivated by endothelial membrane-bound thrombomodulin in cooperation with thrombin in the blood vessels (Conway 2012; Ito et al. 2008) or too little to be detected in the plasma. Considering the previous report showing the delayed increase in serum and/or pancreatic HMGB1 levels in severe pancreatitis models (Choi et al. 2014; Hagiwara et al. 2009; Jo et al. 2013), we assume that a great amount of HMGB1 might be released passively or actively from necrotic or living pancreatic cells that overexpress HMGB1, in addition to macrophages, leading to aggravation of pancreatitis itself in the late stage of acute pancreatitis, followed by the delayed increase in blood HMGB1 levels.

There is affluent evidence that HMGB1 stimulates monocytes or macrophages to release TNF-α, which in turn, induces more HMGB1 release from macrophages (Wang et al. 1999; Yang et al. 2010). The critical role of macrophage-derived HMGB1 in the development of the early stage of pancreatic pain is strongly suggested by the present findings that minocycline, an inhibitor of macrophages/microglias, ethyl pyruvate, known to inhibit HMGB1 release from macrophages, and clodronate liposomes that deplete macrophages, mimicked the antinociceptive effects of HMGB1 inactivation with the neutralizing antibody or rhsTM (see Figs. 1, 2 and 4). Surprisingly, neither minocycline nor clodronate liposomes clearly inhibited the increased pancreatic weight or plasma amylase activity in the early phage of acute pancreatitis (see Fig. 4B, C, G, H), despite the evidence that macrophages are involved in the pathogenesis of severe acute pancreatitis, particularly in the delayed phase (Saeki et al. 2012). HMGB1 may thus play roles as a pronociceptive messenger in the early stage immune-neuronal linkage and as a proinflammatory mediator in the late stage aggravation of acute pancreatitis.

TLR4, RAGE and CXCR4 are considered cell surface targets for extracellular HMGB1 in pain processing (Agalave and Svensson 2014; Kato and Svensson 2015; Yanai et al. 2012). Plenty of evidence indicates the pronociceptive role of the TLR4 complex, particularly in neuropathic pain (Watkins et al. 2009). RAGE is also considered pronociceptive, particularly in diabetic neuropathy (Lukic et al. 2008). CXCL12/CXCR4 signaling is considered to contribute to persistent pain and hypersensitivity (Yang et al. 2015). HMGB1 consists of two positively charged DNA-binding domains, Box A and Box B, and a negatively charged C-tail, and has three conserved cysteines in positions 23 and 45 within Box A, and position 106 in Box B. C23 and C45 can form an intramolecular disulfide bond (disulfide-HMGB1) that is required for activation of TLR4 (Yang et al. 2010). In contrast, the completely reduced form of HMGB1 where all C23, C45 and C106 are in the thiol state (all-thiol-HMGB1)(Yang et al. 2012), does not interact with TLR4, but is capable of activating RAGE, and forms a heterocomplex with CXCL12, leading to accelerated activation of CXCR4 through receptor dimerization (Fiuza et al. 2003; Kato and Svensson 2015; Schiraldi et al. 2012; Venereau et al. 2013; Yang et al. 2010; Yang et al. 2012). Most interestingly, all-thiol-HMGB1 and disulfide-HMGB1 directly activate RAGE and TLR4 expressed in isolated dorsal root ganglion neurons, respectively (Allette et al. 2014), in agreement with our recent report that intraplantar administration of all-thiol-HMGB1 and disulfide-HMGB1 produced RAGE- and TLR4-dependent mechanical hyperalgesia, respectively, in mice (Yamasoba et al. 2016). In the early stage of

acute pancreatitis, all-thiol-HMGB1 rather than disulfide HMGB1 might mediate pancreatic pain, because our results from inhibition experiments indicate greater roles of RAGE and CXCR4 than TLR4 (see Fig. 6), being consistent with our finding that RAGE, CXCR4 and CXCL12, an endogenous agonist of CXCR4, but not TLR4, in the pancreatic tissue were upregulated following the final dose of cerulein (see Fig. 7).

Membrane-bound thrombomodulin expressed on the endothelium consists of 5 distinct domains; an N-terminal lectin-like domain (D1), an EGF-like domain (D2), an *O*-glycosylated serine/threonine-rich domain (D3), a transmembrane domain (D4) and a C-terminal short cytoplasmic domain (D5) (Ito et al. 2016). Like membrane-bound thrombomodulin, rhsTM consisting of D1-D3 retains D2-dependent anticoagulant activity through facilitation of thrombin-mediated activation of protein C, and sequesters HMGB1 with D1 followed by D2-dependent thrombin-mediated degradation of HMGB1 (Conway 2012; Ito et al. 2016; Ito et al. 2008). Indeed, rhsTM has been used for the treatment of disseminated intravascular coagulation (DIC) in Japan (Yamakawa et al. 2013; Yoshimura et al. 2015). We have already demonstrated that rhsTM as well as the anti-HMGB1-neutralizing antibody improves lipopolysaccharide-induced inflammatory hyperalgesia in rats (Tanaka et al. 2013) and bladder pain in cyclophosphamide-induced interstitial cystitis model mice (Tanaka et al. 2014). The most recent clinical study indicates that rhsTM prevents walled-off necrosis in severe acute pancreatitis patients (Eguchi et al. 2015). The present study further suggest that rhsTM may be useful for management of pancreatic pain in patients with acute pancreatitis.

