Purinergic P2X7 receptor antagonist ameliorates intestinal inflammation in postoperative ileus

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Abstract

Postoperative ileus (POI) is gastrointestinal motility dysfunction after abdominal surgery caused by mechanical stress to intestine. It occurs nausea and vomiting, and lead to lower QOL during hospitalization for patients. It is regarded as a problem because it costs medical expenses due to long hospitalization. The intestinal inflammation caused by macrophage and neutrophil is thought to be important of the mechanism of POI. In tissue injury or inflammation, adenosine triphosphate (ATP) is released from injured cell. Purinergic P2X7 receptor (P2X7R) is expressed on inflammatory cells and ATP induces the secretion of inflammatory mediators through P2X7R. P2X7R antagonist is thought to be important in the first step of inflammation, and it is confirmed that P2X7R antagonist showed anti-inflammatory effects in chemically-induced colitis models. Therefore, we hypothesized that P2X7R has important function in POI and examined the effect of P2X7R antagonist in mouse POI model. As a result, P2X7R antagonist A438079 ameliorated macrophage and neutrophil infiltration in POI. The impairment of intestinal transit was improved by P2X7R antagonist. It tended to ameliorate the increase of IL-6, IL-1β and TNF-α mRNA expression in intestinal muscularis caused by POI. P2X7R expressed on both infiltrated and resident macrophages in the inflamed ileal muscle layer. In conclusion, P2X7R antagonist showed the anti-inflammatory effect through P2X7R on macrophages, and it may be the target to treat POI.

Introduction

Postoperative ileus (POI) is transient intestinal dysmotility after abdominal surgery. It induces abdominal distension, nausea, vomiting and may be a risk factor of complications such as pulmonary embolism and thromboembolism [1, 2]. So far, much effort has been conducted for prevention or treatment of POI, but 10 ~ 30% patients after laparotomy develop POI [3]. In addition, much medical cost is necessary for management of POI, and total cost in US for medical economy is estimated over 14.6 billion dollars. Therefore POI seem to give burden for medical system [4, 5]. The development of POI is thought to be divided into three stages: a neurological stage, an inflammatory stage, and a stage of eliminating ileus and vagal nerve over-activation [3]. The inflammatory stage is known to be immune-mediated [1], and POI occurs as follows [1, 6–10]. First, cytotoxicity factor such as ATP is released by mechanical stress during laparotomy, and it activates immune cell, which inflammatory cytokine and chemokine are released. However, detailed identification of cytotoxic factors in POI has not been made. Second, monocytes and macrophages are infiltrated into intestinal muscularis. Third, iNOS and COX-2 are induced and PGE2 and NO are released. Finally, PGE2 and NO decrease contraction of intestinal smooth muscle and intestinal motility is impaired.

Purinergic receptors are receptors for adenosine triphosphate (ATP) and purine nucleotides. It is classified into three groups, P1, P2X and P2Y. P1 and P2Y groups are G protein coupled type receptors (GPCR), whereas P2X receptors are ion channels [11]. P2X receptors are known to have seven subtypes [12, 13]. P2X7 receptor (P2X7R) is expressed mainly on inflammatory cells such as neutrophils, macrophages, dendritic cells, and mast cells [13–15]. When ATP activates P2X7R, potassium flows out from the cell and sodium and calcium flow in [13]. ATP released from infected or stressed cells is thought to be an

endogenous signaling molecule that regulates inflammation and immune responses [16]. When ATP is released extracellularly, it activates the NOD-like receptor protein 2 (NLRP3) inflammasome through activation of P2X7R and secretes inflammatory cytokines IL-1β and IL-18. It also acts as a secondary signal for the production of active oxygen and the activation of nuclear factor kappa B (NF-κB) [14, 17]. This suggests that P2X7R is important in the early stages of inflammation. So far, the anti-inflammatory effect has been confirmed in a model of colitis induced by chemical substances such as Dextran sulfate sodium (DSS) and trinitrobenzene sulfonic acid (TNBS) using P2X7R inhibitor [16, 18]. Macrophages play a central role in the pathogenesis of POI, and P2X7R inhibitors have anti-inflammatory effect in other pathologies of colitis. Therefore it is hypothesized that P2X7R also plays an important role in the pathology of POI. In this study, we aimed to examine the hypothesis that P2X7R plays an important role in POI in which macrophages play a central role using P2X7R inhibitors.

Materials And Methods

Animals

All animal care and experimental procedures complied with the Guide for Animal Use and Care published by the University of Tokyo and were approved by the Institutional Review Board of the University of Tokyo (approval code P16-187). C57BL/6J mice were housed under controlled conditions (6–9 weeks of age, 12 h light/dark cycle).

