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Shin Kashima[1)], Mikihiro Fujiya[1)], Hiroaki Konishi[1)], Nobuhiro Ueno[1)], Yuhei Inaba[1)], Kentaro Moriichi[1)], Hiroki Tanabe[1)], Katsuya Ikuta[1)], Takaaki Ohtake[1)], Yutaka Kohgo[1)]

1) Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical University

Correspondence

To whom correspondence should be addressed:

Mikihiro Fujiya, MD, Ph.D.

Division of Gastroenterology and Hematological Oncology, Department of Medicine, Asahikawa Medical College, Asahikawa, Japan

2-1 Midorigaoka-higashi, Asahikawa, Hokkaido 078-8510, Japan

Tel: +81-166-68-2462

Fax: +81-166-68-2469

e-mail: fjym@asahikawa-med.ac.jp

Running title

Polyphosphate improves colitis-associated fibrosis

Key words

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Author's contributions

S.K., M.F. and H.K. equally contributed to this study.

S.K., M.F. and H.K. provided major input into the conceptual development of the study, wrote the manuscript and supervised all investigations. Y.I., K.M. and H.T. performed the biochemical experiments. N.U. collected and analyzed the mouse samples and performed the isotope studies. K.I. performed the immunocytochemistry. T.O. and Y.K. helped to design the experiments, interpret the data and prepare/review the manuscript. All

authors read and approved the final manuscript.

Conflicts of Interest

There are none to declare.

Abstract

Inflammatory bowel disease frequently causes intestinal obstruction due to extensive fibrosis. This study investigated whether polyphosphate (poly P), an active molecule derived from *Lactobacillus brevis*, could improve the fibrosis in a model of chronic colitis.

In this study, DSS-induced chronic colitis models and TNBS-induced colitis models were used as models of fibrosis. To clarify the mechanism responsible for the observed effects, Caco-2/BBE and THP-1 cells were treated with LPS to induce inflammation. CCD-18 cells, a fibroblast cell line, were treated with TGF-β1 to induce fibrosis. The expression levels of fibrosis- and inflammation-associated molecules were evaluated by both a Western blotting analysis and RT-PCR.

The histological inflammation and fibrosis were significantly improved in the group administered poly P in both the DSS and TNBS colitis models. The levels of IL-1β and TNF-α were significantly decreased by poly P treatment. The expression levels of TGF- β1 and collagens in the colitis mice were decreased by poly P. The LPS-induced expressions of IL-1β and TGF-β1 in Caco-2/BBE cells and of TNF-α in THP-1 cells were reduced by poly P

treatment. Poly P did not affect the expression of collagens and CTGF in the CCD-18 cells.

In conclusion, poly P suppresses intestinal inflammation and fibrosis by down-regulating the expression of inflammation- and fibrosis-associated molecules in the intestinal epithelium. The administration of poly P is therefore a novel option to treat fibrosis due to chronic intestinal inflammation.

Introduction

Inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohn's disease (CD), are characterized by chronic intestinal inflammation whose etiology remains unclear. Many IBD patients frequently exhibit refractory ulcerations and develop severe intestinal fibrosis that eventually causes intestinal obstruction. Abdominal surgeries, such as intestinal resection and strictureplasty, are required to relieve the intestinal obstruction, because no conservative treatments for the obstruction have been established.

The development of intestinal fibrosis is mediated by the activation of cell signaling molecules, including transforming growth factor beta 1 (TGF-β1) and connective tissue growth factor (CTGF). Phosphorylated TGF-β1 produced by intestinal epithelial cells and macrophages facilitates the transfer of smad 2/3 and smad 4 from the cytoplasm to the nucleus and promotes the transcription of collagen mRNA in intestinal fibroblasts, thereby inducing the development of intestinal fibrosis[1-3]. Recent genome-wide studies have identified a TGF- β1 variant to be associated with stricturing CD and UC, suggesting the presence of a strong association

between intestinal fibrosis and abnormalities in TGF-β1[4, 5]. CTGF is associated with wound healing and fibrotic pathology, particularly the induction of sustained fibrosis under cooperation with TGF-β1. Increased expression of CTGF was reported to maintain the intestinal fibrosis, suggesting an important role for CTGF in the induction and maintenance of intestinal fibrosis[6].

The population of commensal bacteria in patients with digestive diseases, including acute enteritis and IBD, is quite different from that observed in healthy people[7]. The maintenance of commensal bacteria and the use of probiotic therapy are therefore thought to be important for the treatment of intestinal inflammation[8]. Indeed, it has been shown that several probiotics, such as *Lactobacillus*, *Bifidobacterium*, *E. coli Nissle* and *Clostridium*, ameliorate intestinal injury caused by acute inflammation, that is often observed in patients with antibiotic–induced colitis[9, 10], necrotizing colitis[11, 12] and IBD[13-18]. We recently isolated an effective molecule, competence and sporulation factor (CSF), from the conditioned media of the probiotic *Bacillus subtilis*, and demonstrated that this molecule enhanced the intestinal barrier function and improved the intestinal injury in an acute enteritis

model[19, 20]. We subsequently showed that the administration of heat-killed *Lactobacillus brevis* SBL88 (*L. brevis* SBL88) helps to maintain intestinal homeostasis and improves intestinal inflammation[21]. Furthermore, the conditioned media from *L. brevis* SBL88 was repeatedly separated by ammonium sulfate precipitation, DEAE anion exchange chromatography and gel filtration, and a fraction that could induce the expression of cytoprotective heat-shock protein (Hsp) 27 in Caco2/bbe cells was isolated. From the Hsp-inducible fraction, polyphosphate was identified as a *L. brevis* SBL88-derived effector that induced Hsp27 expression in the intestinal epithelia.

Thereafter, we chemically synthesized poly P using a poly P-synthesizing enzyme polyphosphate kinase, and found that the synthesized poly P could maintain the intestinal barrier functions via a mechanism involving the intestinal integrin ß1-p38 MAPK pathway[22]. Yan F et al. also identified two bioactive molecules, p40 and p75, produced by *Lactobacillus rhamnosus GG*[23] and reported the effects of p40 in protecting the intestinal epithelia from injury and inflammation in an oxazolone-induced colitis model[24]. Probiotics are therefore thought to be promising to improve intestinal injury

caused by intestinal inflammation by mediating bioactive molecules derived from probiotics. However, it remains unclear whether the intestinal fibrosis induced following chronic inflammation can be improved by treatment with these active molecules. The present study proposes that *L. brevis* SBL88-derived poly P improves intestinal injury as well as fibrosis in a chronic enteritis model.

Materials and Methods

Polyphosphate synthesis

The mixture of 200 mM PEP (2M Tris-HCl pH9.4), 30 mM phosphate buffer (pH 6.0), 30 mM ATP (1M Tris-HCl pH 8.0), 30 mM MgCl2, 600 mM acetic acid buffer (pH 6.0), 0.25mL polyphosphate kinase and 100 unit/mL pyruvate kinase was incubated at 37℃ for 16 to 18 hr. To purify the poly P, the mixture was incubated with the same volume of 2 M acetic acid buffer at 4℃ for 2 hr, and then this was centrifuged at 3000 rpm for 5 min. The precipitate was dissolved in distilled water and incubated with the same volume of 2 M acetic acid buffer at 4℃ for 2 hr. After another round of centrifugation, the precipitate was dissolved in distilled water, and the low

molecular weight components, including ATP and short chain poly P, were removed from the solution by dialysis using a tube equipped with a 3-kDa MWCO membrane (Thermo Fisher Scientific ltd., MA).

Mice

The experiments were approved by the Institutional Animal Care and Use Committee of Asahikawa Medical University. C57Bl/6 mice and BALB/C mice were purchased from Sankyo Labo Service (Tokyo, Japan).

Dextran sodium sulfate (DSS)-induced colitis

DSS with a molecular weight of 25,000 (Tokyo Chemical Industry, Japan) and 36,000 (MP biomedicals, Santa Ana, CA) were dissolved in tap water to obtain a 3% DSS solution. C57Bl/6 mice (18–25 g, 6 weeks old, male) were allowed free access to 3% DSS solution as drinking water for five days, and then the water was replaced with tap water from days 6 to 35 in order to induce the development of chronic DSS-induced colitis[25, 26]. It has also been reported that mice allowed free access to 3% DSS solution as drinking water developed severe inflammatory cell infiltration from day 12 to 26 after the

treatment, and started showing the accumulation of collagen fibers in the intestinal tissues from day 26 to 33[25]. We therefore selected day 25 as the time for the therapeutic intervention, and day 35 as the time to assess the effects of poly P on intestinal fibrosis and inflammation. From day 25, 10 μg of poly P or PBS was trans-anally administered every day until day 35, when the mice were sacrificed. Two 10-mm pieces of the middle parts of the colon were obtained for protein and RNA extraction, and a 5-mm piece was fixed in 10% buffered formalin. Three 4 μm-thick sections were obtained from each sample and used for the subsequent studies.

2.4.6-trinitrobenzene sulphonic acid (TNBS)-induced colitis

TNBS colitis was induced in BALB/C mice (18–25 g, six weeks old, male) by using a modification of the method described in detail in a previous study[27]. In brief, 4 mg of the hapten reagent, TNBS (Sigma Chemical Co, St. Louis, MO), in 50% ethanol (160 μl) was slowly administered into the lumen of the colon (about 30 mm from the anal verge) using a catheter fitted onto a 1-ml syringe under diethyl ether light anesthesia on day 0. The total injection volume was 160 μl for each mouse, and the control mice were administered

50% ethanol in PBS. Mice were then kept in an inverted position for 30 seconds after the intrarectal administration. From day 1, 20 μg of poly P or PBS was trans-anally administered every day until day 7, when the mice were sacrificed. Two 10-mm pieces of colon were obtained for protein and RNA extraction, and a 5-mm piece was fixed in 10% buffered formalin and sectioned at 4 μm for the subsequent studies.