In conclusion, macrophage-derived HMGB1 mediates pancreatic pain by targeting RAGE and CXCL12/CXCR4 signaling axis in the early stage of acute pancreatitis in mice. We thus propose that the inactivation of HMGB1 with rhsTM and pharmacological blockade of RAGE or CXCR4 serve as novel therapeutic strategy for management of pancreatic pain in patients with acute pancreatitis.

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Conflict of interests

A. Kawabata has received research grants from Asahi Kasei Pharma Corporation.

Legend

Fig. 1 Effect of pretreatment with the anti-HMGB1-neutralizing antibody or rhsTM on cerulein-evoked abdominal allodynia/hyperalgesia and pancreatitis in mice. Cerulein (Cer) at 50 μg/kg or vehicle (V) was administered i.p. to mice at 1-h intervals, 6 times in total. The anti-HMGB1-neutralizing antibody (Ab) at 0.1 or 1 mg/kg or the control IgG (IgG) at 1 mg/kg and rhsTM (TM) at 3 or 10 mg/kg or vehicle were administered i.p. to mice 30 min before the first dose of cerulein. The nociception test was performed 30 min after the final administration of cerulein (A), followed by measurement of pancreatic weight (B) and plasma amylase activity (C). (D) Typical microphotographs for the hematoxylin/eosin-stained pancreatic tissue in mice treated with cerulein after administration of the anti-HMGB1 antibody at 1 mg/kg or rhsTM at 10 mg/kg. Arrows indicate acinar cell vacuoles, and arrowheads show inflammatory cell infiltration. Scale bars indicate 50 μm. Data show the mean ± S.E.M. for 7-9 (anti-HMGB1 antibody in panels A-C) or 4-8 (rhsTM in A-C) mice. *P<0.05, **P<0.01, ***P<0.001 vs. V+V. †P<0.05, ††P<0.01 vs. V+Cer.

Fig. 2 Effect of posttreatment with the anti-HMGB1-neutralizing antibody or rhsTM on cerulein-evoked abdominal allodynia/hyperalgesia and pancreatitis in mice. Cerulein (Cer) at 50 μg/kg or vehicle (V) was administered i.p. to the mice at 1-h intervals, 6 times in total. The anti-HMGB1-neutralizing antibody (Ab) at 1 mg/kg, the control IgG (IgG) at 1 mg/kg, rhsTM (TM) at 10 mg/kg or vehicle was administered i.p. to mice 5 min after the final administration of cerulein. The nociception test was performed 30 min after the antibody treatment and 30 or 90 min after the rhsTM treatment. Data show the mean ± S.E.M. for 7-8 (A) or 10-12 (B) mice. **P<0.01, ***P<0.001 vs. V+V. †P<0.05, ††P<0.01 vs. Cer+V.

Fig. 3 Protein levels of HMGB1 in the plasma and pancreatic tissue of mice with cerulein-induced acute pancreatitis. Cerulein (Cer) at 50 μg/kg or vehicle (V) was administered i.p. to mice at 1-h intervals, 6 or 12 times in total. Lipopolysaccharide (LPS) at 30 mg/kg was administered i.p. to mice. The blood and pancreatic tissue were collected 1 h after the final dose of cerulein or 18 h after LPS challenge. Plasma (A) and pancreatic (B) HMGB1 levels were determined by ELISA and Western blotting, respectively. Typical

photographs for Western blotting are shown on the top, and protein levels were quantified by densitometry (B). Data show the mean ± S.E.M. for 4-6 mice.

Fig. 4 Effect of pretreatment with minocycline, an inhibitor of macrophages/microglias, ethyl pyruvate, known to reduce HMGB1 release from macrophages, or liposomal clodronate that depletes macrophages on cerulein-evoked abdominal allodynia/hyperalgesia and pancreatitis in mice. Cerulein (Cer) at 50 μg/kg or vehicle (V) was administered i.p. to mice at 1-h intervals, 6 times in total. Minocycline (Mino) at 30 mg/kg and ethyl pyruvate (EP) at 80 mg/kg were administered i.p. to mice 1 h before the first dose of cerulein (A-B). Liposomal clodronate (Cld) at 1.05 mg/mouse or control liposome (Ctl) was given i.p. 24 h before cerulein treatment (D-H). The nociception test was performed 30 min after the final administration of cerulein (A, F) followed by measurement of pancreatic weight (B, G) and plasma amylase activity (C, H). In the isolated splenic tissue from mice treated with Cer after administration of liposomal clodronate, the CD11b- and F4/80-positive cells were determined by flow cytometry (D, E). Data show the mean ± S.E.M. for 4-6 (A-C), 4 (E) or 5-6 (F, G, H) mice. *P<0.05, **P<0.01, ***P<0.001 vs. V+V (A-C) or Ctl+V (F-H). †P<0.05, ††P<0.01, †††P<0.001 vs. V+Cer (A) or Ctl+Cer (E, F).

Fig. 5 Macrophage infiltration into the pancreatic tissue in the early phage of acute pancreatitis in mice treated with cerulein. Cerulein (Cer) at 50 μg/kg or vehicle (V) was administered i.p. to mice at 1-h intervals, 6 times in total. (A) Typical microphotographs for the immunofluorescence staining of macrophages in the pancreatic tissue 1 h after the final dose of cerulein in mice. The pancreatic tissues was stained for F4/80 (red), a marker for macrophages, or nucleus (DAPI, blue). The bottom photos show the non-immune IgG control. Scale bars indicate 50 μm. Arrows show F4/80-positive macrophages. (B) The number of F4/80 positive cells in the pancreatic tissue 1 h after the final dose of cerulein in mice. Data show the mean ± S.E.M. for 4 mice. **P<0.01 vs. V.