Postoperative ileus model

Postoperative ileus model was made by surgical intestinal manipulation of distal ileal part by using C57BL/6J after 16–24 h fasting. All mice were anaesthetized with sodium pentobarbital 50 mg/kg i.p. (Somnopentyl; Kyoritsu Seiyaku Corp., Tokyo, Japan) and the animal model of postoperative ileus was made by intestinal manipulation previously reported [4]. Briefly, the distal ileum (10 cm from the ileocecal region) was exposed and scratched three times with a sterile moist cotton applicator. In the present study, laparotomy with intestinal manipulation treatments was considered as a postoperative ileus model. The mice were randomly assigned to the following groups in WT mice. WT; no treatment with fasting, IM; intestinal manipulation, IM + A438079, P2X7R antagonist (34 mg/kg) was subcutaneously injected 30 minutes before and 2 hours after IM. A438079 was dissolved in sterilized physiological saline. The administration concentration of A438079 was decided following previous reports [16, 18].

Whole mount immunohistochemistry

For ileal muscle layer immunohistochemistry, physiological salt solution was used as follows. (mM): NaCl 136.9, $NaHCO_3$ 23.8, glucose 5.5, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, EDTA 0.01. Mice were exsanguinated and manipulated ileal parts were isolated at 24h after intestinal manipulation. The ileum was opened along mesenteric attachment, and the mucosal and submucosal layers were removed with incisive scissors and tweezers. The ileal smooth muscle layer was cut into pieces and fixed in acetone for 5–10 min. The preparations were washed with Tris-buffered saline (TBS) three times. Next they were blocked

with 2%BSA/TBS for 30 min, first antibodies were treated overnight at 4 ^{o}C. Second antibodies were treated after washing three times. Then preparations were washed three times with TBS and immunohistochemically analyzed using a confocal microscope (EZ-C1, Nikon). CD68 positive cells in the myenteric plexus layer of four randomly selected areas in each preparation were counted and averaged value was calculated. The same experiment was performed at least four times to calculated means ± SEM. First and second antibodies are listed in Table 1.

Table 1
Antibodies used in this study.

Type	Target	Host	Clone	Label	Dilution	Supplier (catalogue No.)
Primary antibody	PGP9.5	Rabbit polyclonal			1:200	UltraClone Limited, Isle of Wight, UK (RA95101)
	CD68	Rat monoclonal	FA-11		1:500	AbD Serotec, Oxford, UK (MCA1957)
	P2X7	Rabbit polyclonal			1:250	Alomone labs, Israel
Secondary antibody	Rat IgG (H + L)	Donkey		Alexa Fluor 488	1:500	Life Technologies, Gaithersburg, MD, USA (A21208)
	Rat IgG (H + L)	Goat		Alexa Fluor 594	1:500	Life Technologies, Gaithersburg, MD, USA (A11007)
	Rabbit IgG (H + L)	Donkey		Alexa Fluor 488	1:500	Life Technologies, Gaithersburg, MD, USA (SA5-10038)
	Rabbit IgG (H + L)	Donkey		Alexa Fluor 594	1:500	Life Technologies, Gaithersburg, MD, USA (A21207)

Table 2
Primers used in this study.

Gene name	Forward / Reverse	Sequences (5' to 3')
18S rRNA	Forward	AAACGGCTACCACATCCAAG
	Reverse	CCTCCAATGGATCCTCGTTA
TNF-α	Forward	CAAACCACCAAGTGGAGGAG
	Reverse	GTAGACAAGGTACAACCCATCG
IL-1β	Forward	GACGGACCCCAAAAGATGAA
	Reverse	ACAGCTTCTCCACAGCCACA
IL-6	Forward	GCCAGAGTCCTTCAGAGAGATACA
	Reverse	CTTGGTCCTTAGCCACTCCTTC

Myeloperoxidase staining

Whole mount preparations were fixed with 5 or 10 % formalin neutral buffer for 30 min. They were washed three times with TBS for 1.5 h. They were incubated in 10 mL TBS containing 10 mg Hanker-Yates reagent (Polysciences, Warrington, Pennsylvania, USA) and 30 ~ 35.5 % hydrogen peroxidase (Mitsubishi Gas Chemical Company, Tokyo, Japan) 10 µL for 5 min and then washed in TBS for over 10 min. Myeloperoxidase (MPO)-positive neutrophils were counted under a microscope (Nikon ACT-1C for DXM1200; Nikon, Tokyo, Japan) in four randomly selected areas of each preparation.

