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Research paper

Polysaccharides derived from *Ganoderma lucidum* fungus mycelia ameliorate indomethacin-induced small intestinal injury via induction of GM-CSF from macrophages

Kenta Nagai[a], Yoshitaka Ueno[b,*], Shinji Tanaka[b], Ryohei Hayashi[b], Kei Shinagawa[a], Kazuaki Chayama[a]

[a] Department of Medicine and Molecular Science, Hiroshima University, Japan
[b] Department of Endoscopy, Hiroshima University, Japan

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ABSTRACT

Non-steroidal anti-inflammatory drugs often cause ulcers in the human small intestine, but few effective agents exist to treat such injury. *Ganoderma lucidum* Karst, also known as “Reishi” or “Lingzhi”, is a mushroom. We previously reported that a water-soluble extract from *G. lucidum* fungus mycelia (MAK) has anti-inflammatory effects in murine colitis induced by trinitrobenzene sulfonic acid, and induction of granulocyte macrophage colony-stimulating factor (GM-CSF) by MAK may provide anti-inflammatory effects. However, its effects on indomethacin-induced small intestinal injuries are unknown. The present study investigated the preventative effects of MAK via immunological function and the polysaccharides from MAK on indomethacin-induced ileitis in mice. Peritoneal macrophages (PMs) were stimulated in vitro with MAK and adoptively transferred to C57BL/6 mice intraperitoneally, which were then given indomethacin. Intestinal inflammation was evaluated after 24 h. We performed in vivo antibody blockade to investigate the preventive role of GM-CSF, which derived from PMs stimulated with MAK. We then used PMs stimulated with MAK pre-treated by pectinase in an adoptive transfer assay to determine the preventive role of polysaccharides. Indomethacin-induced small intestinal injury was inhibited by adoptive transfer of PMs stimulated in vitro with MAK. In this transfer model, pre-treatment with anti-GM-CSF antibody but not with control antibody reversed the improvement of small intestinal inflammation by indomethacin. Pectinase pretreatment impaired the anti-inflammatory effect of MAK. PMs stimulated by MAK appear to contribute to the anti-inflammatory response through GM-CSF in small intestinal injury induced by indomethacin. The polysaccharides may be the components that elicit the anti-inflammatory effect.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin and aspirin, are commonly used worldwide for the treatment of musculoskeletal pain and inflammation. However, NSAIDs can cause serious adverse reactions in the form of gastrointestinal lesions [1,2]. Recent advancements in capsule and double-balloon endoscopy have contributed to the increased diagnosis of NSAID-induced small intestinal lesions such as ulcers, bleeding, perforation, and strictures [3–6]. It has become clear that NSAID-induced small intestinal lesions are not as rare as previously thought [7]. For example, Graham et al. reported that 71% of chronic users of NSAIDs have lesions of the small intestine [8]. However, in contrast with upper gastrointestinal injury, few effective agents can prevent and treat small intestinal injury. Therefore, the exploration of preventive and therapeutic agents for NSAID-induced small intestinal injury remains an urgent priority.

Recently obtained data have shown that GM-CSF plays an important role in maintaining intestinal homeostasis. The effect of GM-CSF has been studied in murine models of dextran sodium sulfate-induced colitis, which can be ameliorated by administration of GM-CSF [9,10]. Recent studies have suggested that autoantibodies to GM-CSF are associated with progressive ileal disease in Crohn’s disease patients [11]. Furthermore, a recent phase II, randomized, double-blind, placebo-controlled trial of sargramostim (yeast-derived recombinant human GM-CSF) found that it was effective in the treatment of patients with moderate to severely active Crohn’s disease [12].

Abbreviations: Ab, antibody; mAb, monoclonal antibody; MAK, water-soluble extract from a cultured medium of Reishi mycelia; MLNs, mesenteric lymph nodes; NSAIDs, non-steroidal anti-inflammatory drugs; PM, peritoneal macrophage; TNBS, trinitrobenzene sulfonic acid

* Corresponding author at: Department of Endoscopy, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
E-mail address: yueno@hiroshima-u.ac.jp (Y. Ueno).

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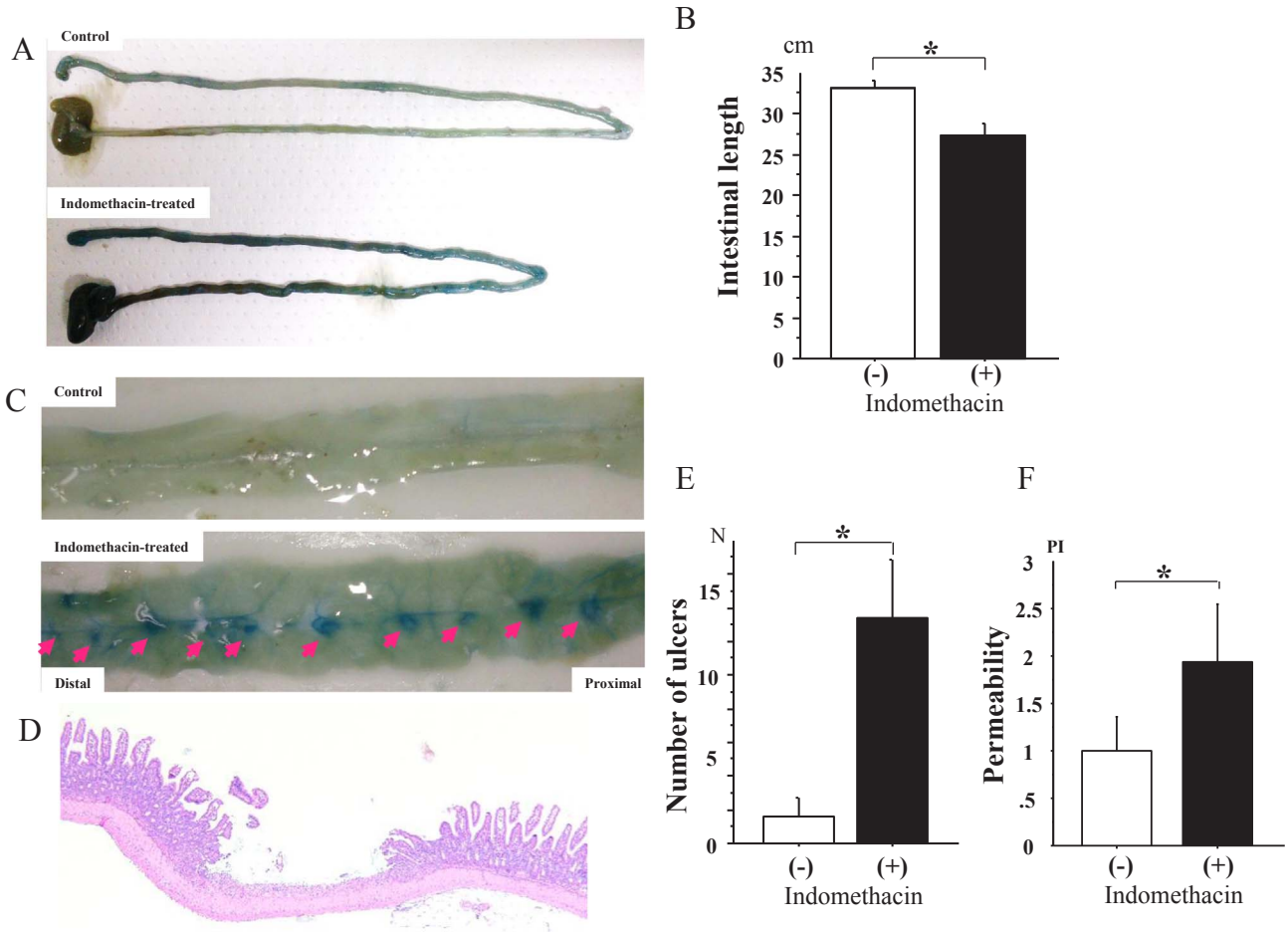