Fig. 6 Effect of pretreatment or posttreatment with FPS-ZM1, a RAGE antagonist, low molecular weight heparin, known to inhibit RAGE, LPS-RS, a TLR4 antagonist, and AMD3100, a CXCR4 antagonist, on cerulein-evoked abdominal allodynia/hyperalgesia and pancreatitis in mice. Cerulein (Cer) at 50 μg/kg or vehicle (V) was administered i.p. to mice at 1-h intervals, 6 times in total. FPS-ZM1 (FPS)

at 0.3 or 1 mg/kg, low molecular weight heparin (LH) at 2.5 mg/kg, LPS-RS at 0.5 mg/kg, AMD3100 (AMD) at 2.7 or 8 mg/kg, or vehicle was administered i.p to mice 30 min before the first dose of cerulein (A-C) or 5 min after the final dose of cerulein (D). The nociception test was performed 30 min after the final dose of cerulein (A, D), followed by measurement of pancreatic weight (B) and plasma amylase activity (C). Data show the mean ± S.E.M. for 8-10 (FPS-ZM1or LPS-RS in A-C), 4-6 (LH in A-C), 6-8 (AMD3100 in A-C) or 4-8 (D) mice. *P<0.05, **P<0.01, ***P<0.001 vs. V+V. †P<0.05, ††P<0.01, †††P<0.01 vs. V+Cer.

Fig. 7 The protein levels of RAGE, TLR4, CXCR4 and CXCL12 in the pancreatic tissue in the early phage of acute pancreatitis in mice treated with cerulein. Cerulein (Cer) at 50 μg/kg or vehicle (V) was administered i.p. to mice at 1-h intervals, 6 times in total. The pancreatic tissue was isolated 1 h after the final dose of cerulein. The protein levels of RAGE, TLR4, CXCR4 and CXCL12 were determined by Western blotting. Typical photographs for Western blotting are shown on the top of each panels, and protein levels were quantified by densitometry. Data show the mean ± S.E.M. from 6-7 mice. *P<0.05 vs. V.

Fig. 8 A hypothetical scheme for the pronociceptive role of macrophage-derived HMGB1 in the early stage of acute pancreatitis in mice treated with cerulein. HMGB1 is a nuclear protein that has C23 and C45 within Box A, and C106 within Box. The cerulein-induced activation of pancreatic enzymes followed by the early stage of tissue damage or inflammation possibly triggers macrophage infiltration into the pancreatic tissue and upregulates RAGE, CXCL12 and CXCR4. The macrophage-derived HMGB1 may cause pancreatic pain via activation of RAGE and CXCL12/CXCR4 signaling axis.

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Figure1

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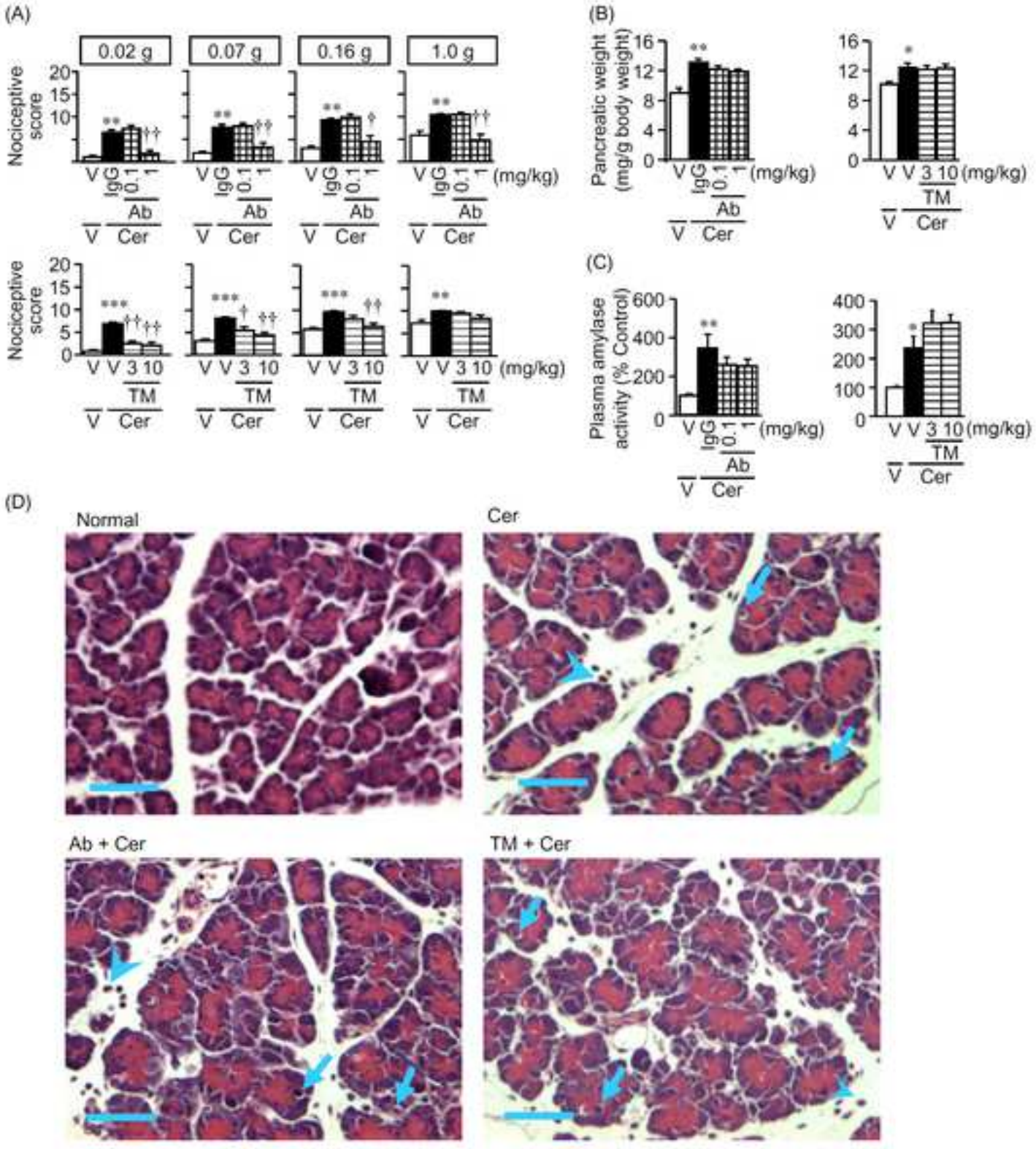