Cell culture

Caco-2/bbe colonic epithelial cells, THP-1 cells as human macrophage like cell line, CCD-18 cells as human colonic myofibroblast cell lines were purchased from ATCC. Caco-2/BBE cells were grown in high-glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2 mM L-glutamine , 50 U/ml penicillin, 50 μg/ml streptomycin and 10 μg/ml transferrin (all from Invitrogen/GIBCO, Grand Island, NY) in a humidified atmosphere of 5% CO_2. The THP-1 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10 μg/ml transferrin. To obtain the monocyte-derived macrophage cells, THP-1 cells were differentiated by treatment with 200 nM

phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) for three days[28]. CCD-18 cells were grown in Minimum Essential Medium (MEM) supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10 μg/ml transferrin. The cells were plated on six-well plates at a density of 10^5 cells/cm^2. For the induction of an inflammatory condition, the Caco-2/bbe cells and THP-1 cells were treated with 1 μg/mL lipopolysaccharide (LPS) from *Escherichia coli* (Wako Pure Chemical Industries, Ltd). CCD-18 cells were treated with human TGF-β1 (hLatent TGF-β1; cell signaling technology, Beverly, MA.) for the induction of fibrosis-associated molecules, including CTGF and collagens.

Western blotting

The mouse colonic tissue samples were rinsed with ice-cold saline, and the epithelium was gently sheared off with glass slides. The samples were washed with PBS, and total protein was extracted using a Mammalian Cell Extraction Kit (BioVision, Mountain View, CA). Five to 30 μg of protein in each sample was resolved using SDS-PAGE (12.5%) and immediately transferred to a nitrocellulose membrane using a transfer buffer (25 mM Tris, pH 8.8, 192

mM glycine with 20% (vol/vol) methanol). The nitrocellulose membranes were incubated in PBS with 0.05% (vol/vol) Tween 20 (T-PBS) containing 1% (wt/vol) bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) for one hour at room temperature to block any nonspecific binding. The blots were incubated overnight at 4℃ with anti-CTGF polyclonal antibody (Abcam), anti-TGF-β1 monoclonal antibody (Abcam), anti-SMAD4 monoclonal antibody (Abcam) and anti-Collagen IV polyclonal antibody (Abcam) as the primary antibodies. The blots were washed three times in T-PBS at room temperature, incubated for 60 minutes in species-appropriate horseradish peroxidase-conjugated secondary antibodies (R&D systems, Minneapolis, MN) in T-PBS, washed three times in T-PBS, then developed using either the Super-Signal West Pico or femto-enhanced chemiluminescence system (Thermo Science) according to the manufacturer's protocols. The average protein expression was normalized to the actin expression (BD Transduction Laboratories, Lexington, KY).

Real-time PCR

Total RNA was extracted using an RNeasy mini kit (Qiagen, Tokyo,

Japan) according to the manufacturer's instructions. The mRNAs of TNF-α, IL-1β, IFN-γ, IL-4, IL-6, IL-10, IL-17A, TGF-β1, CTGF and collagen types 1 and 4 were reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The gene expression was measured with real-time PCR using specific primers of TNF-α, IL-1β, IFN-γ, IL-4, IL-6, IL-10, IL-17A, TGF-β1, CTGF and collagen types 1 and 4 (Applied Biosystems Foster City, CA). The average mRNA expression was normalized to the 18S rRNA expression.

Histological assessment

The histology was scored as follows in the DSS chronic colitis model[29]: Epithelium (E): 0, normal morphology; 1, loss of some goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts and 4, loss of crypts in large areas. Infiltration (I): 0, no infiltrate; 1, infiltrates around crypt bases; 2, infiltration reaching to the muscularis mucosae; 3, extensive infiltration reaching the muscularis mucosae and thickening of the mucosa with abundant edema; 4, infiltration of the submucosa. The total histological score represented the sum of the epithelium and infiltration scores (total score = E

+ I). In the TNBS colitis model, the histological activity was assessed according to the following scoring system[30]: Extent: 0, none; 1, focal; 2, limited to one segment; 3, involving more than one segment. Inflammation: 0, none; 1, mild; 2, moderate; 3, severe. Damage: 0, none; 1, mild (superficial); 2, moderate (involving the muscularis mucosae); 3, severe (transmural). Regeneration: 0, complete re-epithelialization; 1, broad, multifocal re-epithelialization; 2, focal migration and mitotic figures; 3, none. A maximum score of 12 indicated severe colitis, with marked inflammation, ulceration and necrosis, without evidence of regeneration. The grade of intestinal inflammation was assessed in representative sections of the colon, which were selected from three sections obtained from each sample, based on the multifocal and variable severity of the intestinal lesions. The grade of intestinal fibrosis was evaluated using Masson's trichrome staining. Areas stained with Masson's trichrome in one field (x 200) were traced and measured with NIH imaging (Image J).

Immunohistochemistry

After deparaffinization and rehydration, the endogenous peroxidase activity

was blocked with 0.6% H_2O_2 in methanol for 25 min. The slides were then treated by means of the antigen-retrieval technique based on microwave oven heating in 10 mM citrate buffer (pH 6.0) for 20 min. After blocking any nonspecific reaction with 10% goat serum in PBS, the sections were then incubated with anti-collagen IV monoclonal antibody (Abcam) at 4℃ overnight. This step was followed by sequential incubation with Alexa 594-conjugated secondary antibody (Invitrogen-Molecular Probes) diluted at 1:100 for 1 hr at room temperature. The nuclei were counterstained with DAPI and 4',6'-diamidino-2-phenylindole dihydrochloride (Lonza). Images were obtained with a confocal microscope. Parallel sections were incubated in PBS as a primary antibody for the negative control. The fluorescence intensity of the stained areas was measured using an image analytical software program.

Statistical analysis

All values were expressed as the standard error of the mean (SEM). The statistical evaluation of the results was performed by the one-way analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD).

Results

Poly P improves intestinal injury in mice with DSS-induced chronic colitis

On day 35, the weights of the mice were not significantly different among the groups **(Figure 1A)**. Because it has been reported that the shortening of the colon is caused by a chronic inflammation in the DSS model due to both the inflammation and fibrosis[26], the colon length was assessed as a marker of the intestinal fibrosis and inflammation. The length of the colon was significantly longer in the poly P-administered mice than in the PBS-administered mice ($p<0.01$) **(Figure 1B)**. The grade of inflammation in the mice administered poly P was significantly lower than that in the mice that received PBS mice ($p<0.01$) **(Figure 2)**, suggesting that poly P improves intestinal damage in mice with DSS-induced chronic colitis. Poly P had a significant effect on the inflammation in the middle part of the colon, but not in the distal colon, due to the immediate leakage of poly P after the transanal administration. These results were reproduced when using a molecular weight of 36,000 DSS for the development of intestinal inflammation **(Supplemental Figure 1)**.

Poly P decreases intestinal fibrosis in mice with DSS-induced chronic colitis

The amount of intestinal fibrosis evaluated by Masson's trichrome staining was significantly increased in the colons of the mice with DSS-induced chronic colitis. In contrast, the submucosal fibrosis observed in the colons of the mice administered poly P was extremely mild, similar to that noted in the control mice **(Figure 3A-C)**. The areas stained by Masson's trichrome in the poly P-administered mice were significantly smaller than those in the mice that received PBS ($p<0.01$) **(Figure 3D)**. Immunoreactivity for collagen IV was significantly increased in the mucosal and submucosal layers of the colon in the mice with DSS-induced chronic colitis, while that in the mice treated with poly P was similar to that noted in the control mice **(Figure 3E-G)**. The intensity of fluorescence in the colons of the poly P-administered mice was significantly lower than that observed in the mice administered PBS mice ($p<0.001$) **(Figure 3H)**. Western blotting revealed that the expression of collagen IV was increased in the colons of the mice with DSS-induced chronic colitis ($p<0.001$) and recovered in the mice administered poly P ($p<0.01$) **(Figure 3I)**. This suggests that the

administration of poly P during the chronic phase of inflammation (from 25 to 35 days) improves intestinal fibrosis.

Poly P suppresses the increased expression of inflammation-associated cytokines in the colons of mice with DSS-induced chronic colitis

The mRNA expression levels of pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ were significantly increased in the colons of the mice with DSS-induced chronic colitis ($p<0.01$ or 0.05). The administration of poly P suppressed the expression of these mRNAs ($p<0.01$ or 0.05). The mRNA expression levels of IL-10 and IL-17A were significantly increased in the colons of the mice with DSS-induced chronic colitis ($p<0.05$), while the increased these mRNA expression levels were not suppressed by the administration of poly P **(Figure 4)**.

Poly P inhibits the upregulation of TGF-β1 and CTGF in DSS-induced chronic colitis

In the colons of the mice with DSS-induced chronic colitis, a Western blotting analysis revealed that the expressions of TGF-β1, smad4 and CTGF were

increased ($p<0.05$ or 0.001). TGF-β1, smad4 and CTGF are key molecules involved in the development and persistence of intestinal fibrosis caused by inflammation. This suggests that our DSS colitis model is applicable to investigate the pathogenesis of the fibrotic changes in Crohn's disease patients. Poly P suppressed the increased expressions of TGF-β1, smad4 and CTGF ($p<0.05$ or 0.01) in this model. As CTGF plays a role in maintaining fibrosis,[6] and since TGF-β1 contributes to both the initial development and the persistence of fibrosis, poly P is considered to act by inhibiting both the development and persistence of fibrosis **(Figure 5)**.

Poly P improves the intestinal injury and fibrosis in mice with TNBS-induced colitis

To confirm the anti-inflammatory and fibrotic effects of poly P, the TNBS-induced colitis model was selected, because this model is well known to develop severe inflammation and fibrosis in the colon. The grade of inflammation in the mice administered poly P was significantly lower than that in the mice administered PBS ($p<0.01$) **(Figures 6A-D)**. The immunohistochemical reactivity for the anti-collagen IV antibody was

increased in the colons of mice with TNBS-induced colitis compared to the control mice, and that in the mice treated with poly P was repressed to a similar level as was observed in the control mice. The intensities of fluorescence in the colons of the poly P-administered mice were significantly lower than those observed in the PBS-administered mice ($p<0.05$) **(Figures 6E-H)**. This suggests that the administration of poly P improves the intestinal inflammation, as well as the fibrosis induced by TNBS treatment.

Poly P inhibits the upregulation of inflammation- and fibrosis-associated mediators in the colons of mice with TNBS-induced colitis

The mRNA expression levels of pro-inflammatory cytokines IL-1β and TNF-α were significantly increased in the colons of the mice with TNBS-induced colitis ($p<0.01$ or 0.05). The administration of poly P suppressed the expression of these mRNAs ($p<0.05$). The mRNA expression of TGF-β and collagen type 1 were significantly increased in the colons of the mice with TNBS-induced colitis ($p<0.05$), while the mRNA expression of CTGF was not changed among the groups **(Figure 7)**.