Fig. 1. Small intestinal damage after indomethacin administration. (A) The whole small intestine after indomethacin administration. (B) The length of the small intestine was shorter. Date are expressed as means ± SD, n = 5. $^{*}P < 0.05$ versus untreated controls. (C) Injured mucosa stained dark blue with ulcer formations by injection of 1% Evans blue (arrows). (D) Histological findings in the small intestine. Destruction and necrosis of intestinal epithelium extending to the base of the crypts was observed. (E) The number of macroscopic ulcers and (F) the permeability of blood vessels in the small intestine. Data are presented as mean ± SD (n = 4–5 for each group). $^{*}P < 0.05$ versus untreated controls. Data are presented as mean ± SEM of three independent experiments.

Ganoderma lucidum Karst, belonging to the Basidiomycetes class of fungi, is well known as "Reishi", a traditional food in China and Japan [13]. It contains various bioactive substances, including polysaccharides, proteins, nucleotides, fatty acids, terpenoids, sterols, and cerebrosides [14]. Reishi has multiple immunologic functions, such as activation of natural killer cells in BALB/c mice [15], induction of CD40/CD86 on human peripheral blood monocytes [16], and cytokine-induced killer cells in C57BL/6 mice [17]. Recently, it was reported that Reishi contains a fraction named "F3", which stimulates mouse spleen cell proliferation and cytokine production, especially that of GM-CSF [18]. Although a water-soluble extract from a cultured medium of Reishi mycelia (MAK) and F3 are purified by different methods, there appears to be a strong likelihood that components of Reishi may contribute to GM-CSF-mediated immune responses.

We previously reported that murine trinitrobenzene sulfonic acid (TNBS)-induced colitis was prevented via GM-CSF production by feeding with MAK [19]. So far, however, there have been no reports showing the effect of MAK on small intestinal inflammation. In this work, we investigated the role of MAK in indomethacin-induced small intestinal injury. This study shows that peritoneal macrophages (PMs) stimulated by MAK are effective in the prevention of intestinal inflammation. In vitro, MAK stimulated PMs to produce GM-CSF in a dose-dependent manner. Finally, the protective effect of PMs on intestinal inflammation is dependent on GM-CSF. Therefore, GM-CSF may be a candidate for the treatment of small intestinal inflammation.

2. Materials and methods

2.1. Mice

Specific pathogen-free C57BL/6(B6) mice were purchased from CLEA Japan (Tokyo, Japan). All mice were housed under specific pathogen-free conditions in micro-isolator cages in the animal facility at Hiroshima University, and only male mice (9–14 weeks old) were used. The animals were maintained in accordance with the "Guidelines for the Care and Use of Laboratory Animals" established by Hiroshima University. Normal tap water was also provided *ad libitum*. The MAK was provided by Noda Shokkin-Kogyo Co., Ltd. (Chiba, Japan). The preparation of MAK (overall yield ≈ 10%) was as follows: a pure culture of G. lucidum mycelia was inoculated into a solid culture medium that was composed of bagasse and defatted rice bran and cultured until just before the formation of the fruit body (for 3–4 months); subsequently, the entire medium overgrown with G. lucidum mycelia was extracted with hot water, and then the extract was sterilized by filtration and lyophilized for powderization.

2.2. Preparation of peritoneal macrophages

Peritoneal cells were collected by washing the peritoneal cavity with ice-cold PBS. The cells were seeded at 1×10^6 cells/well in 96-well plates to allow them to adhere to the surface and incubated in humidified 5% CO_2 at 37 °C for 1–2 h in RPMI 1640 medium