Measurement of intestinal transit

23 hours after IM, 100 µL FITC-dextran solution (5 mg/mL) was administered orally. An hour after injection, mice were euthanized and intestine from stomach to the end of colon was dissected. The intestine was separated to 15 section, from Sto; Stomach, S1-S10; small intestine, C1-C3; colon. The sections were cut in PSS, shaked vigorously for 10 seconds. centrifuged at 1500 g, 4 ℃, 5 min, and each sup was transferred to a new tube. The sup was additionally centrifuged at 11000 g, 4℃, 5 min, and 200 uL of sup was transferred to 96 well plate. Absorbance of FITC was measured by plate reader (EMC-427, JASCO Corporation).

Ratio of each absorbance to total absorbance was calculated. In addition, Geometric Center (GC) of FITC-dextran distribution is calculated.

GC = sum (each absorbance ratio % × section number) / 100

Real-time RT-PCR

Total RNA was extracted from the ileal muscularis using Trizol Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Total RNA was reverse transcribed using ReverTra Ace and

random primer at 30℃ for 10 min, 42℃ for 60 min, 99℃ for 5 min. Real-time PCR analysis was performed using SYBR qPCR Mix. The cDNA were amplified via 95℃ for 1 min, 40 cycles of 95℃ for 15 sec and 60℃ for 45 sec. Relative expression value was shown against mRNA expression of control sample.

Statistical analyses

Results are expressed as means ± SEM. Data were statistically evaluated using one way analysis of variance and Tukey's test. Values of $p < 0.05$ were considered statistically significant.

Results

P2X7 blockage inhibits macrophage and neutrophil infiltration by IM

We immunohistochemically observed CD68-positive macrophages (Fig. 1A). Some resident macrophages were detected in the myenteric plexus regions of ileum in WT mice. Many macrophages were infiltrated into the muscle layer 24h after intestinal manipulation. Inhibition of P2X7R strongly inhibited macrophage infiltration into myenteric plexus.

We further investigated MPO-positive neutrophils infiltration (Fig. 1A). There were almost no neutrophils in the myenteric plexus region of control mice. Many neutrophils were infiltrated after intestinal manipulation and inhibition of P2X7R attenuated the infiltration (Fig. 1A and C). Administration of A438079 alone did not change movements of macrophages and neutrophils in the myenteric plexus region in the ileum (n = 2, data not shown).

P2X7R inhibition ameliorates intestinal dysmotility by IM

We measured the gastrointestinal transit capacity 24 hours after IM. In control group, 90 % of FITC was located in S6-S10. Otherwise, 72 % of FITC was located in Sto-S5 in IM-treated group, which suggested that IM decreased the intestinal transit capacity. Compared to it, 69 % of FITC was located in S6-S10 in A438079-treated group. Taken together, it was suggested that A438079 improved the impairment of intestinal transit capacity induced by IM (Fig. 2A). In addition, we analyzed these graphs geometrically and calculate Geometric center of FITC distribution (GC value). High GC value means high capacity of intestinal transit. IM-treated group showed decrease of transit, and A438079 improved it significantly (Fig. 2B).

Inflammatory mediators tend to be attenuated by P2X7R antagonist

We examined mRNA expression of inflammatory mediator in ileal muscle layer three hours after IM. The expression levels of TNF-α, IL-1β and IL-6 were increased by IM. A438079 showed weak effects to decrease these expression (Fig. 3).

P2X7R is expressed on macrophages in small intestinal muscle layer

P2X7R antagonist A438079 showed anti-inflammatory effect in POI model, therefore we examine the expression of P2X7R in intestinal muscle layer. As a result, P2X7R was expressed on resident macrophages in normal mice intestine. At 24 hours after IM, P2X7R was also expressed on infiltrated macrophages in IM-treated mice intestine.

Discussion

POI is a transient postoperative gastrointestinal dysfunction that occurs primarily after laparotomy [5]. In the current prevention and treatment of POI, lidocaine, COX-2 inhibitor, Mosapride, and Daikenchuto have been shown to be effective [1, 19–20]. Lidocaine acts on the adrenal glands to reduce catecholamine release and directly stimulate smooth muscle to enhance gastrointestinal motility. Mosapride and Daikenchuto are distributed in the cholinergic nerves of the gastrointestinal wall. It has preventive and therapeutic effects on POI by directly or indirectly activating the 5-HT_4 receptor to enhance gastrointestinal motility. On the other hand, COX-2 inhibitors show preventive and therapeutic effects on POI by suppressing inflammation locally in the gastrointestinal tract, but have a risk of NSAIDs-induced gastric ulcer and small intestinal ulcer. Therefore, it is considered that promoting gastrointestinal motility is effective in preventing and treating POI because it prevents gastrointestinal adhesion due to stagnation of gastrointestinal motility.