Poly P suppresses the increased expression of inflammatory cytokines in epithelial cells and macrophages

To assess the anti-inflammatory effects of poly P on each component of intestinal tissue, Caco-2/bbe and THP-1 cells were selected as representative epithelial cells and macrophages, respectively. To induce inflammation, 1 μg/mL LPS was administrated to the Caco-2/bbe cells. The expression of TNF-α and IL-1β mRNA in the cells treated with LPS was significantly increased in comparison to that in the cells without the treatment ($p<0.05$ or 0.001). Treatment with 20 μg/mL of poly P significantly decreased the expression of IL-1β in the cells ($p<0.001$), but did not significantly affect the TNF-α expression **(Figure 8A)**.

The THP-1 cells pre-treated with PMA were stimulated with 1 μg/mL LPS. The mRNA expression levels of IL-1β, TNF-α and IL-10 were increased in the LPS-treated THP-1 cells ($p<0.05$ or 0.001). The expression of TNF-α was significantly decreased by the administration of 20 μg/mL poly P in the cells ($p<0.001$) **(Figure 8B)**.

Poly P suppresses the expression of TGF-β1 in epithelial cells.

To assess the anti-fibrotic effects of poly P on each component of intestinal tissue, Caco-2/bbe, THP-1 and CCD-18 cells were used in the experiments. The Caco-2/bbe and THP-1 cells were treated with 1 μg/ml of LPS to induce inflammation. The mRNA expression of TGF-β1 was induced by LPS, and the induction was reduced by treatment of the Caco-2/bbe cells with poly P ($p<0.01$) **(Figure 9A)**, but this did not occur in THP-1 cells **(Figure 9B)**. In the CCD18 cells treated with 100 ng/mL of TGF-β1, the mRNA expression levels of CTGF and collagens type 1 and 4 were increased ($p<0.001$), and poly P did not change the mRNA expression levels of these molecules **(Figure 9C)**. These data suggest that poly P downregulates the release of TGF-β1 from epithelial cells, but it cannot change the release of TGF-β1 from macrophages and fibrosis-associated molecules from intestinal fibroblasts.

Discussion

The present study demonstrated for the first time that probiotic Lactobacillus brevis-derived poly P relieves intestinal inflammation as well as intestinal fibrosis in a mouse colitis model. While some strains of *Bifidobacterium, Lactobacillus, Streptococcus, Escherichia coli* Nissle and

VSL#3 (a mixture of eight probiotic bacteria) are known to exhibit beneficial effects in the treatment of intestinal inflammation, including IBDs [11, 12, 13, 15-18, 31-33], the effects of probiotics, as well as probiotic-derived molecules, in improving intestinal fibrosis have not been fully elucidated. We previously identified poly P as an effective molecule derived from Lactobacillus brevis and showed the effect of poly P on the improvement of intestinal barrier function. The present study expanded our previous achievement and demonstrated the effect of poly P on the improvement of the intestinal inflammation and fibrosis. The results of the present study may facilitate the development of a novel therapeutic strategy targeting intestinal fibrosis by using a probiotic-derived bioactive, molecule poly P.

The present study also investigated the mechanisms underlying the effects of poly P on improving intestinal fibrosis. TGF-β1 is known to promote the transcription of fibrosis-associated molecules, such as collagens[3], and leads to the development of intestinal fibrosis. The serum levels of TGF-β1 and its mRNA expression level[31] are known to increase in IBD patients. A polymorphism of the TGF-β1 gene was found to be associated with stricturing CD[4] and UC[5]. Therefore, this study examined the expression

of TGF-β1, and demonstrated that poly P treatment downregulated the expression of TGF-β1 in both the DSS- or TNBS-induced models of colitis. The expression of CTGF, which is known to play a pivotal role in the persistence of fibrosis[6], was also decreased by poly P treatment in the DSS-induced colitis model. These results indicate that poly P inhibits both the development and persistence of intestinal fibrosis through the downregulation of TGF-β1, collagens and CTGF. Therefore, poly P, which downregulates the TGFβ1-smad pathway and the expression of collagens and CTGF, is a potential agent that could be useful for the treatment of intestinal fibrosis due to the chronic inflammation in patients with IBDs.

Although the beneficial effects of some probiotics as well as probiotic-derived molecules in improving intestinal injury have been demonstrated in acute enteritis models[19, 20, 22, 24, 34-38], the effects of probiotic-produced bioactive molecules on chronic enteritis remain unclear. The present study revealed that *Lactobacillus brevis*-derived poly P improves the shortening of and the grade of the histological severity in the colons of enteritis model mice. The expression levels of pro-inflammatory cytokines, including IL-1β, TNF-α and IFN-γ were dramatically decreased in

the mice administered poly P. Poly P did not decrease the expression of the anti-inflammatory cytokine, IL-10, suggesting that the administration of poly P contributes to relieving the intestinal inflammation in mice with chronic enteritis through the downregulation of pro-inflammatory mediators. While some biological drugs targeting pro-inflammatory cytokines are useful for inducing remission and for maintenance in Crohn's disease patients, a loss of response may occur in 20-50% of such patients[39-41]. The administration of poly P may help to maintain the remission in such cases with a loss of response to biological drugs.

In order to identify the target cell(s) of poly P that lead to an improvement in both intestinal fibrosis and inflammation, we investigated the function of poly P in each type of cell composing the intestinal tissue by using representative cell lines. The overexpression of TGF-β1 mRNA in the intestinal epithelia-derived cells, Caco-2/bbe cells, by the stimulation with LPS was significantly decreased when they were treated with poly P, while the mRNA expression of TGF-β1 in THP-1 cells, which were derived from macrophages, was not changed by the treatment with poly P. The overexpression of collagens by the stimulation with TGF-β1 was also

unchanged in CCD-18 cells after treatment with poly P, suggesting that the intestinal epithelia is the target for the inhibition of intestinal fibrosis by poly P. Conversely, poly P inhibited the mRNA expression of IL-1β in Caco-2/bbe cells and TNF-α in THP-1 cells, which were induced by the treatment of LPS. This suggests that poly P targets both the intestinal epithelia and macrophages, but in a different manner.

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Figure legends

Figure 1. Poly P prevents intestinal shortening in mice with DSS-induced chronic colitis

On day 35 after the initial DSS treatment, the mice were sacrificed and the length and histological findings of the colon were assessed. The weights of the mice were not significantly different between the PBS-administered mice and the poly P-administered mice **(A)**. The colons of the mice were photographed. The left, middle and right images are of the control, DSS + PBS and DSS + poly P groups, respectively. The length of the colon was significantly longer in the poly P-administered mice than in the DSS+PBS mice **(B)** (control: n=11, DSS+PBS: n=16, DSS+poly P: n=18). **p<0.01 ***p<0.001

Figure 2. Poly P relieves the severity of intestinal inflammation in mice with

DSS-induced chronic colitis

Photographs of representative histological findings are shown. The left (A), middle (B) and right (C) images are of the control, DSS + PBS and DSS + poly P groups, respectively. A loss of crypts in large areas and the infiltration of the L. submucosa were observed in middle image (B). No loss of crypts and less infiltration were detected in the right image (C). The grade of inflammation of the colon in the mice treated with poly P was significantly lower than that observed in the PBS-treated mice (D) (control: n=11, DSS+PBS: n=16, DSS+poly P: n=18). **p<0.01 *** p<0.001

Figure 3. Poly P decreases the amount of intestinal fibrosis in mice with DSS-induced chronic colitis

Photographs of the histological findings of Masson's trichrome staining are shown. The left (A), middle (B) and right (C) images are of the control, DSS + PBS and DSS + poly P groups, respectively. The areas stained with Masson's trichrome were calculated using an image analytical software program (D). The stained areas of the colon in the DSS + poly P group were significantly smaller than those observed in the DSS + PBS group (control: n=11,

DSS+PBS: n=16, DSS+poly P: n=18). Immunoreactivity for anti-collagen IV antibodies is indicated by the magenta areas in the photographs. The left **(E)**, middle **(F)** and right **(G)** images are of the control, DSS + PBS and DSS + poly P groups, respectively. The intensity of fluorescence in the colon was measured using an image analytical software program **(H)**. The expression of collagen IV was significantly increased in the mucosal and submucosal layers of the colon in the mice with DSS-induced chronic colitis, while that observed in the mice treated with poly P was almost similar to that noted in the control mice. Western blotting revealed that the expression of collagen IV was increased in the colons of the mice with DSS-induced chronic colitis. Poly P suppressed the expression of collagen IV to a similar level as that observed in the control mice **(I) (control: n=5, DSS+PBS: n=7, DSS+poly P: n=8)**. *p<0.05 **p<0.01 ***p<0.001

Figure 4. Poly P suppresses the increased upregulation of proinflammatory mediators in mice with DSS-induced chronic colitis

The mRNA expression levels of inflammation-associated cytokines, including IL-1β, IL-4, TNF-α, IFN-γ, IL-10 and IL-17A, were examined with real-time

PCR. The expression levels of IL-1β, TNF-α, IFN-γ and IL-17A were significantly increased in the colons of the mice with DSS-induced chronic colitis. The administration of poly P suppressed the excess expression of the IL-1β, TNF-α and IFN-γ, but not IL-17A, mRNA **(control: n=5, DSS+PBS: n=7, DSS+poly P: n=8).** *p<0.05 **p<0.01 ***p<0.001

Figure 5. Poly P suppresses the increased upregulation of fibrosis-associated molecules in mice with DSS-induced chronic colitis

The expressions of TGF-β1, smad4 and CTGF were examined using Western blotting with a densitometric analysis. The expressions of TGF-β1, smad4 and CTGF were increased in the colons of the mice with DSS-induced chronic colitis in comparison to those observed in the control mice. Poly P suppressed the excess expressions of TGF-β1, smad4 and CTGF **(control: n=5, DSS+PBS: n=7, DSS+poly P: n=8)**. * p<0.05 ** p<0.01 ***p<0.001

Figure 6. Poly P reduces the severity of intestinal inflammation in mice with TNBS-induced colitis

Photographs of representative histological findings are shown. The left

(A), middle **(B)** and right **(C)** images are of the control, TNBS + PBS and TNBS + poly P groups, respectively. The grade of inflammation of the colon in the mice treated with poly P was significantly lower than that observed in the PBS-treated mice **(D)**. Immunoreactivity for anti-collagen IV antibodies is indicated by the magenta areas in the photographs. The left (E), middle (F) and right (G) images are of the control, TNBS + PBS and TNBS + poly P groups, respectively. The intensity of fluorescence in the colon was measured using an image analytical software program (H). Immunohistochemical reactions for anti-collagen IV antibody was increased in the colon of mice with TNBS-induced colitis than those in control mice, and those in the mice treated with poly P was repressed to the similar levels of the control mice. The intensities of fluorescence in the colons of the poly P-administered mice was significantly lower than those observed in the PBS-administered mice **(control: n=10, TNBS+PBS: n=6, TNBS+poly P: n=7)**. **p<0.01 *** p<0.001

Figure 7. Poly P suppresses the upregulation of inflammation-associated cytokines, fibrosis-associated molecules and collagen in mice with TNBS-induced colitis

The mRNA expression levels of inflammation-associated cytokines (IL-1β, IL-4, IL-6, TNF-α, IFN-γ, IL-10 and IL-17A), fibrosis-associated molecules (TGF-β and CTGF) and collagen were examined by real-time PCR.