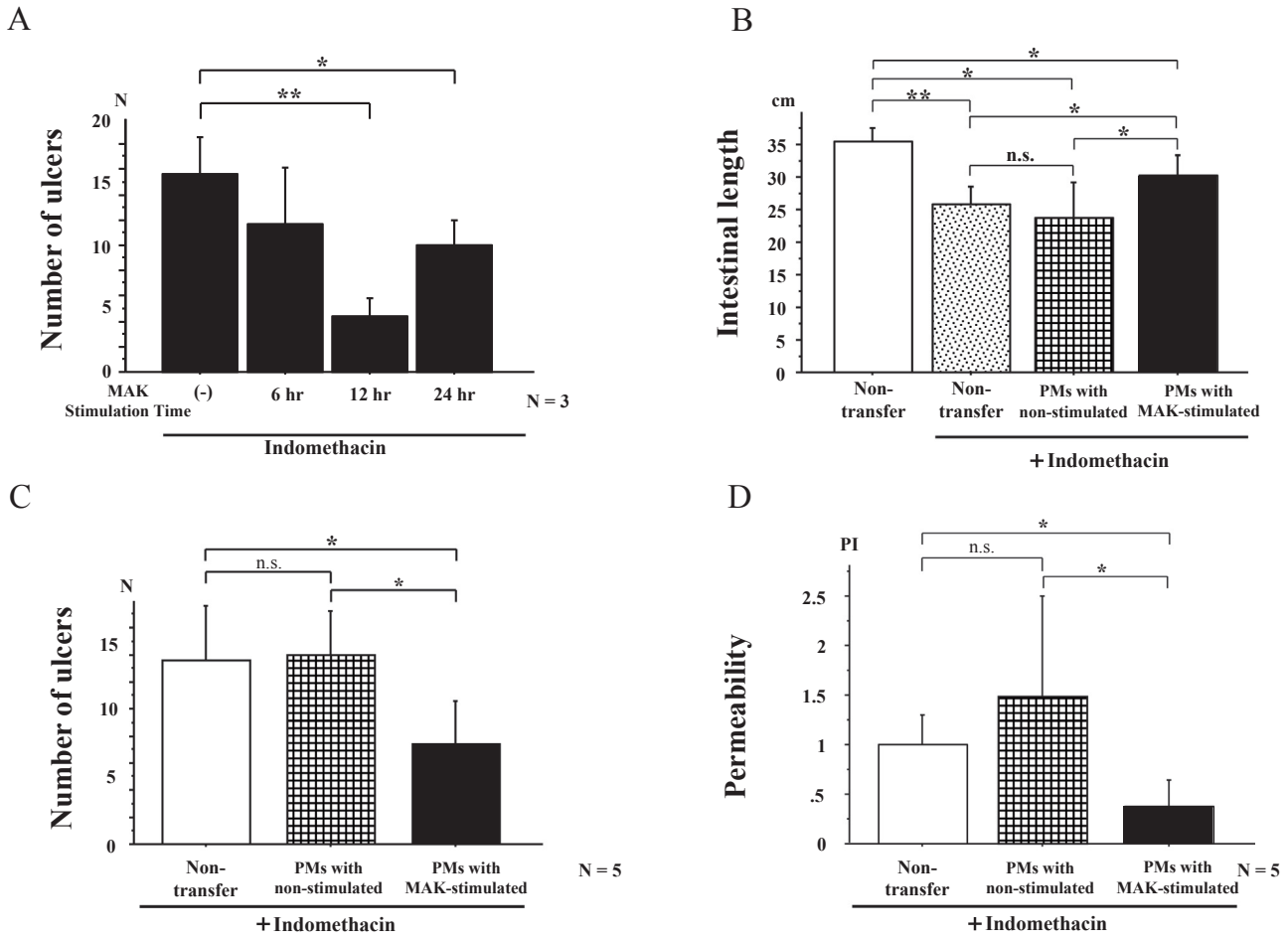

Fig. 2. Adoptive transfer of PMs stimulated with MAK prevented indomethacin-induced small intestinal injuries. (A) PMs were stimulated in vitro with MAK at the indicated times. PMs stimulated with MAK for 12 h, but not 6 or 24 h, decreased the number of ulcers. Data are expressed as mean ± SD. n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$. (B) The relative small intestinal length was measured. The length of the small intestine was significantly shorter in the group treated with indomethacin and transferred non-treated PMs than the group fed chow only. As compared with the group treated with indomethacin and transferred non-treated PMs, a reduction in shortness of the small intestine length was prevented in the group with transferred PMs stimulated with MAK for 12 h. Data are presented as mean ± SD (n = 3–5 for each group) of two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. (C) PMs stimulated with MAK for 12 h significantly decreased the number of small intestinal ulcers induced by indomethacin. Data are presented as mean ± SD of two independent experiments (n = 5 for each group). $^{*}P < 0.05$. (D) The permeability of the blood vessel was improved after transfer of PMs stimulated with MAK. Data are presented as mean ± SD (n = 5 for each group). $^{*}P < 0.05$. Data are presented as mean ± SEM of three independent experiments.

supplemented with 10% FCS. Non-adherent cells were washed with PBS, and the attached cells were designated as PMs.

2.3. Cytokine ELISA assay

PMs of C57BL/6 mice were stimulated in vitro with 500 ng/ml PMA (Sigma, St. Louis, MO) and 50 ng/ml ionomycin (Sigma) or LPS and MAK powder at different concentrations for 24 h. The culture supernatant was collected, and the concentration of cytokine was determined by ELISA. GM-CSF and IL-10 were measured with OptEIA Kits (BD, San Jose, CA). All samples were analyzed in duplicate.

2.4. Induction and assessment of small intestinal injury

To induce small intestinal injury, 10 mg/kg indomethacin (EC number 200-186-5, Sigma) was administered by gavage to fasted animals that were sacrificed 24 h later. In each case, to delineate the damage, 1% Evans blue was injected i.v. into the tail vein of each mouse 30 min before sacrifice, and the small intestine was opened along the anti-mesenteric attachment and examined for injury under a dissecting microscope with square grids. The number of ulcers of macroscopically visible lesions was measured, summed per small intestine, and used as the lesion score. The length of the small intestine was measured after sacrifice. Intestinal permeability was assessed by a modified protocol [20]. Briefly, whole small intestine containing Evans blue was incubated at 37 °C for 16 h. A mixture of 0.6 N H_3PO_4 and acetone (5:13 ratio) was added to this solution, which was centrifuged at 3000 rpm for 15 min followed by measurement of the OD at 620 nm. Permeability Index (PI) was defined as each concentration of Evans blue divided by mean of control group.

2.5. In vivo adoptive transfer model

To test the therapeutic efficacy of GM-CSF, PMs were used for the adoptive transfer study. PMs stimulated with 100 μg/ml MAK or medium only for 0, 3, 6, 12, and 24 h were collected and washed twice with Hanks' solution. The PMs (1-2x10^6) were adoptively transferred intraperitoneally to each mice, which were then given indomethacin. The mice were sacrificed 24 h later and evaluated for small intestinal inflammation.

2.6. Assessing accumulation of transferred PMs in vivo

To determine whether transferred PMs have the ability to migrate to the organs of mice, PMs were labeled with red fluorescent linker dye (PKH26 Red Fluorescent Cell Linker Kit; Sigma). PMs (2×10^6)