In this study, the P2X7R selective inhibitor A438079 significantly improved the gastrointestinal transport capacity suppressed by IM in POI model mice (Figs. 2). That is, it was suggested that the administration of P2X7R inhibitors may be effective in the prevention and treatment of POI. In POI, decreased gastrointestinal motility is caused by macrophage-based inflammation in the muscularis of the gastrointestinal tract. They produce PGE_2 and NO, which effect on smooth muscle of the gastrointestinal tract [7, 21–22]. It has also been reported that suppression of infiltration of inflammatory cells such as macrophages and neutrophils restore hypokinesia of the gastrointestinal tract [8, 23]. Therefore, in order to clarify whether the improvement effect of gastrointestinal transport ability by the P2X7R inhibitor A438079 is due to the suppression of inflammatory cell infiltration in the POI model, we performed immunohistochemical staining. As a result, the P2X7R inhibitor significantly suppressed the infiltration of neutrophils and macrophages into the inflamed area of the gastrointestinal tract by IM (Fig. 1). From these results, it was clarified that P2X7R inhibition by A438079 has an anti-inflammatory effect in the POI model, which in turn restored the delayed intestinal transit. In addition, in POI model mice, the mRNA expression of the inflammatory mediators TNF-α, IL-1β, and IL-6 in the ileal muscularis 3 hours after IM was measured, and all tended to be increased by IM. This is thought to be because the resident macrophages activated by IM produced inflammatory mediators. Similarly, the mRNA expression of this inflammatory mediator tended to be suppressed by P2X7R inhibition using A438079, but no significant

difference was obtained in this experiment (Fig. 3). Regarding this result, it is necessary to be scrutinized in the future.

When P2X7R is activated by extracellular ATP, immune cells such as monocytes and macrophages expressing P2X7R are activated and release IL-1β [24–26]. On the other hand, macrophages from P2X7R or NLRP3 inflammasome knock out mice don't release IL-1β [27–29]. P2X7R activation by extracellular ATP is one of the strongest signal transduction system to activate NLRP3 inflammasome and release IL-1β [29, 30]. Also, Recent studies have shown that outflow of K^+ to extracellular is involved in the activation of NLRP3 inflammasome [30–32]. Therefore, anti -inflammatory effect of P2X7R antagonist may be induced by suppression of NLRP3 inflammasome followed by the decrease of IL-1β release. It is reported that P2X7R activation induces NF-κB phosphorylation and neuronal inflammation [33]. In inflammatory bowel syndrome, NF-κB in macrophages is activated [34] and inhibition of NF-κB transcription decreases release of TNF-α and IL-1β [35]. In addition, NF-κB is activated by intracellular Ca^{2+} increase [36, 37]. As a result, iNOS is induced and NO is released, which is related to inflammatory response [7, 9–10]. From these reports and this study, P2X7R induces influx of C^{a2+} [31, 38] and P2X7R antagonist is thought to ameliorate release of inflammatory cytokine and NO from macrophages through iNOS.

Next, we investigated the point of action of the anti-inflammatory effect of P2X7R inhibitor on POI. A previous report revealed that macrophages play an important role in the pathogenesis of POI [8], but the mechanism of macrophage activation in POI is unknown. Therefore, in order to clarify the role of P2X7R in the pathological expression of POI and the relationship of macrophages, immunohistochemical staining was performed on both P2X7R and macrophages. As a result, P2X7R was expressed in both the relatively large, dendritic-shaped resident macrophages in the gastrointestinal muscularis and the small, circular infiltrated macrophages infiltrated by IM (Fig. 4). This suggests that the target cells for P2X7R-mediated anti-inflammatory action in POI are macrophages. Because POI is caused by non-infectious cytotoxic inflammation, it is likely due to damage to cells in the gastrointestinal wall and abdominal wall, such as serosal lining cells and smooth muscle cells that have been surgically damaged. It was considered that ATP leaked extracellularly and activated P2X7R to cause local inflammation of the gastrointestinal tract and contribute to the development of POI. As described above, it was thought to be that the anti-inflammatory effect of P2X7R inhibitors on POI is due to a series of anti-inflammatory effects caused by inhibiting P2X7R expressed in both resident macrophages and infiltrating macrophages in the gastrointestinal muscularis. The conclusions are supported by reports that P2X7R stimulation increases IL-1β secretion in macrophages [39–41], IL-1β induces iNOS [42–44], and NO production from iNOS is responsible for the pathogenesis of POI [1, 3–4, 21–22, 45].