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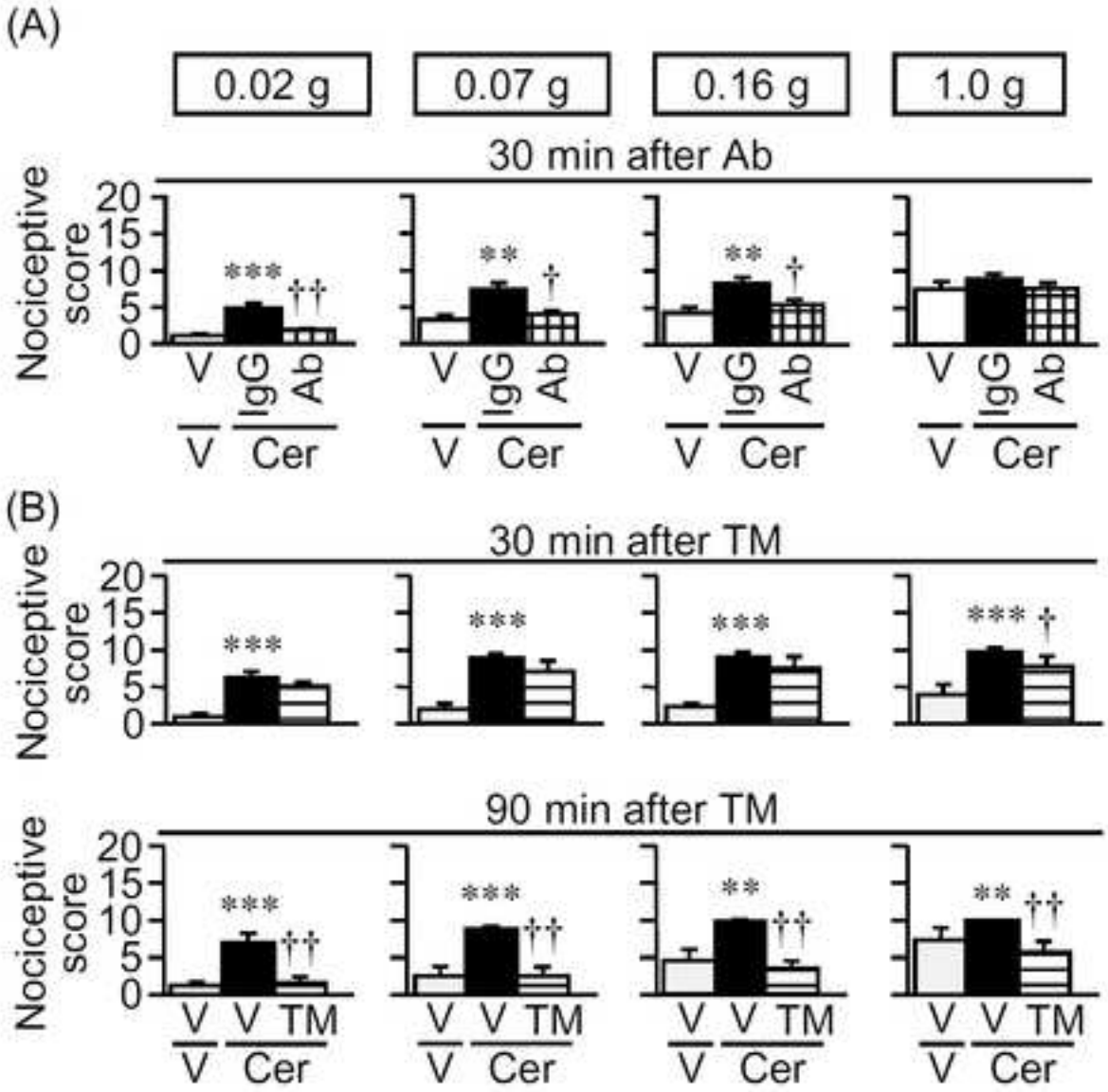