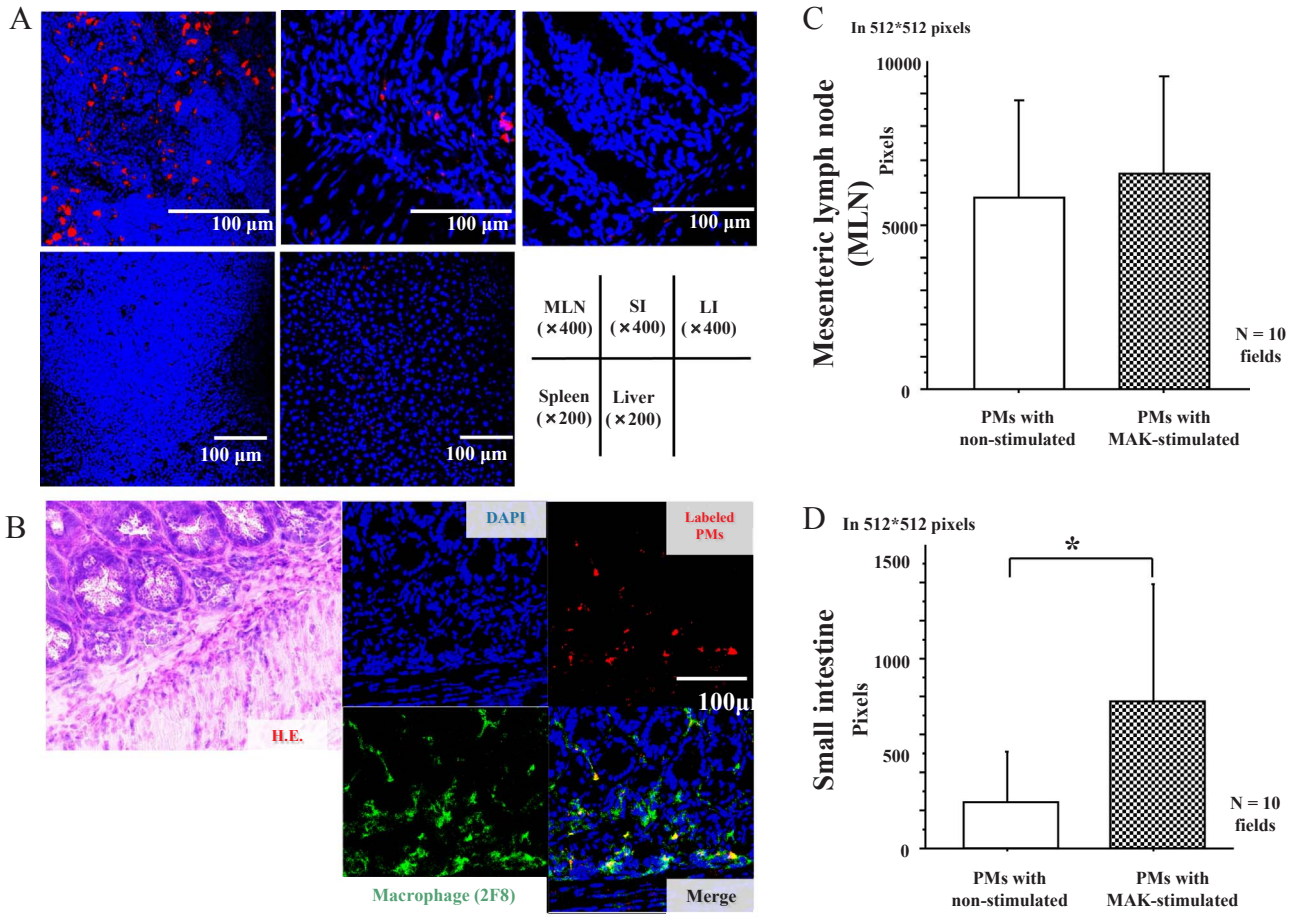

Fig. 3. PKH-labeled PMs stimulated with MAK were adoptively transferred intraperitoneally to mice, which were then given indomethacin. (A) Immunostaining showed that the transferred labeled PMs (red) were detected in the small intestinal (SI) tissues and mesenteric lymph nodes (MLNs). Labeled PMs were not found in the spleen, liver, or large intestine (LI), which showed no inflammation. (B) Immunostaining of small intestinal tissues showed that the transferred labeled PMs were detected in the submucosa and lamina propria (red). Immunofluorescence staining for macrophages (green) was performed in indomethacin-induced small intestinal injuries that transferred PKH26-labeled PMs (red) stimulated with MAK intraperitoneally. DAPI staining for cell nuclei (blue). Scale bars: 100 μm. (C) The area of the labeled PMs was assessed in MLNs. There was no difference in the frequency of transferred PMs in MLNs regardless of MAK stimulation. (D) The area of the labeled PMs was assessed in small intestine. In the small intestine, the frequency of PMs stimulated with MAK was significantly higher than that of PMs not stimulated with MAK. Data are expressed as means + SD. n = 10 fields for each group. $^{*}P < 0.05$ versus unstimulated PMs. Data are presented as mean ± SEM of two independent experiments.

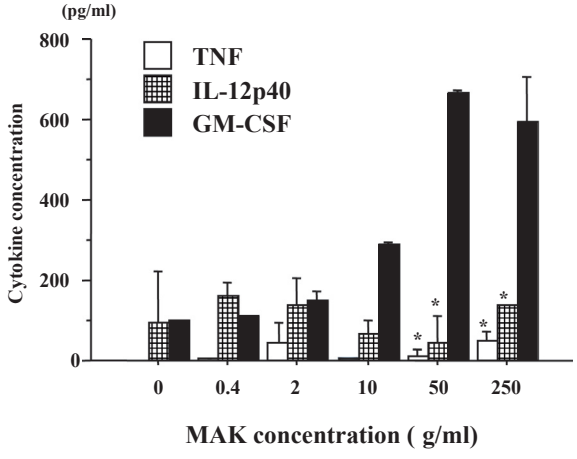

Fig. 4. Effect of MAK on GM-CSF production in vitro. PMs stimulated in vitro with MAK produced GM-CSF in a dose-dependent manner. GM-CSF, TNFα, and IL12p40 on the same supernatant were assayed. MAK selectively induced a large amount of GM-CSF in a dose-dependent manner. However, neither TNFα nor IL-12p40 was induced by MAK. Data are expressed as means ± SD. n = 3. $^{*}P < 0.05$ versus GM-CSF. Data are presented as mean ± SEM of three independent experiments.

stimulated with MAK or medium for 12 h were incubated with PKH26 for 3 min at 25 °C. The PKH26-labeled PMs were then purified and adoptively transferred intraperitoneally to each mouse, which were then given indomethacin orally. After 24 h, the mice were sacrificed. Liver, spleen, mesenteric lymph nodes (MLNs), and small and large intestines were removed and then snap-frozen in liquid nitrogen and stored at 80 °C until tissue processing. Sections of PKH26-labeled tissues were analyzed by means of fluorescence confocal microscopy. For quantification of the transferred PMs, 10 random fields were captured for each tissue, and areas of red fluorescence were measured. The areas were then calculated with the use of NIH ImageJ software.