Based on the above, the mechanism of action of anti-inflammatory action in POI via P2X7R is considered to be due to the suppression of secretion of the inflammatory cytokine IL-1β in macrophages. However, such suppression of P2X7R-mediated secretion of the inflammatory cytokine IL-1β has also been reported in neutrophils [38]. Neutrophil infiltration also plays an important role in the pathogenesis of POI [1, 7, 23]. Therefore, although immunohistochemical staining of P2X7R and neutrophils was not

performed in this study, it is possible that P2X7R is also expressed in neutrophils and is involved in the pathogenesis of POI. P2X7R is also highly expressed in nerves [46], and P2X7R-mediated neural signals have also been reported to be responsible in other intestinal disease models [47]. From these reports, it was considered that P2X7R expressed in nerves may also be involved in the anti-inflammatory action and gastrointestinal motility promoting action in POI.

In order to investigate the above possibilities, it is necessary to investigate the expression of P2X7R other than macrophages and the point of action on the prevention and treatment of POI. There is no denying the possibility that other cells excepting macrophages and nerve plexus are involved in the pathogenesis of POI. However, as a result of immunohistochemical staining, P2X7R-expressing cells and CD68-positive gastrointestinal wall macrophages were co-stained almost completely, and P2X7R was not found in cells other than macrophages including intramural plexus. Therefore, it was considered that P2X7R on intestinal macrophages had important role in POI.

In summary, it was shown that A438079 significantly suppresses inflammation of the ileal muscularis and improves gastrointestinal motility by inhibiting P2X7R in the pathophysiology of POI. This indicates that P2X7R inhibitors may be effective in preventing POI. It was also shown that P2X7R-mediated signals play an important role in the pathogenesis of POI in gastrointestinal resident and infiltrating macrophages. Since P2X7R inhibitors suppress inflammation in POI and significantly improve gastrointestinal transport capacity, they are considered to be effective drug discovery targets for the treatment and prevention of gastrointestinal insufficiency.

Declarations

Compliance with ethical standards

All animal experiments were performed according to the Guide for Animal Use and Care published by the University of Tokyo and were approved by the Institutional Review Board of the University of Tokyo (approval code P16-187).

Consent for publication

All authors have given consent for publication.

Availability of data and materials

Not Applicable.

Competing Interests

No conflicts of interest, financial or otherwise, are declared by the authors.

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Author Contributions

M.H. and N.K. designed the experiments. T.Y. and K.H. mainly performed the research, and T.M. and N.K. helped the research. H.K., T.Y. and M.H. wrote and revised the manuscript. H.T., T.M., N.K. and M.H. discussed the research study.