(A) The mRNA expression levels of pro-inflammatory cytokines IL-1β and TNF-α were significantly increased in the colons of the mice with TNBS-induced colitis ($p<0.01$ or 0.05). The administration of poly P suppressed the expression of these mRNAs ($p<0.05$). The expression of IL-4, IL-6, IFN-γ, IL-10 and IL-17A mRNA was not detected in this study.

(B) The mRNA expression levels of TGF-β and collagen type 1 were significantly increased in the colons of the mice with TNBS-induced colitis ($p<0.05$). The administration of poly P suppressed the expression of these mRNAs ($p<0.05$). The expression of CTGF was not increased in this TNBS-induced colitis model **(control: n=10, TNBS+PBS: n=6, TNBS+poly P: n=7)**. * $p<0.05$ ** $p<0.01$

Figure 8. The expression of proinflammatory mediators *in vitro*

The mRNA expression levels of inflammation-associated cytokines were examined by real-time PCR. The expression levels of IL-1β and TNFα were

significantly increased in Caco-2/bbe cells stimulated with LPS. The administration of poly P suppressed the expression of IL-1β (A). The expression levels of IL-1β, IL-6, IL-10 and TNF-α all significantly increased in THP-1 cells stimulated with LPS, and the expression of TNF-α was suppressed by the administration of poly P (B). (n=5) *p<0.05, **p<0.01 ***p<0.001

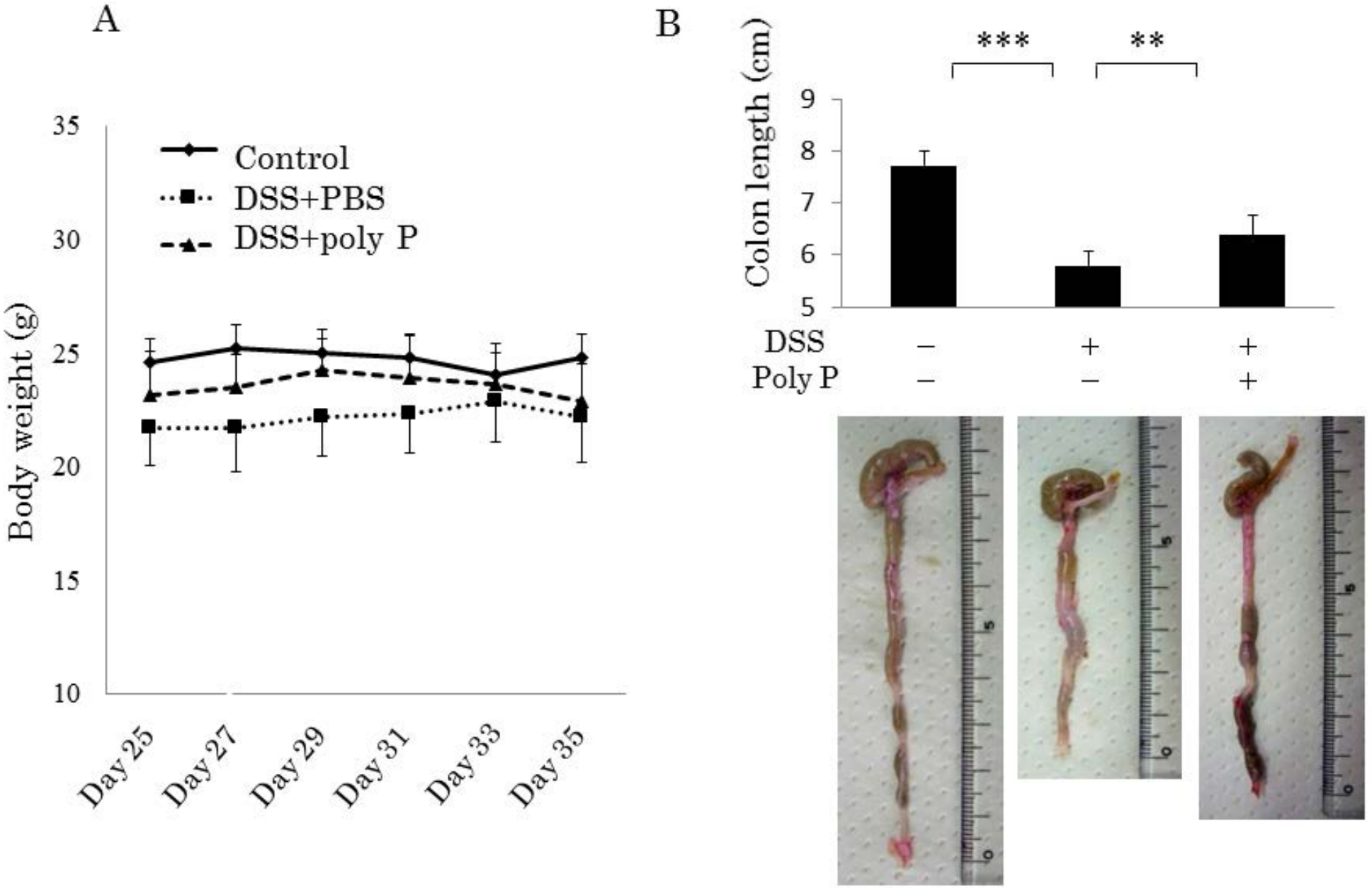
Figure 9. The expression of fibrosis-associated molecules and collagen *in vitro*

The mRNA expression levels of fibrosis-associated molecules (TGF-β1 and CTGF) and collagens were examined by real-time PCR. The expression of TGF-β1 significantly increased in Caco-2/bbe cells stimulated with LPS. The administration of poly P suppressed the expression of TGF-β1 (A). The expression of TGF-β1 was not increased in THP-1 cells stimulated with LPS, and the expression of TGF-β1 in these cells was not suppressed by the administration of poly P (B). The mRNA expression levels of CTGF and collagens type 1 and 4 were evaluated after treatment of the CCD-18 cells with TGF-β1, but the expression levels of these molecules were not

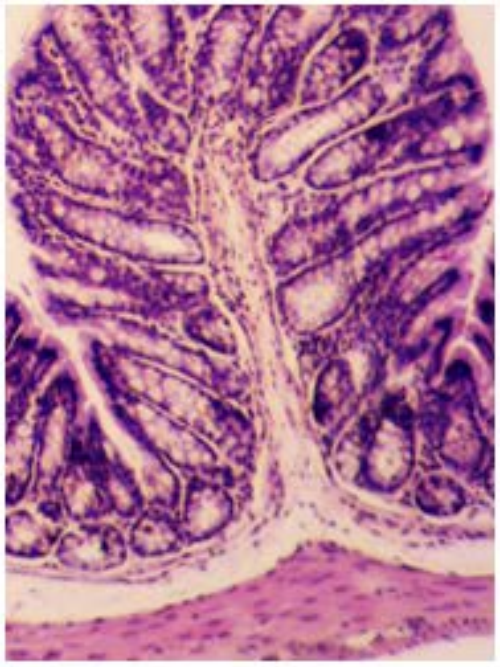
suppressed by the administration of poly P (C). (n=5) *p<0.05, **p<0.01 ***p<0.001

Supplemental Figure 1. The effects of poly P on the improvement of intestinal inflammation and fibrosis in each part of the colon, induced by DSS at a molecular weight of 25,000 or 36,000

The severities of intestinal inflammation and fibrosis in the proximal, middle and distal colon of the mice treated with a molecular weight of 25,000 (A) (n=9) or 36,000 (B) (n=6) of DSS were assessed. Inflammation and fibrosis were observed in the middle and distal colon in the mice treated with DSS, regardless of the molecular weight. Poly P improved the inflammation and fibrosis in the middle colon, but not in the distal colon, of the mice treated with a molecular weight of 25,000 (C) (control: n=6, DSS+PBS: n=9, DSS+poly P: n=10) or 36,000 (D) (control: n=5, DSS+PBS: n=6, DSS+poly P: n=6) of DSS. * p<0.05, ** p<0.01, ***p<0.001


A
Body weight (g)
35
30
25
20
15
10
Control
DSS+PBS
DSS+poly P
Day 25
Day 27
Day 29
Day 31
Day 33
Day 35
B
Colon length (cm)
9
8
7
6
5
***
**
DSS
Poly P
–
+
+
–
–
+