2.7. Immunofluorescence staining

Frozen specimens cut into 8-μm sections on glass slides were fixed for 15 min in 4% paraformaldehyde in PBS. The slides were blocked briefly in protein blocking solution and incubated overnight at 4 °C with anti-macrophage antibody (Serotec Ltd., Kidlington, England). The slides were washed with PBS and then incubated for 1 h at room temperature with Alexa Fluor® 488-labeled secondary antibody (Ab). Nuclear counterstain with DAPI was applied for 10 min, and mounting medium was placed on each specimen with a glass coverslip. Macrophages were identified by green fluorescence, whereas PKH26 on transferred PMs was identified by red fluorescence. Co-localization of

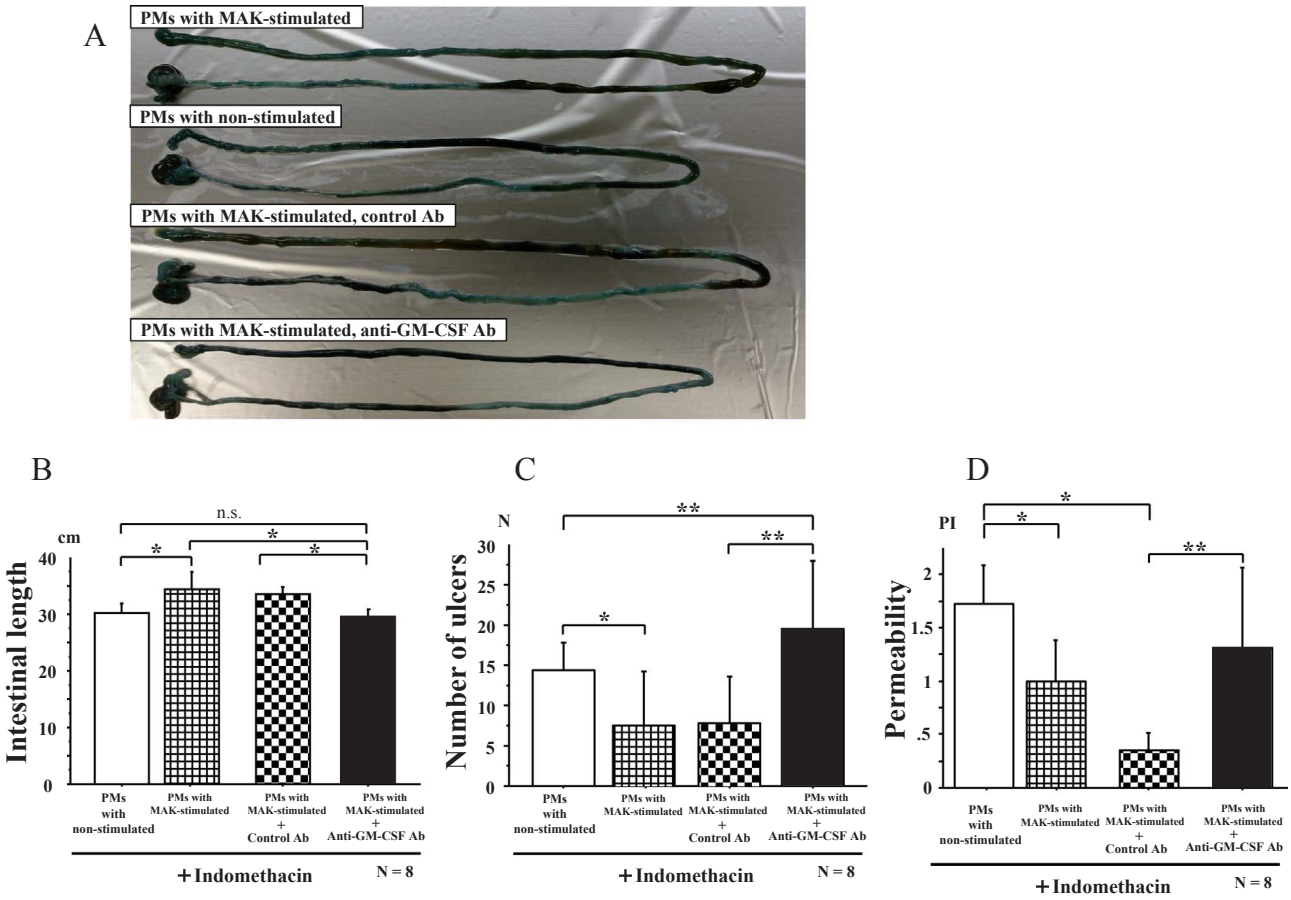

Fig. 5. Blocking of GM-CSF negates the improvement in small intestinal injuries induced by indomethacin. (A) The representative whole small intestines. (B) The relative small intestinal length was measured. Shortening of small intestinal length by indomethacin was improved by transfer of PMs stimulated with MAK. However, anti-GM-CSF but not control Ab pre-treatment diminished the improvement in small intestinal length. Data are presented as mean ± SD (n = 8 for each group). $^{*}P < 0.01$ between anti-GM-CSF Ab-treated mice and control Ab-treated mice exposed to indomethacin. (C) PMs stimulated with MAK significantly decreased the number of the small intestinal ulcers induced by indomethacin (MAK(−), 14.3 ± 3.4 versus MAK(+), 7.5 ± 6.7; $^{*}P < 0.05$). Control Ab pre-treatment did not alter this effect (MAK(+), versus control Ab, 7.8 ± 5.8; n.s.). The number of ulcers induced by indomethacin was significantly increased in anti-GM-CSF Ab-treated mice (MAK(+) versus anti-GM-CSF Ab, 19.5 ± 8.5; $P < 0.01$). Data are presented as mean ± SD (n = 8 for each group). $^{**}P < 0.01$ between anti-GM-CSF Ab-treated mice and control Ab-treated mice exposed to indomethacin. (D) Pretreatment with anti-GM-CSF Ab but not with control Ab reversed the improvement of blood vessel permeability. Data are presented as mean ± SD (n = 6 for each group). $^{*}P < 0.01$, $^{**}P < 0.05$. Data are presented as mean ± SEM of two independent experiments.

PKH26 and macrophages was detected as yellow staining.

2.8. Confocal microscopy

Confocal fluorescence images were captured with a 20× or 40× objective lens on an LSM 510 laser scanning microscopy system (Carl Zeiss, Thornwood, NY) equipped with a motorized Axioplan microscope, argon laser (458/477/488/514 nm, 30 mW), HeNe laser (543 nm, 1 mW), HeNe laser (633 nm, 5 mW), LSM 510 control and image acquisition software, and appropriate filters (Chroma Technology Corp., Brattleboro, VT). Confocal images were exported to Adobe Photoshop software, and image montages were prepared.

2.9. Administration of monoclonal antibodies (mAb)

Mice were administered anti-GM-CSF Ab, 50 μg intraperitoneally (clone number MP1-22E9; Endogen, Rockford, IL) or isotype control antibody (clone R35-95; BD Pharmingen, San Diego, CA) [11]. Two weeks later, PMs stimulated with MAK for 12 h were purified and adoptively transferred intraperitoneally to each mice, which were then given indomethacin orally. After 24 h, the mice were sacrificed and evaluated for small intestinal inflammation.