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Figures

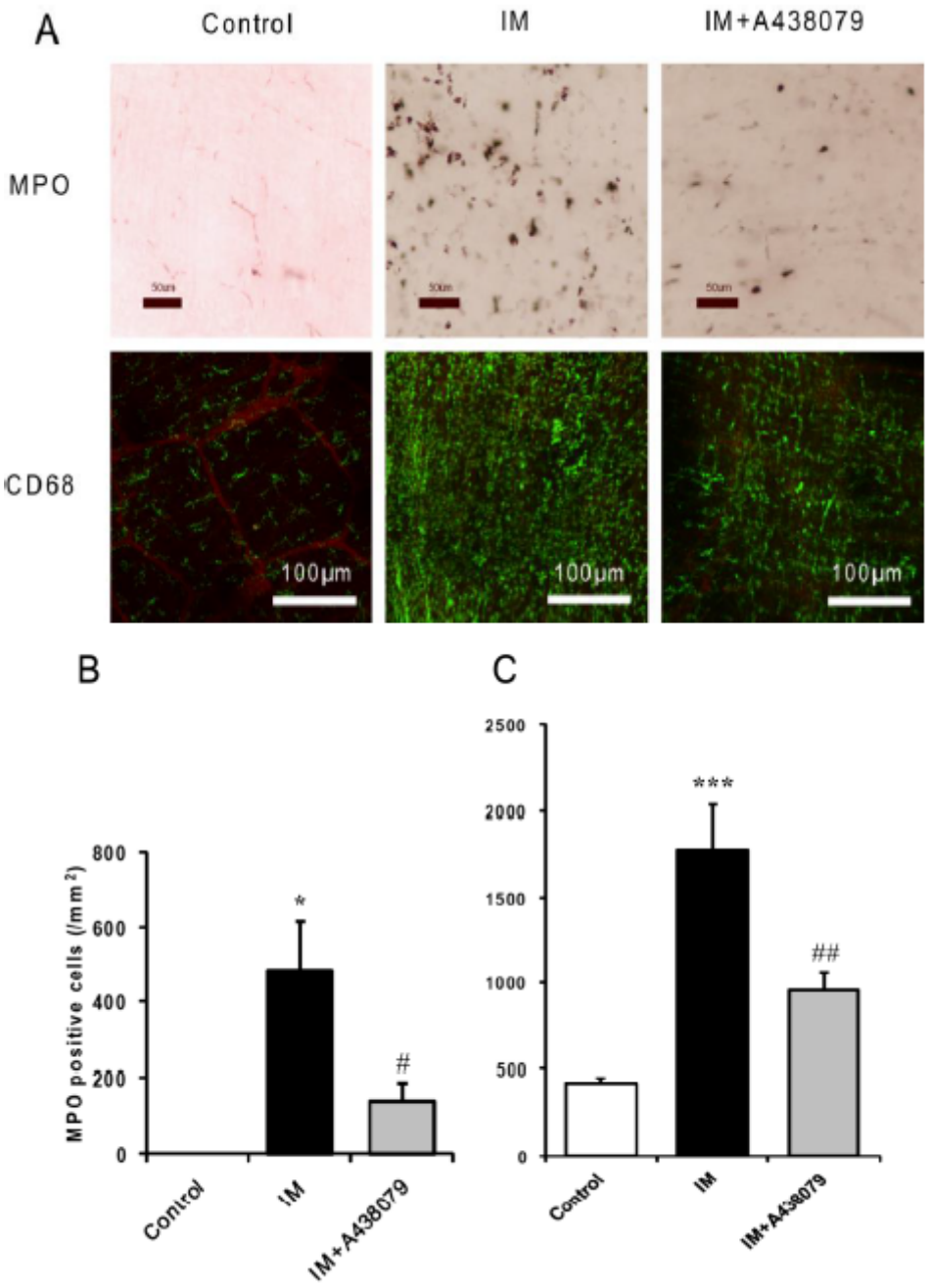

Figure 1

P2X7R blockage inhibited leukocytes infiltration in postoperative ileus model of WT mice. Effects of P2X7R blockage on leukocytes infiltration by intestinal manipulation (IM) in postoperative ileus model of WT mice. Myenteric nerve plexus inhibition via P2X7R blockage was performed by administration of A438079 (34 mg/kg, s.c.) as described in Materials and Methods. A: Immunohistochemical or histochemical staining of CD68-positive macrophages or MPO-stained neutrophils in WT mice intestine. Representative pictures from 4 independent experiments were shown. Bar indicated 100 □m. Red and green signals indicated PGP 9.5-positive myenteric neuron and CD68-positive macrophages, respectively. B and C: Quantified results of infiltrated macrophages (B) and neutrophils (C) cell number from A. ***,

significantly different from control at p<0.001; ## or ###, significantly different from IM at p<0.01 or 0.001, respectively (n=4 each). Each column shows the mean ± SEM.