A



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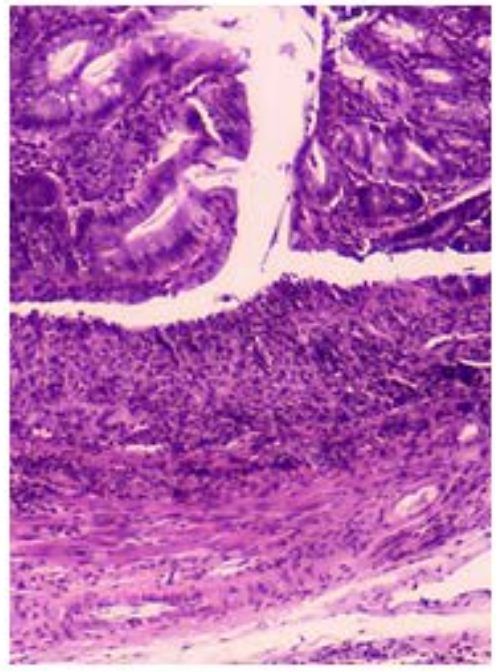
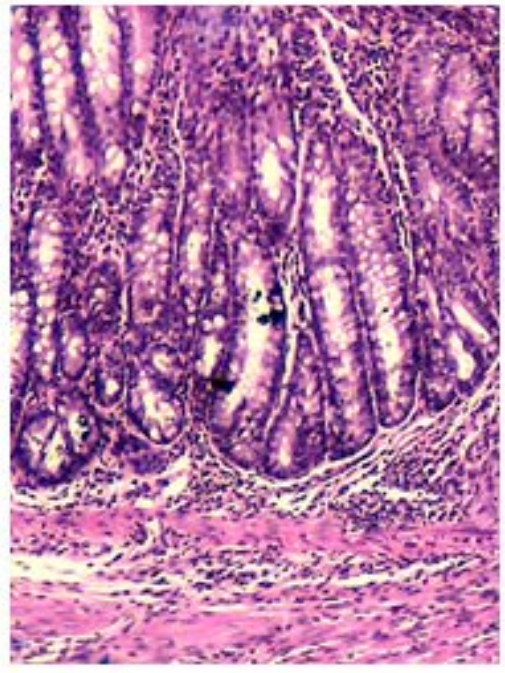


C



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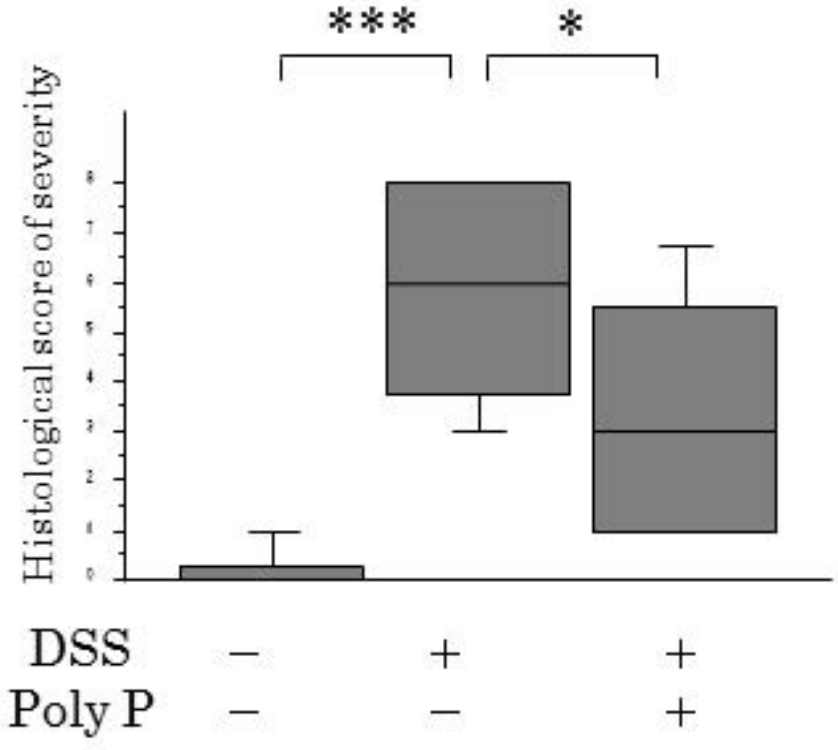
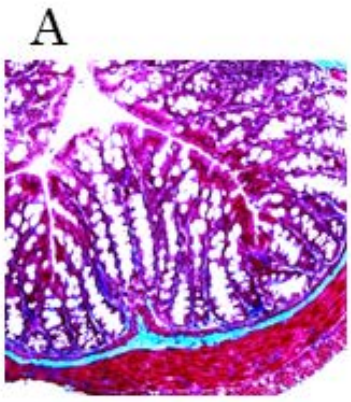
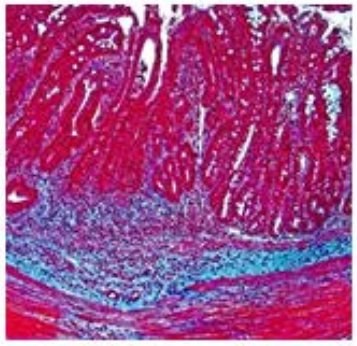



A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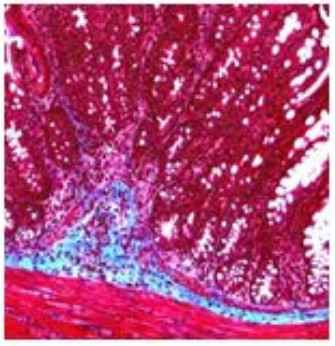
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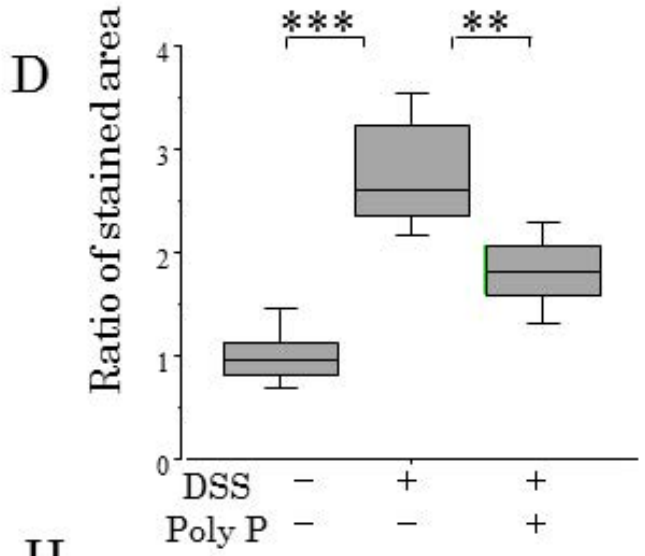
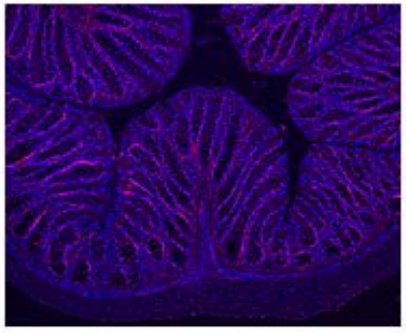