2.10. Enzymatic digestion of polysaccharides

Pectinase (polygalacturonase, EC 3.2.1.15; Sigma) was used for enzymatic digestion of the polysaccharides from *G. lucidum* [21]. To investigate the role of the polysaccharides from MAK in the induction of GM-CSF, MAK (including polysaccharides, 25 μg/ml, 1 ml) were mixed with pectinase at final concentrations of 1, 2.5, 10, and 50 U/ml in 1.5-ml Eppendorf tubes and digested overnight (12 h) under optimum conditions (buffer solution: 50 mM sodium acetate, pH 5.5, 40 °C). Then the mixture was boiled at 85 °C for 30 min to stop the enzyme activity. The PMs were stimulated in vitro with LPS and the MAK pre-treated by pectinase for 24 h. The culture supernatant was collected, and the amount of GM-CSF and IL-10 was measured by ELISA. To determine the preventive effect of polysaccharides, PMs stimulated with MAK pre-treated or not treated with pectinase for 12 h were purified and adoptively transferred intraperitoneally to each mice, which were then given indomethacin orally. After 24 h, the mice were sacrificed and evaluated for small intestinal inflammation.

2.11. Statistical analysis

Assessment of statistical differences was determined by a parametric Student *t*-test and nonparametric Mann-Whitney test as appropriate. Data were analyzed with Stat-View software (Hulinks, Tokyo, Japan).

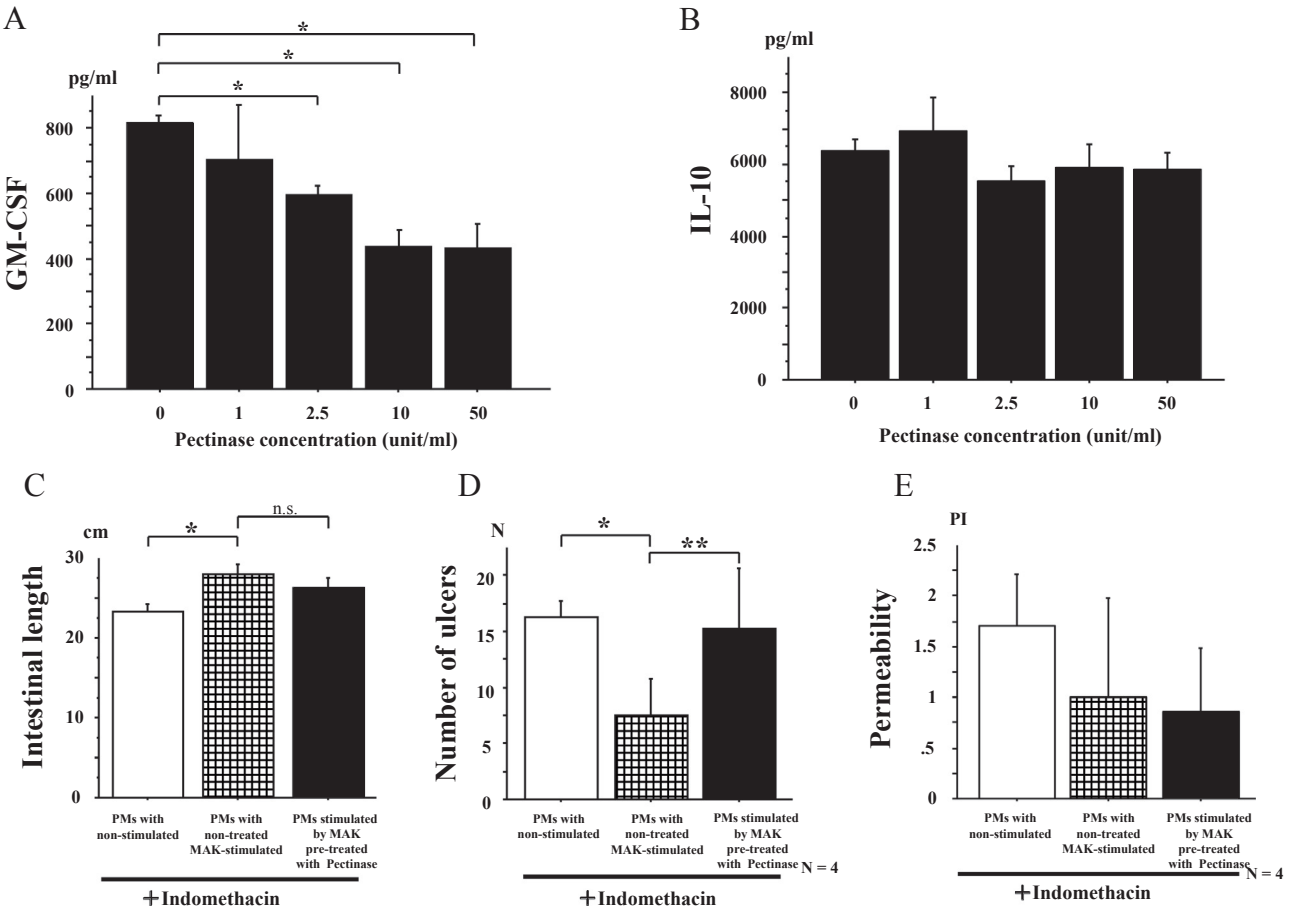

Fig. 6. Pectinase reversed the ability of MAK inhibited development of indomethacin-induced small intestinal injuries. Pectinase reversed the ability of MAK to produce GM-CSF in vitro. PMs were co-stimulated in vitro with LPS plus MAK (25 μg/ml) treated with different concentrations of pectinase in a 96-well plates in triplicate for 24 h. The concentrations of GM-CSF and IL-10 in the supernatants were measured by ELISA. Pectinase treatment reduced the concentration of GM-CSF (A) but not IL-10 (B). $^{*}P < 0.05$ between pectinase-treated (2.5, 10, and 50 U/ml) and untreated PMs. (C) The relative small intestinal length was measured in indomethacin treatment model mice with adaptive transfer of PMs stimulated with control and pectinase-treated MAK. The length of the small intestine shortened by was significantly improved in mice with transferred PMs stimulated with MAK. Data are presented as mean ± SD (n = 4 for each group). $^{*}P < 0.05$ between MAK-treated and untreated PMs. (D) PMs stimulated with non-treated MAK significantly reduced the number of small intestinal ulcers induced by indomethacin (PMs with non-stimulated, 16.3 ± 2.3 versus PMs with non-treated MAK-stimulated, 7.5 ± 3.3; $^{*}P < 0.01$). The number of ulcers induced by indomethacin was significantly increased with mice that transferred PMs stimulated with pectinase-treated MAK (PMs with non-treated MAK-stimulated, 7.5 ± 3.3 versus PMs stimulated by MAK pre-treated with pectinase, 15.3 ± 5.4; $^{**}P < 0.05$). Data are presented as mean ± SD (n = 4 for each group). (E) Blood vessel permeability was not significantly different between non-treated MAK and MAK pre-treated with pectinase. Data are presented as mean ± SD (n = 4 for each group). Data are presented as mean ± SEM of two independent experiments.