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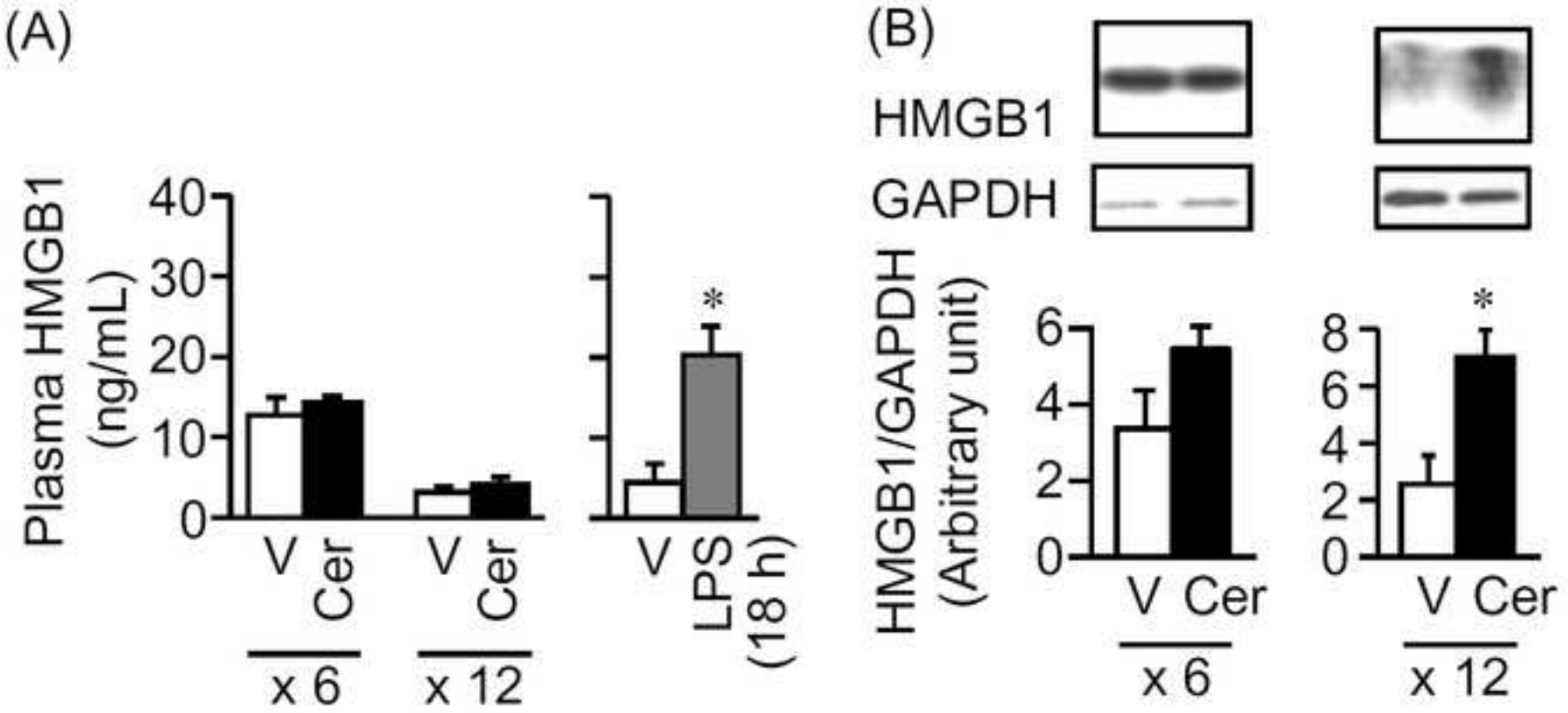

Figure 3

Figure4

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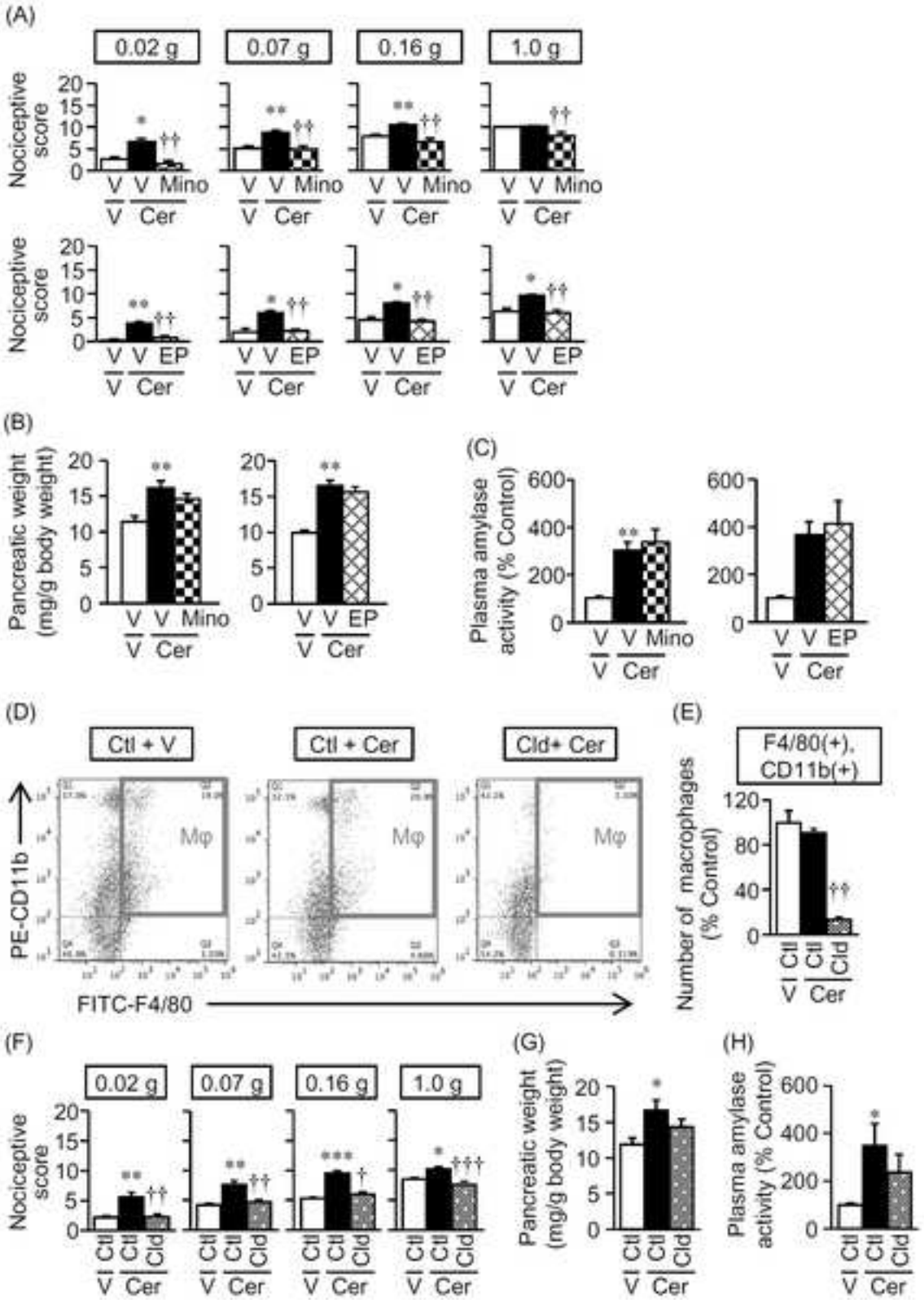