D
Ratio of stained area
***
**
0
1
2
3
4
DSS – + +
Poly P – – +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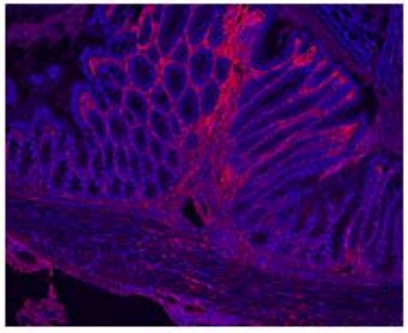
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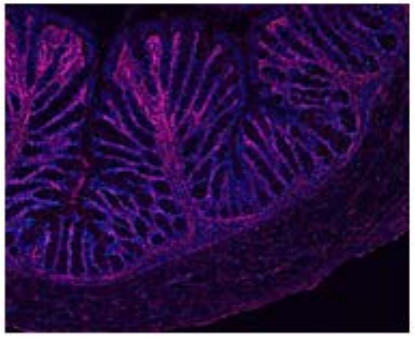
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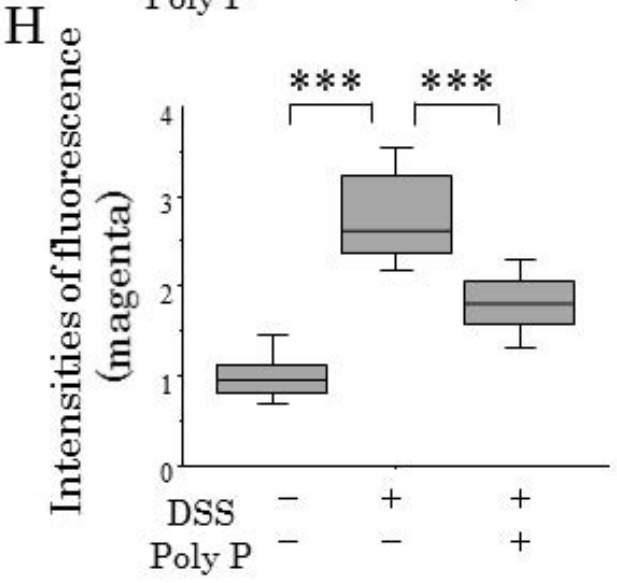
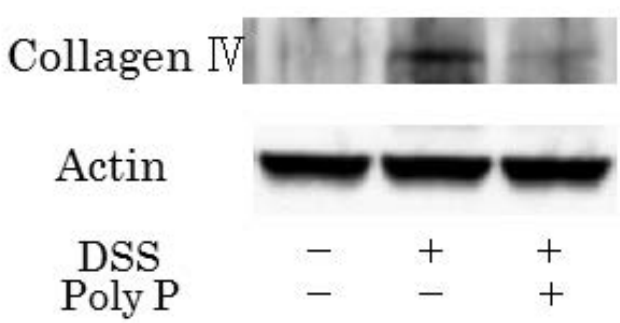
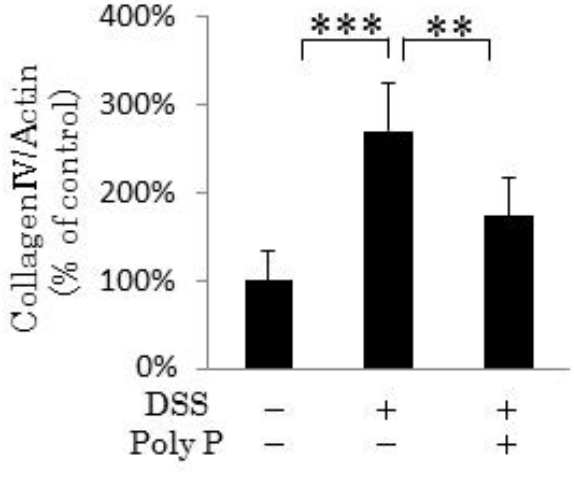
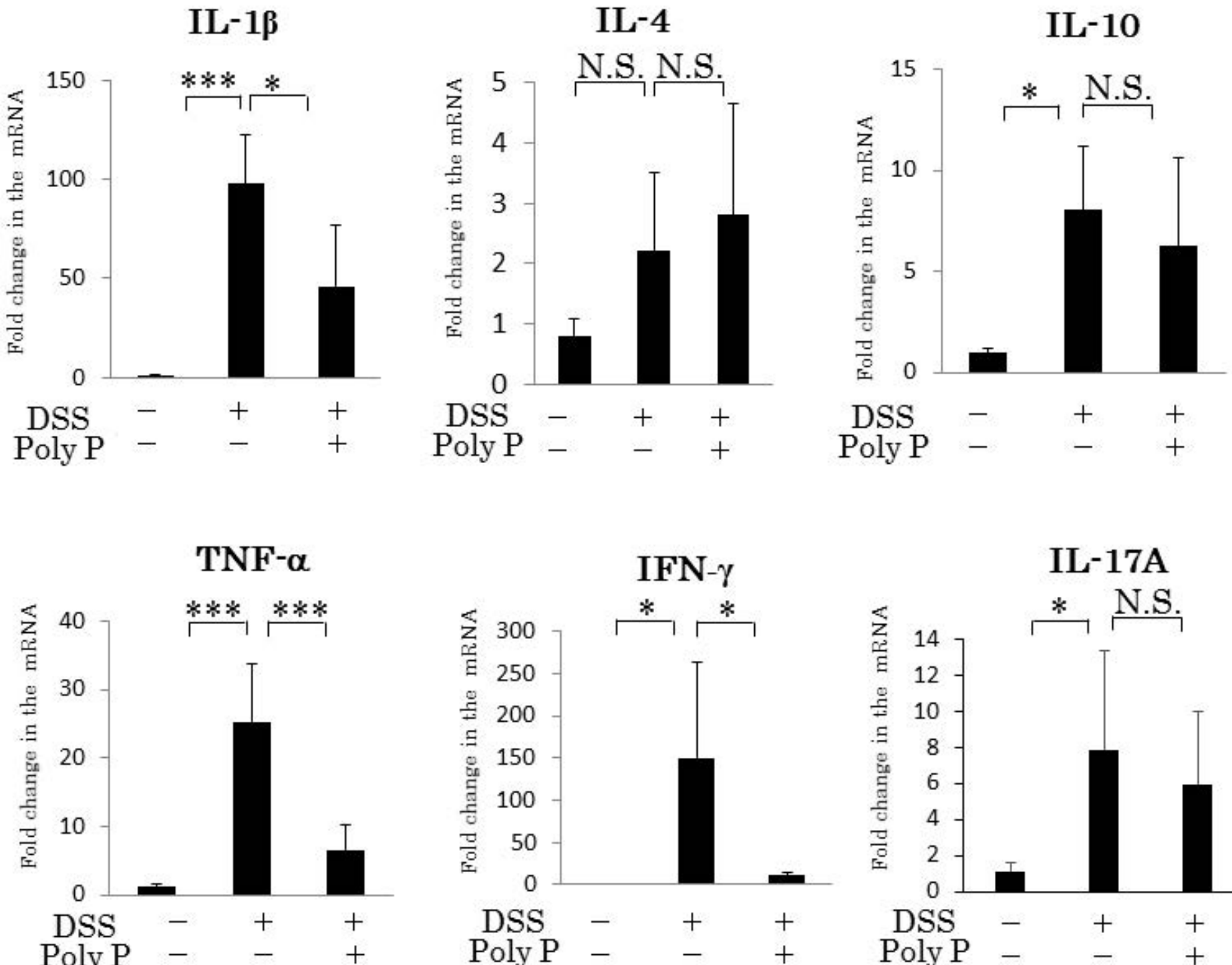
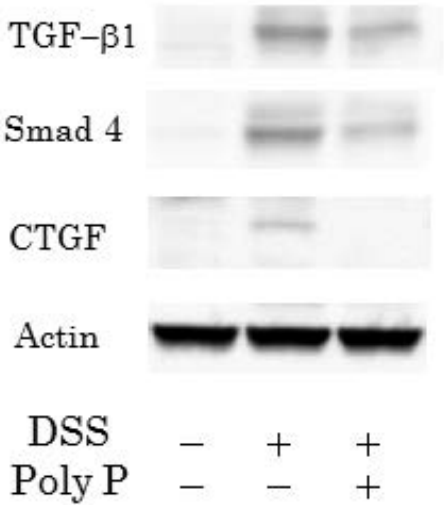
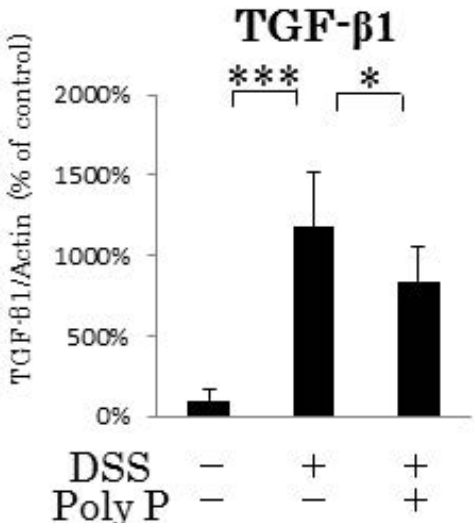

H
Intensities of fluorescence (magenta)
***
***
0
1
2
3
4
DSS – + +
Poly P – – +

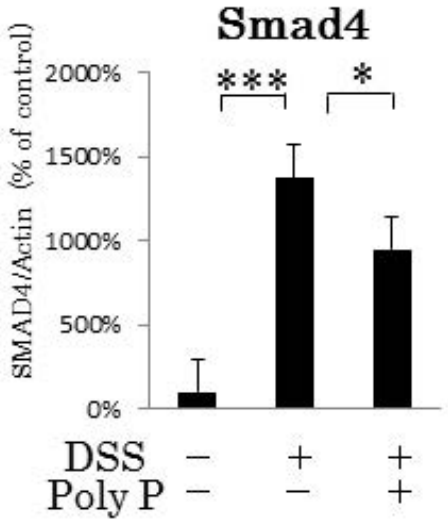

I
Collagen Ⅳ
Actin
DSS – + +
Poly P – – +

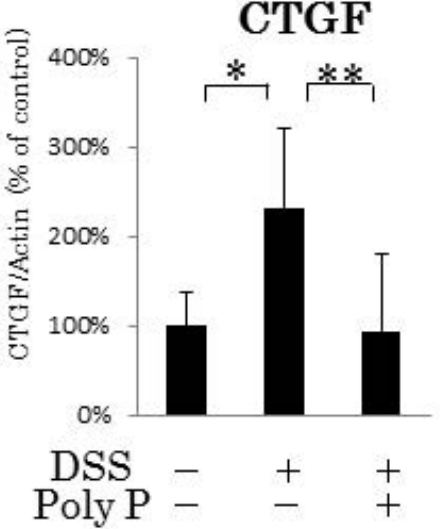

Collagen Ⅳ/Actin (% of control)
***
**
0%
100%
200%
300%
400%
DSS – + +
Poly P – – +

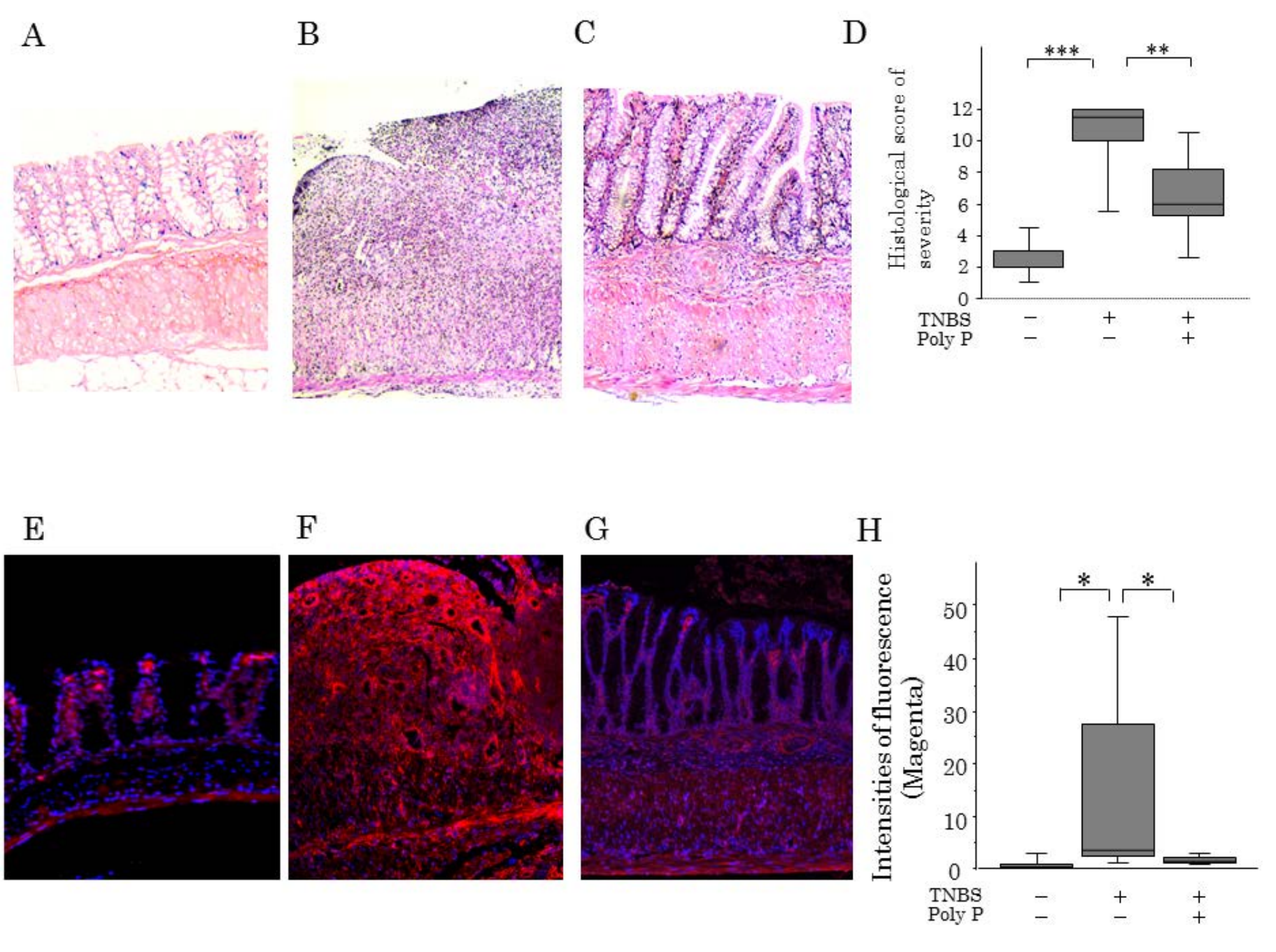

IL-1β
Fold change in the mRNA
150
100
50
0
***
*
DSS – + +
Poly P – – +
IL-4
N.S. N.S.
Fold change in the mRNA
5
4
3
2
1
0
DSS – + +
Poly P – – +
IL-10
Fold change in the mRNA
15
10
5
0
*
N.S.
DSS – + +
Poly P – – +
TNF-α
Fold change in the mRNA
40
30
20
10
0
***
***
DSS – + +
Poly P – – +
IFN-γ
Fold change in the mRNA
300
250
200
150
100
50
0
*
*
DSS – + +
Poly P – – +
IL-17A
Fold change in the mRNA
14
12
10
8
6
4
2
0
*
N.S.
DSS – + +
Poly P – – +