The data are expressed as the mean ± SD. Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Indomethacin-induced small intestinal injury

To induce and evaluate macroscopic small intestinal damage, we orally administered 10 mg/kg of indomethacin to each mouse followed by i.v. injection of 1% Evans blue dye into the tail vein of each mouse 24 h after indomethacin administration. The length of the small intestine was significantly shorter in mice administered indomethacin than in those not administered indomethacin (Fig. 1A). As shown in Fig. 1B, the shortened small intestinal length was observed from 12 h (control, 35.3 ± 2.1 cm versus at 12 h, 30.2 ± 1.3 cm; $P < 0.05$) after indomethacin administration and reached a peak by 48 h (control versus 48 h, 23.0 ± 4.6 cm; $P < 0.05$). Macroscopic small intestinal ulcers were detected (Fig. 1C), and the ulcers were confirmed histologically (Fig. 1D). Furthermore, an increase in both the number of ulcers and in blood vessel permeability were observed after indomethacin administration (Fig. 1E, F).

3.2. Adoptive transfer of PMs stimulated with MAK prevented indomethacin-induced small intestinal injury

We hypothesized that PMs stimulated with MAK would have an anti-inflammatory effect on small intestinal injuries caused by indomethacin. Preliminarily, to determine the optimal stimulation time, we stimulated PMs with MAK in vitro at different times. Then, the PMs were adoptively transferred intraperitoneally to other mice, which were then given indomethacin. PMs stimulated with MAK for 12 h, but not 6 or 24 h, decreased the number of ulcers (Fig. 2A). The length of the small intestine was shorter in the mice treated with indomethacin and transferred non-treated PMs than that in the mice fed chow only. However, shortening of the small intestine was prevented in the mice with transferred PMs stimulated with MAK for 12 h (Fig. 2B). These PMs significantly prevented the small intestinal ulcers induced by indomethacin (indomethacin, 13.6 ± 4.0 versus PMs stimulated with MAK + indomethacin, 7.4 ± 3.2; $P < 0.05$ and non-treated PMs + indomethacin, 14.0 ± 3.2 versus PMs stimulated with MAK + indomethacin; $P < 0.05$) (Fig. 2C). Blood vessel permeability was also improved by the transfer of PMs stimulated with MAK for 12 h but not by non-treated PMs (Fig. 2D). These results suggest that PMs stimulated with MAK contribute to the prevention of intestinal inflammation

induced by indomethacin.

3.3. Transferred PMs accumulated in the inflammatory tissues

Next, we examined whether the transferred PMs accumulated in the small intestine. PKH-labeled PMs stimulated with MAK were adoptively transferred intraperitoneally to mice, which were then given indomethacin. As shown in Fig. 3A, labeled PMs were detected in MLNs and the small intestine. Immunostaining of small intestinal tissues showed that the transferred labeled PMs were detected in the submucosa and lamina propria (Fig. 3B). PMs were not found in the spleen, liver, or large intestine, none of which showed inflammation (Fig. 3A). There was no difference in the number of transferred PMs in MLNs regardless of MAK stimulation. In contrast, in the small intestine, the number of PMs stimulated with MAK was significantly higher than that not PMs stimulated with MAK (Fig. 3C and D). These results suggest that the transferred PMs stimulated with MAK preferably accumulated in the inflammatory tissues.

3.4. PMs stimulated with MAK produced GM-CSF but not TNFa or IL-12p40

We previously reported that PMs stimulated in vitro with MAK produce GM-CSF in a dose-dependent manner [19]. To confirm whether GM-CSF is selectively induced, we measured GM-CSF and other inflammatory cytokines such as TNF-α and IL12p40 in the same supernatant. MAK selectively induced a large amount of GM-CSF in a dose-dependent manner (Fig. 4). However, neither TNFα nor IL-12p40 was induced by MAK. These data suggest that MAK selectively induced GM-CSF from PMs.

3.5. Blocking of GM-CSF negates the improvement in indomethacin-induced small intestinal injury

To investigate the preventive role of GM-CSF on indomethacin-induced small intestinal injury, Ab blockade was performed. Shortening of the small intestinal length by indomethacin was improved by transfer of PMs stimulated with MAK. However, pre-treatment anti-GM-CSF Ab but not control Ab diminished the improvement in small intestinal length (Fig. 5A, B). PMs stimulated with MAK significantly prevented the number of small intestinal ulcers induced by indomethacin (indomethacin, 14.4 ± 3.4 versus MAK + indomethacin, 7.5 ± 6.7; $P < 0.05$). Control Ab pre-treatment did not alter this effect (MAK + indomethacin versus MAK + control Ab + indomethacin, 7.8 ± 5.8; n.s.). However, pre-treatment with anti-GM−CSF Ab reversed the therapeutic effect of MAK (MAK + indomethacin versus MAK + anti-GM-CSF Ab + indomethacin, 19.5 ± 8.5; $P < 0.01$) (Fig. 5C). Pretreatment with anti-GM-CSF Ab but not with control Ab also reversed the improvement of blood vessel permeability (Fig. 5D). These results support the possibility that GM-CSF derived from PMs might have an anti-inflammatory role in indomethacin-induced small intestinal injury.

3.6. Preventive effect of MAK was negated by pretreatment with pectinase

We used pectinase to examine which component of MAK induces GM-CSF. Pectinase is known to digest the polysaccharides that are the major component of MAK [21]. PMs were stimulated with MAK treated by pectinase. GM-CSF and IL-10 in the supernatants were measured by ELISA. Pectinase treatment reduced the concentration of GM-CSF. However, the concentration of IL-10 was not affected by the pectinase treatment (Fig. 6A, B). Adaptive transfer assay of PMs stimulated with pectinase-treated-MAK diminished the improvement in small intestinal length (Fig. 6C). PMs stimulated with non-treated MAK significantly reduced the number of small intestinal ulcers induced by indomethacin (indomethacin, 16.3 ± 1.5 versus MAK + indomethacin, 7.5 ± 3.3; $P < 0.01$). However, pectinase pre-treatment reversed the therapeutic effect of MAK (MAK + indomethacin versus pectinase-treated MAK + indomethacin, 15.3 ± 5.4; $P < 0.05$) (Fig. 6D). There was no significant difference in blood vessel permeability between non-treated MAK and MAK pre-treated with pectinase (Fig. 6E). These results supported the notion that the polysaccharides from *G. lucidum* induce GM-CSF from PMs, which may partially prevent the development of indomethacin-induced small intestinal injury.