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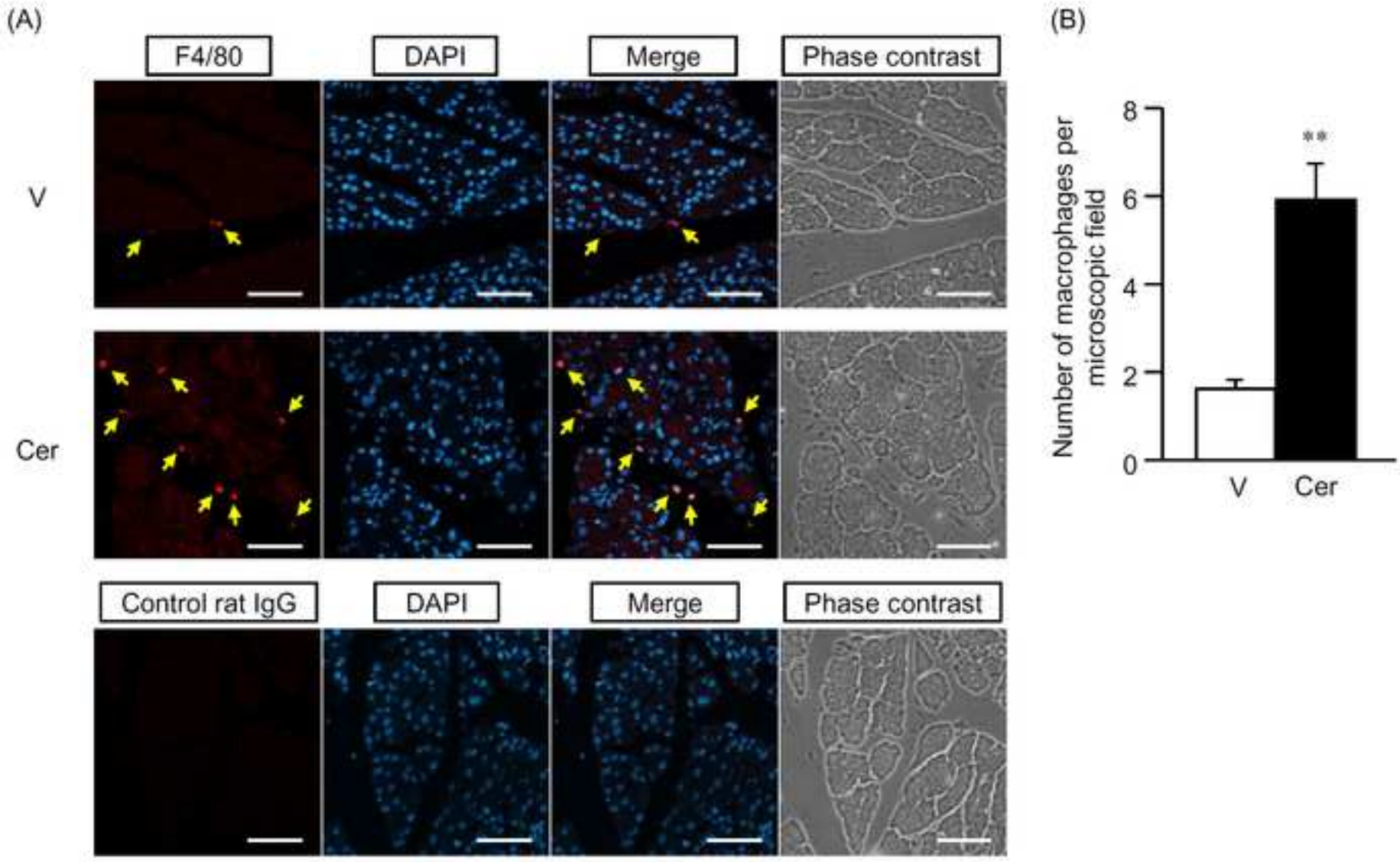