TGF–β1
Smad 4
CTGF
Actin
DSS – + +
Poly P – – +

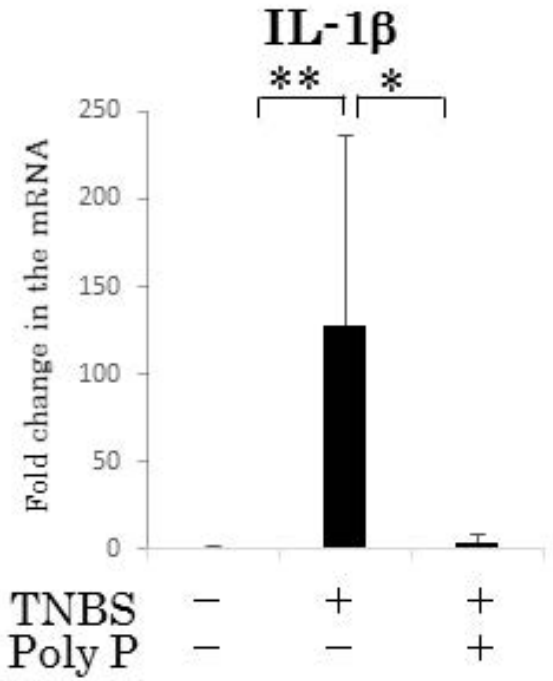

TGF-β1
TGF-β1/Actin (% of control)
2000%
1500%
1000%
500%
0%
***
*
DSS – + +
Poly P – – +

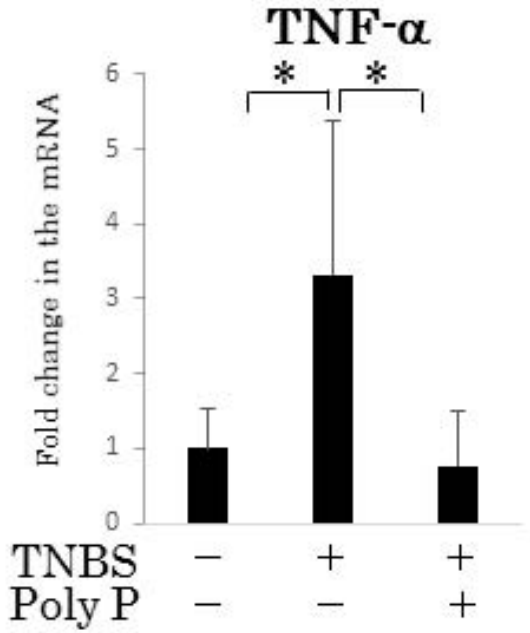

Smad4
SMAD4/Actin (% of control)
2000%
1500%
1000%
500%
0%
***
*
DSS – + +
Poly P – – +


CTGF
CTGF/Actin (% of control)
400%
300%
200%
100%
0%
*
**
DSS – + +
Poly P – – +

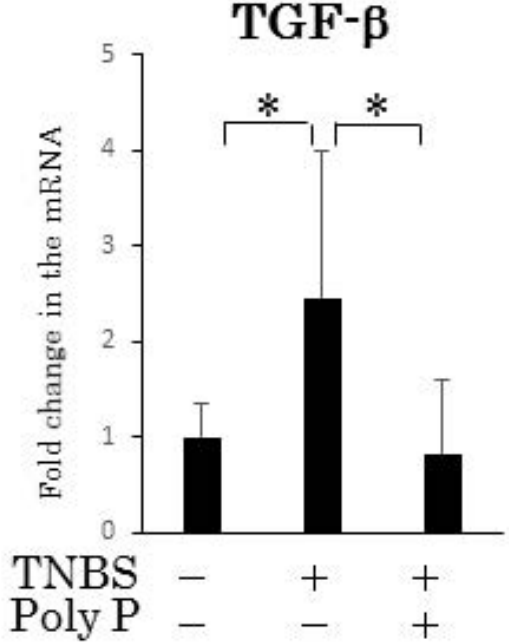

A
B
C
D
***
**
Histological score of severity
0
2
4
6
8
10
12
TNBS
Poly P
E
F
G
H
*
*
Intensities of fluorescence (Magenta)
0
10
20
30
40
50
TNBS
Poly P

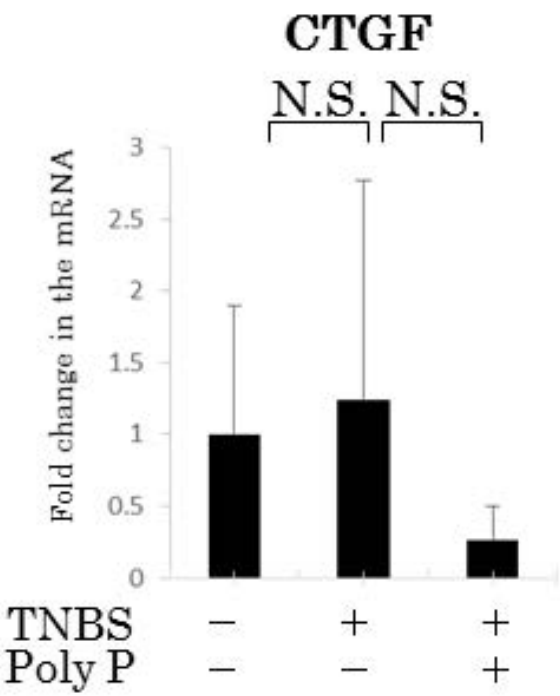
A

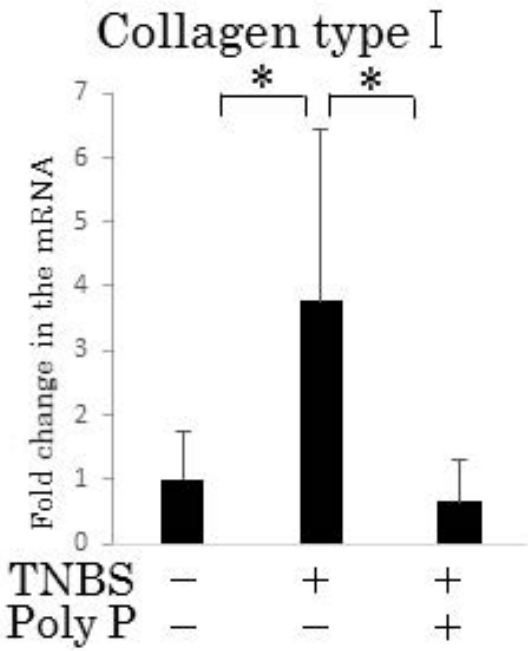

IL-1β
**
*
Fold change in the mRNA
250
200
150
100
50
0
TNBS − + +
Poly P − − +


TNF-α
*
*
Fold change in the mRNA
6
5
4
3
2
1
0
TNBS − + +
Poly P − − +

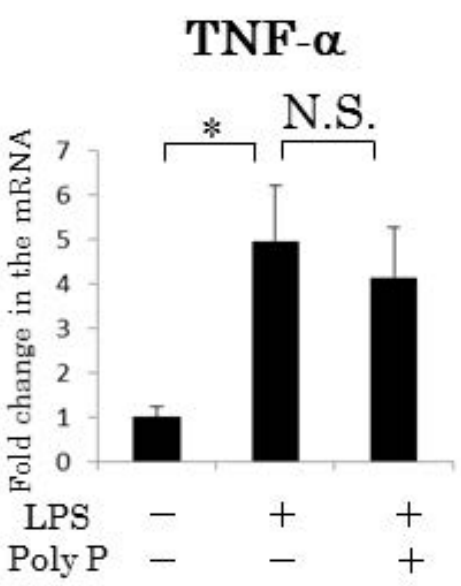
B

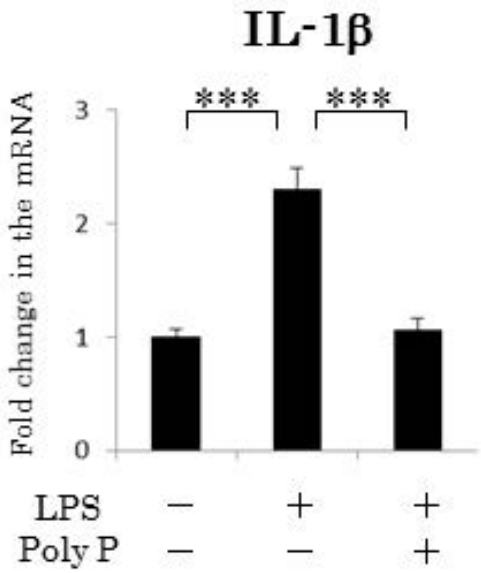

TGF-β
*
*
Fold change in the mRNA
5
4
3
2
1
0
TNBS − + +
Poly P − − +

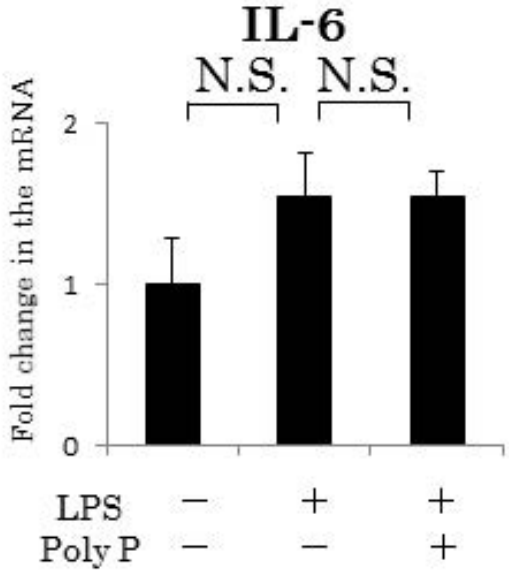

CTGF
N.S. N.S.
Fold change in the mRNA
3
2.5
2
1.5
1
0.5
0
TNBS − + +
Poly P − − +