4. Discussion

Here, we have shown that MAK induced GM-CSF from PMs, and transferred PMs stimulated with MAK significantly attenuated small intestinal damage in mice following indomethacin administration. This preventive effect was blocked by the administration of Ab against GM-CSF. In addition, the effect of PMs on the prevention of small intestinal damage was negated by stimulation with polysaccharide-digested MAK. These results suggest that local GM-CSF supplementation ameliorates indomethacin-induced small intestinal injury.

G. lucidum (Reishi or Lingzhi) has been used as a health-promotion supplement owing to its anti-tumor and immunomodulating effects [22]. We recently reported that MAK suppressed the development of colorectal adenomas [23]. The anti-tumor effects of *G. lucidum* may be attributed to the activity of β-glucans [24]. The immunomodulating activity of the β-glucans is mainly related to their effects on immune effector cells, such as macrophages, mononuclear cells, and neutrophils, resulting in the production of cytokines [25,26]. Therefore, stimulation of immune effector cells leads to the subsequent production of cytokines and contributes to the anticancer activity of *G. lucidum*. We recently reported that MAK prevented the development of TNBS-induced colitis [19]. However, the anti-inflammatory role of MAK in indomethacin-induced small intestinal injury has never been examined, to our knowledge. This is the first study to reveal that MAK, and especially the pectinase-sensitive component of polysaccharides from MAK, may have a potential immunological role in indomethacin-induced small intestinal injury. Furthermore, macrophages play an important role in both the host-defense mechanism and inflammation [27,28], and the overproduction of inflammatory mediators by macrophages has been implicated in several inflammatory diseases and cancer [29]. Moreover, macrophages may be a key player in the pathogenesis of indomethacin-induced enteropathy [30].

First, we confirmed examined the effect of MAK on cytokine production in PMs. Previous studies have suggested that in vitro Reishi-treatment induces GM-CSF from murine splenocytes [18], and we previously reported that MAK induces GM-CSF from PMs in a dose-dependent manner, whereby we administered MAK in vivo and observed GM-CSF production in PMs. In vivo administration of MAK dramatically increased GM-CSF protein in a dose-dependent manner [19]. In addition, we investigated cytokines such as IFN-γ, IL12p40, TNF-α, GM-CSF, and IL-10. No production of IFN-γ, IL12p40, or TNF-α was seen, and production of IL-10 was not affected by MAK (data not shown).

Several mechanisms by which NSAIDs induce small intestinal injury have been reported. The decrease in prostaglandin production is considered to be the main cause of such injury [2,31–34]. The involvement of the following has also been reported important in small bowel injury: the reduction of intestinal mucus due to NSAIDs, microcirculatory disturbances accompanying abnormally increased intestinal motility, nitric oxide derived from inducible nitric oxide synthase, inflammatory cytokines, neutrophil infiltration, and reactive oxygen species [35–40]. M2 macrophages were recently reported to prevent indomethacin-induced intestinal injury in mice [41]. PMs stimulated with MAK produce GM-CSF. Therefore, we focused on the role of macrophages in NSAID-induced small intestinal injury. Several time points after the administration of indomethacin have been chosen to evaluate the pathogenesis of indomethacin-induced small intestinal injury [42–44]. We chose the

24-h time point after indomethacin administration because body weight loss was most evident at this time.

To examine the anti-inflammatory role of PMs stimulated by MAK in vitro, we used mAb to block the biological activity of GM-CSF. The mAb against GM-CSF, clone MP1-22E9, is known to block the biological activity of GM-CSF [11]. The mAb treatment assay with GM-CSF revealed that the protective effect of PMs stimulated by MAK on indomethacin-induced intestinal injuries was inhibited by treatment with the anti-GM-CSF Ab. In terms of indomethacin-induced injuries, the shortening of small intestinal length was completely similar to that in the mice not treated with MAK, suggesting that the preventive effect of MAK on indomethacin-induced ileitis is mainly contributed by GM-CSF. Therefore, GM-CSF induced by MAK appears to be involved in the prevention of indomethacin-induced injuries.

We have shown that transferred PMs accumulated in the inflammatory tissues. Although the mechanisms by which the transferred PMs accumulated only in the inflamed tissues are unclear, these PMs might produce GM-CSF in the local inflamed mucosa and ameliorate inflammation.

To investigate the mechanism by which MAK induces GM-CSF, an enzymatic digestion assay was performed. We showed that pectinase-treated MAK significantly reduced the production of GM-CSF from PMs. Furthermore, adaptive transfer of PMs stimulated with pectinase-treated-MAK partially diminished the improvement in small intestinal injuries. MAK contains various types of high-molecular-weight constituents, such as polysaccharides with protein or water-soluble lignin, and low-molecular-weight constituents, such as triterpenes. In addition, the immunological properties of polysaccharides and immunomodulatory protein derived from Lingzhi (*G. lucidum*) have been studied [45–48]. Triterpenes and polysaccharides are usually considered to be the main active components in *G. lucidum*. Recent pharmacologic studies have revealed that polysaccharides have multiple pharmacologic activities [18,48–50]. Polysaccharides are digested by several enzymes, such as dextranase, pectinase, cellulase, β-manannase, xylanase, lichenase, and β-glucanase. Previous studies found that polysaccharides from *G. lucidum* showed positive responses to pectinase and digestion but not to β-manannase, xylanase, lichenase, and β-glucanase [51]. Polysaccharides from *G. lucidum* usually consist of arabinose, galactose, glucose, xylose, and mannose. These data suggest that polysaccharides of MAK play a critical role in preventing small intestinal injury induced by indomethacin. Masuda et al. reported that β-glucans derived from *Grifola frondosa* (an oriental edible mushroom) can directly stimulate GM-CSF production in resident macrophages through activation of dectin-1-independent ERK and p38 MAPK [52]. Although we did not investigate whether β-glucans from MAK produce GM-CSF, pectinase-sensitive polysaccharides may at least induce GM-CSF from PMs to prevent intestinal damage from indomethacin. Further analysis will be performed to obtain direct evidence that polysaccharides themselves can directly induce GM-CSF from macrophages.

In conclusion, MAK treatment was shown to prevent indomethacin-induced intestinal injury. Endogenous GM-CSF may contribute to the protective effect of MAK. This activity contributes to the prevention of indomethacin-induced intestinal injury, suggesting that GM-CSF could be a promising therapeutic target for the prevention of NSAID-induced intestinal damage.

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