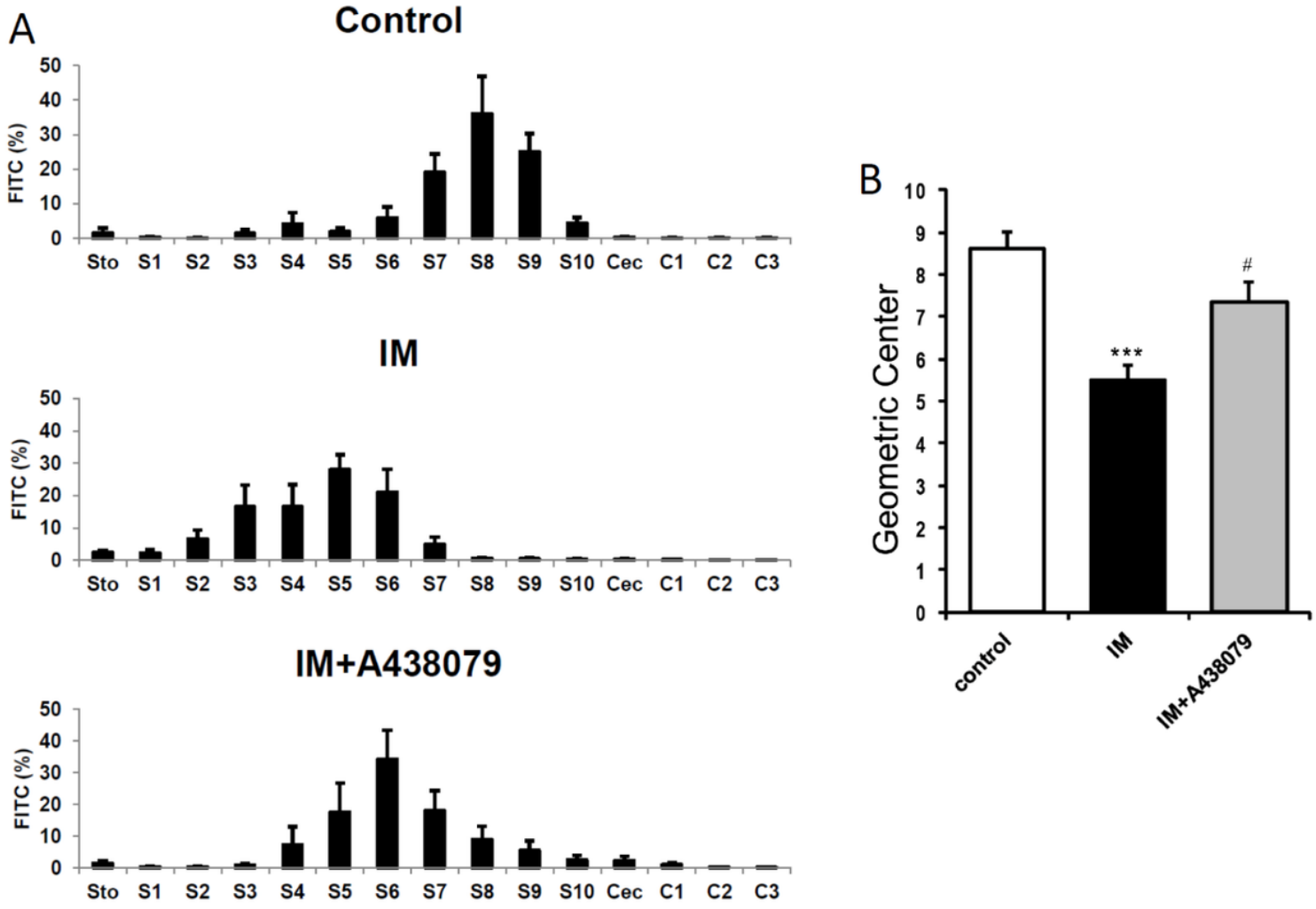

Figure 2

P2X7R inhibition ameliorates gastrointestinal transit in a mouse model of POI A: Data shown are means±SEM of ratio (%) of FITC content B: Geometric center calculated from A **P < 0.01; significantly different from control. ##P < 0.01; significantly different from POI. Data shown are means ± SEM from four independent experiments.