Collagen type Ⅰ
*
*
Fold change in the mRNA
7
6
5
4
3
2
1
0
TNBS − + +
Poly P − − +

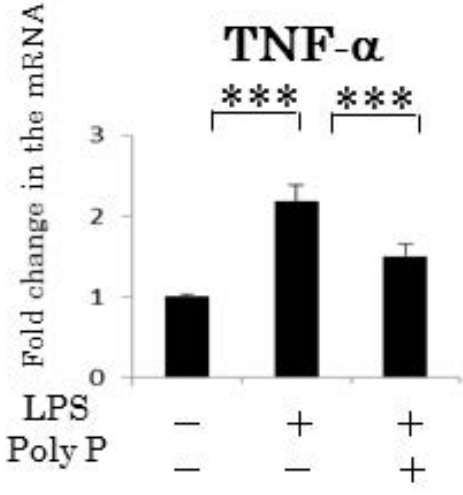
A

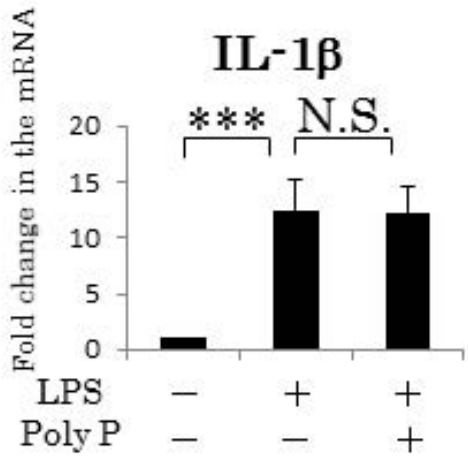

TNF-α
Fold change in the mRNA
*
N.S.
LPS
Poly P

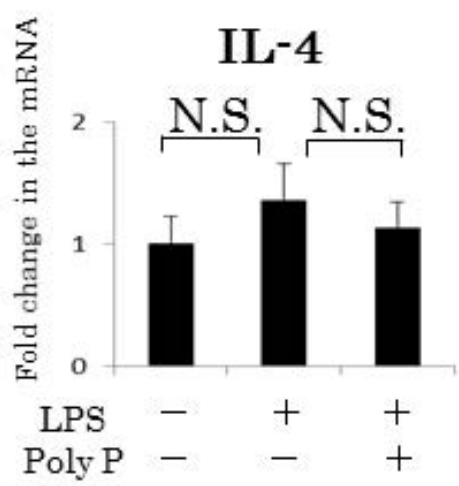

IL-1β
Fold change in the mRNA
***
***
LPS
Poly P

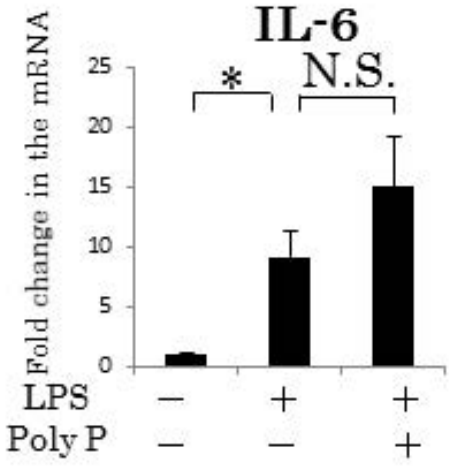

IL-6
N.S.
N.S.
Fold change in the mRNA
LPS
Poly P

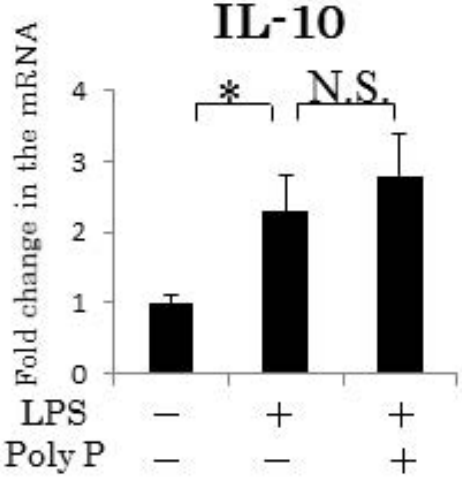
B

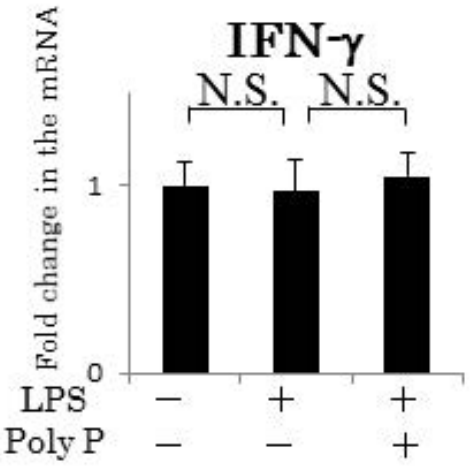

TNF-α
***
***
Fold change in the mRNA
LPS
Poly P

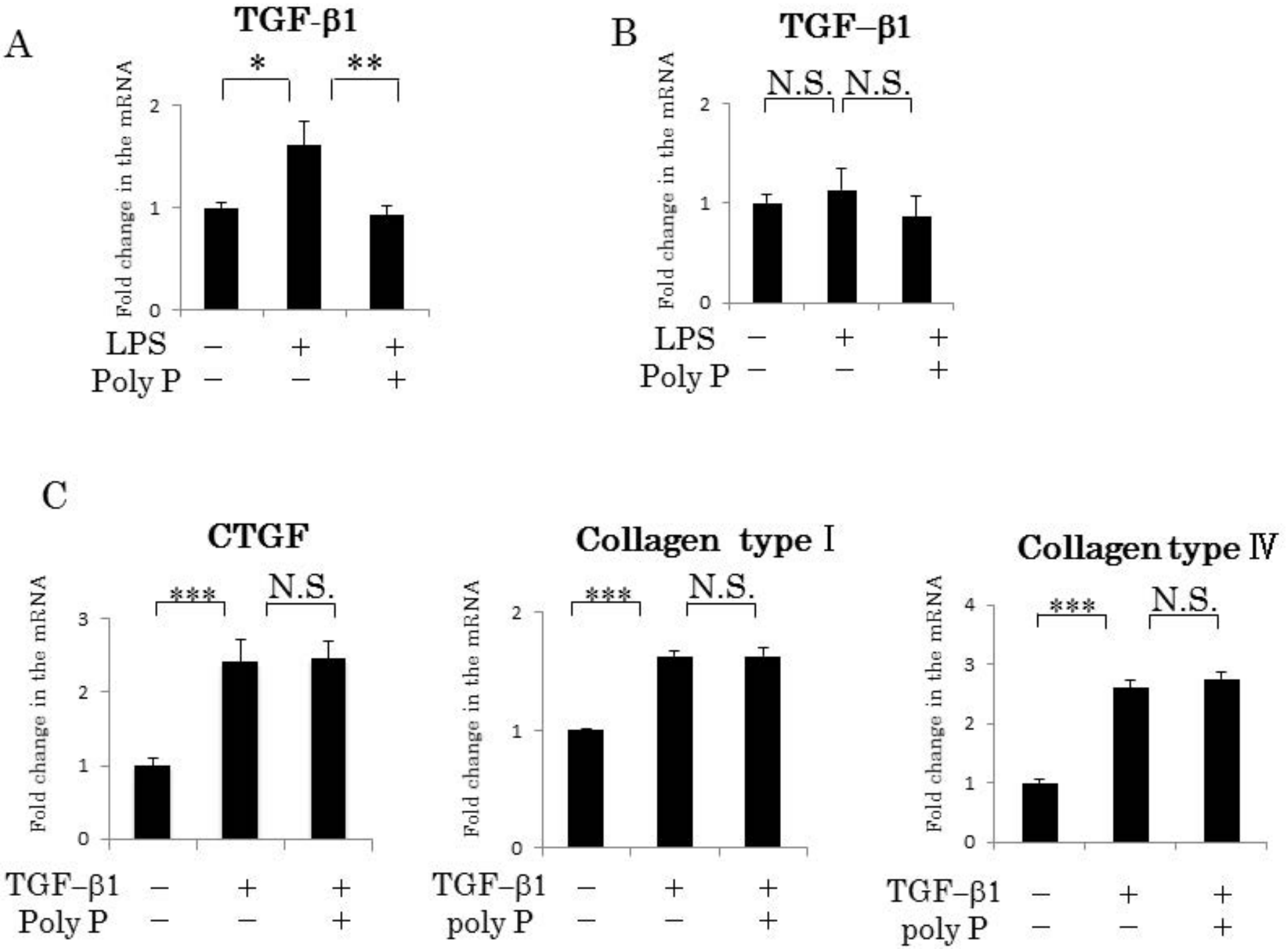

IL-1β
***
N.S.
Fold change in the mRNA
LPS
Poly P


IL-4
N.S.
N.S.
Fold change in the mRNA
LPS
Poly P


IL-6
*
N.S.
Fold change in the mRNA
LPS
Poly P


IL-10
*
N.S.
Fold change in the mRNA
LPS
Poly P


IFN-γ
N.S.
N.S.
Fold change in the mRNA
LPS
Poly P


A
TGF-β1
Fold change in the mRNA
*
**
LPS – + +
Poly P – – +
B
TGF–β1
Fold change in the mRNA
N.S. N.S.
LPS – + +
Poly P – – +
C
CTGF
Fold change in the mRNA
***
N.S.
TGF–β1 – + +
Poly P – – +
Collagen type Ⅰ
Fold change in the mRNA
***
N.S.
TGF–β1 – + +
poly P – – +
Collagen type Ⅳ
Fold change in the mRNA
***
N.S.
TGF–β1 – + +
poly P – – +