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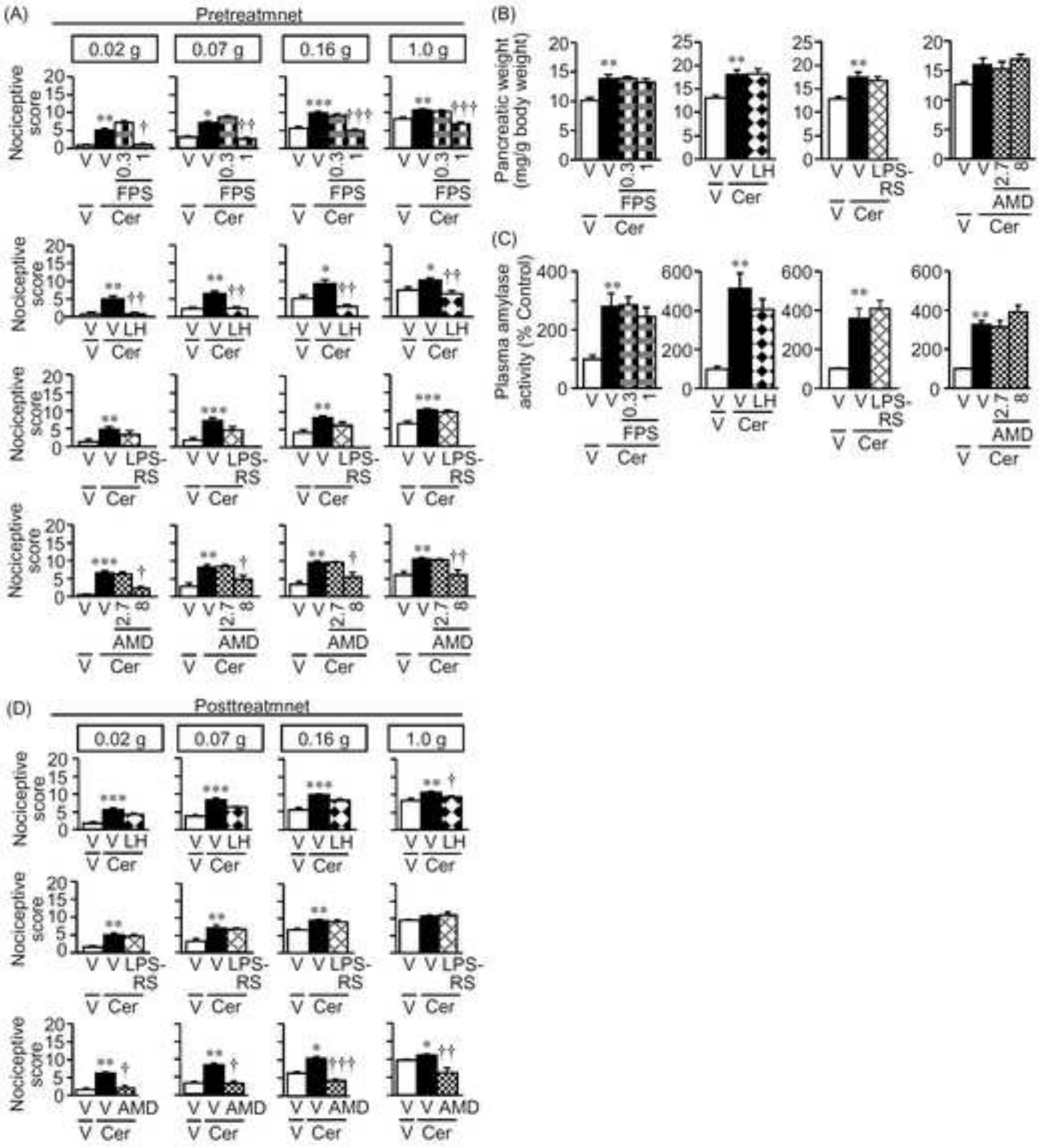

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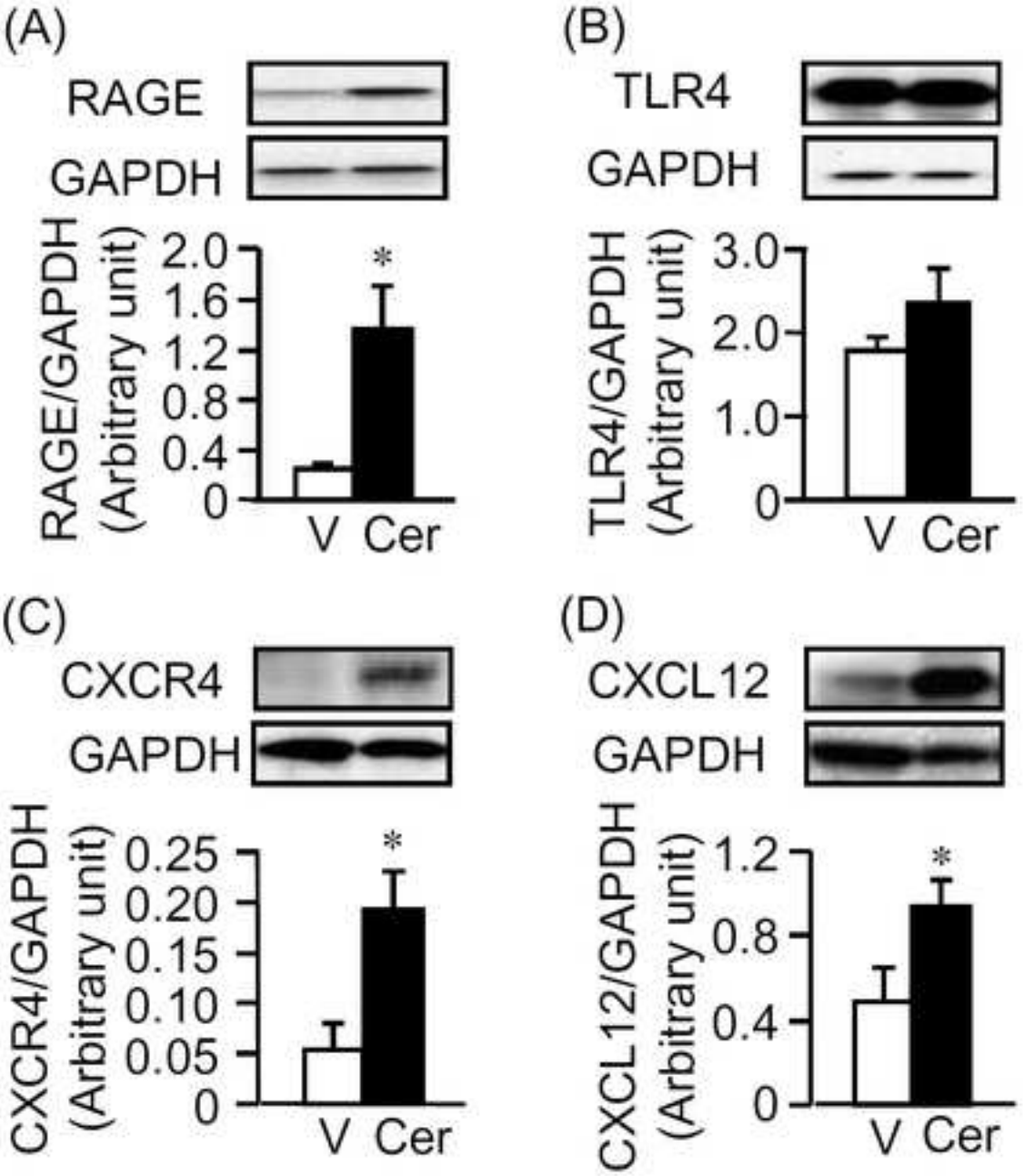