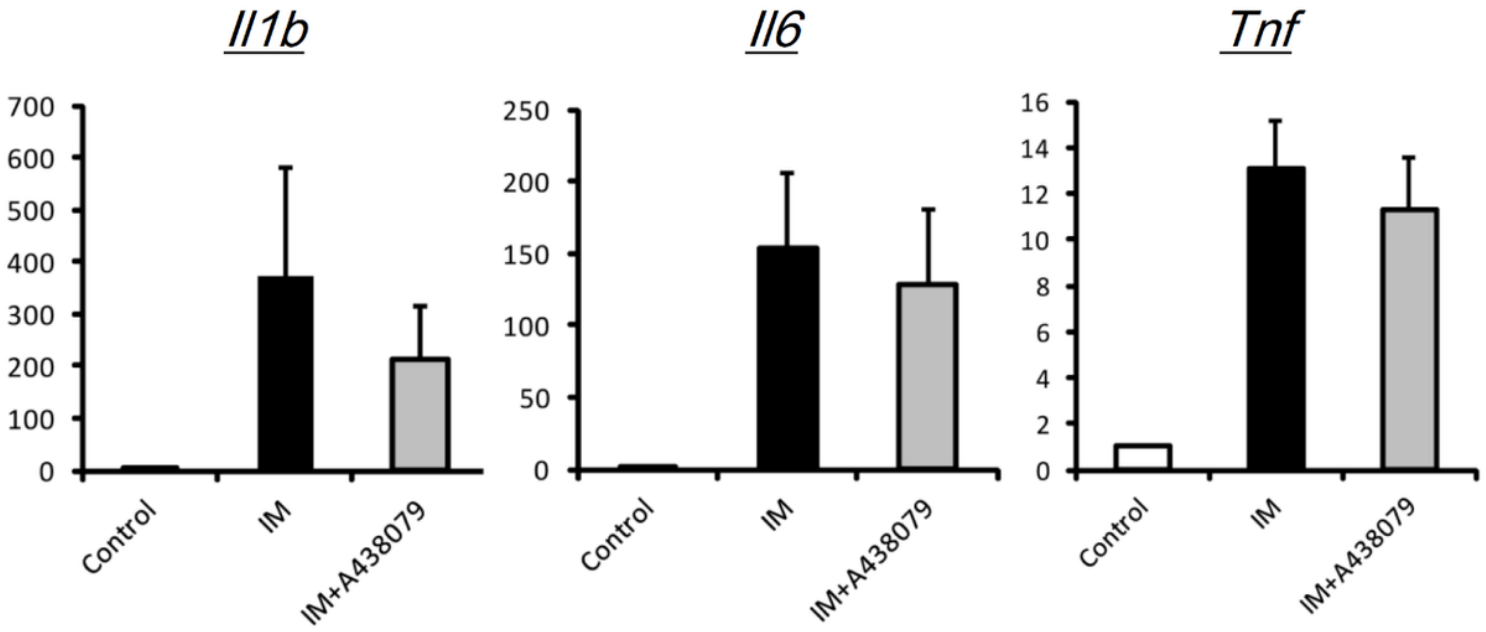

Figure 3

Effects of A438079 on pro-inflammatory cytokine expression in ileal muscle layer after IM mRNA expression levels of IL-1β (A), IL-6 (B) or TNF-α (C). Each column shows mean ± SEM (n = 7).

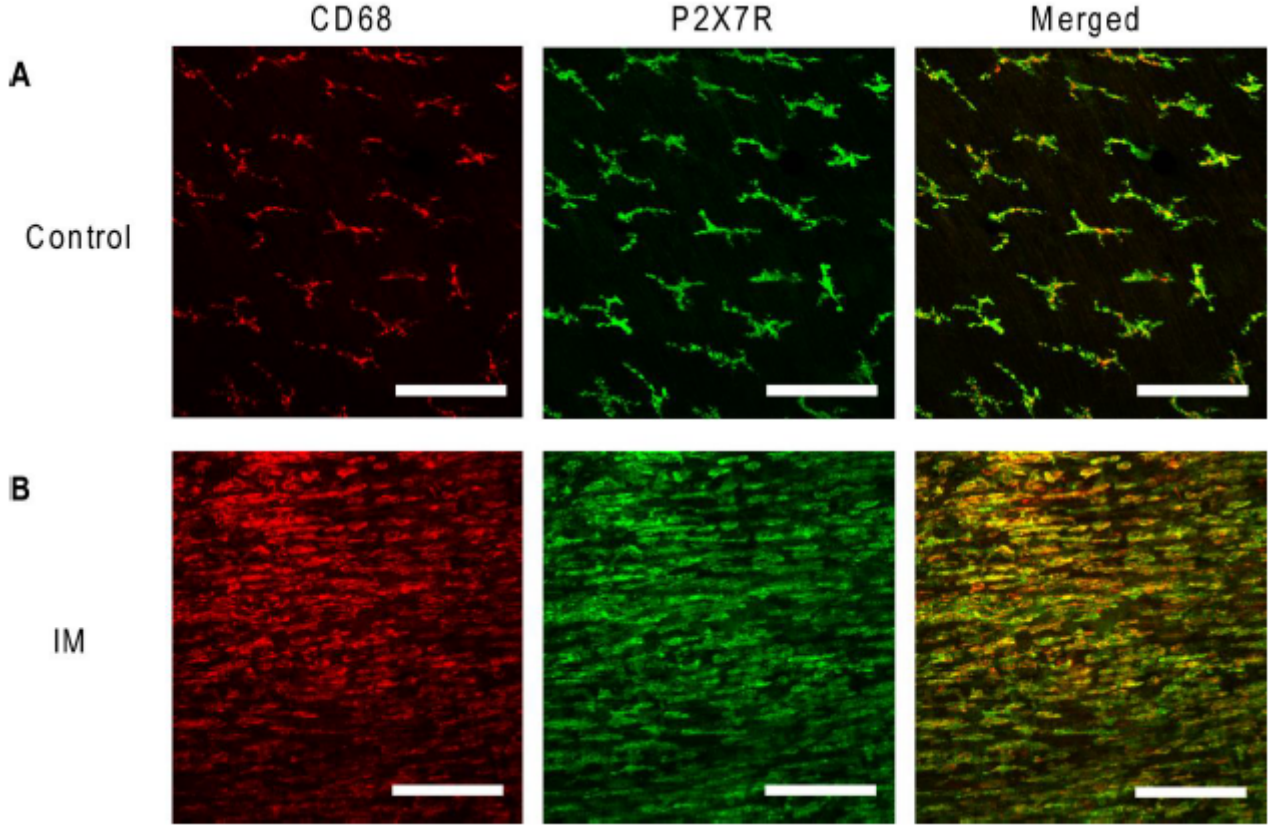

Figure 4

Expression of P2X7R on ileal muscle layer of control and IM-treated mice. Double-immunostaining of CD68 (red) and P2X7R (green) in ileal muscle layer of control and IM-treated mice. Scale bar, 100 μm. Typical results are shown out of 3 independent experiments.