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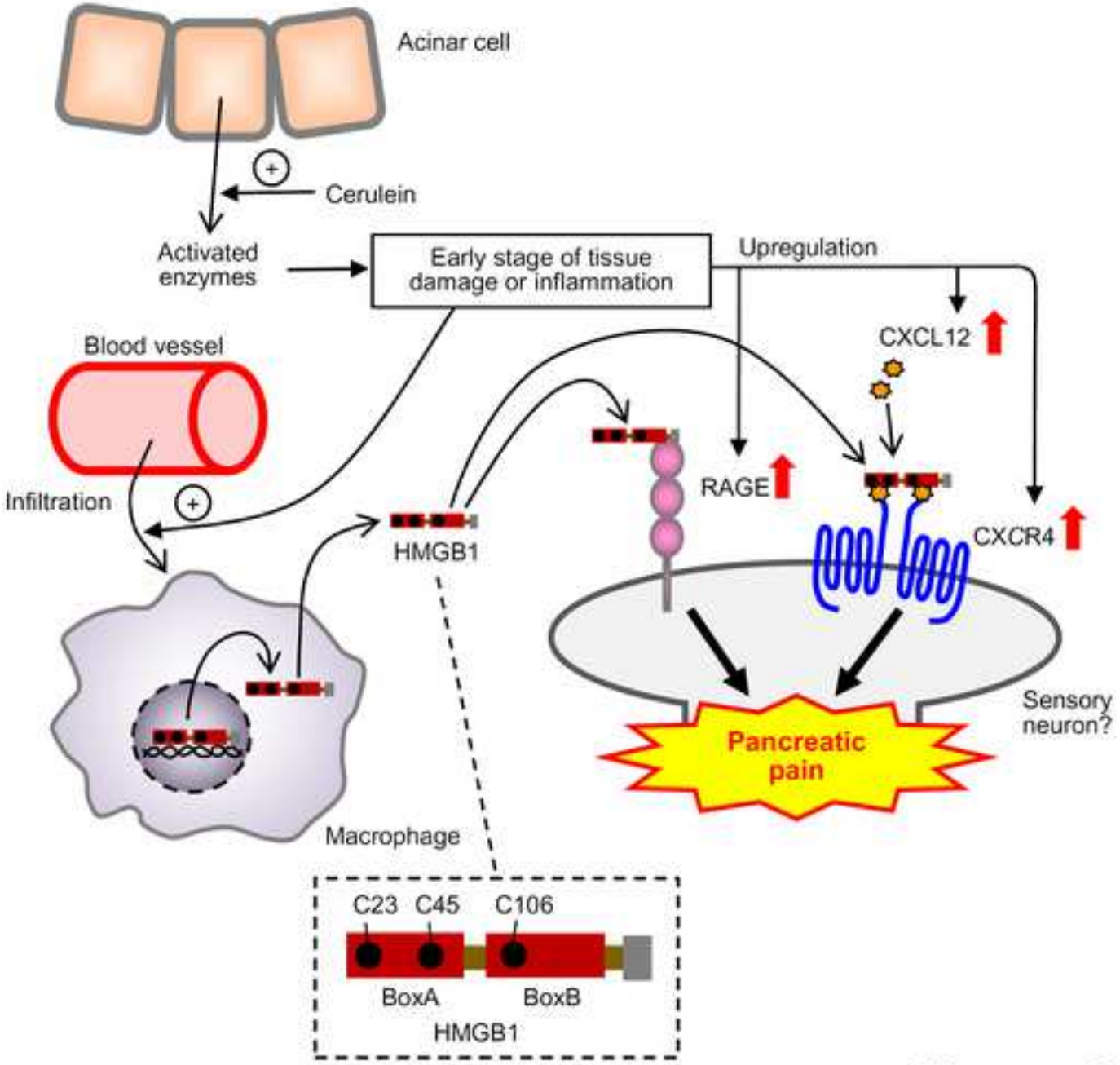

Figure 8