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***In vitro* model to estimate gut inflammation using co-cultured Caco-2 and RAW264.7 cells**

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Abstract

A system for assessing the anti-inflammatory effect of food factors was developed by establishing a co-culture system with intestinal epithelial Caco-2 cells (apical side) and macrophage RAW264.7 cells (basolateral side). In this system, the stimulation of RAW264.7 cells with lipopolysaccharide was followed by a decrease in transepithelial electrical resistance, which is a marker of the integrity of the Caco-2 monolayer and an increase in TNF- α production from RAW264.7 cells and IL-8 mRNA expression in Caco-2 cells. Treatment with anti-TNF- α antibodies or budesonide suppressed the increase in TNF- α production and IL-8 mRNA expression. These results indicated that this co-culture model could imitate the gut inflammation *in vivo*. In addition, fucoidan, sulphated polysaccharides from brown algae, was employed as a candidate of evolution and added to the apical side of this model. Fucoidan suppressed IL-8 gene expression through a reduction in TNF- α production from RAW264.7 cells stimulated with lipopolysaccharide.

Key Word; IL-8, TNF- α , TER, co-culture, gut inflammation, fucoidan, Caco-2 cells,

Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are characterized by chronic inflammation of the gastrointestinal tract. While the precise etiology still remains unknown, understanding of the pathophysiology of IBD has advanced, and the typical features of these diseases have been shown in various studies and especially for intestinal immune cells and intestinal epithelial cells of IBD patients. It has been reported that intestinal epithelial cells and macrophages secrete large amounts of chemokines and pro-inflammatory cytokines in the inflamed intestine of IBD patients [1]. Clayburgh et al. reported that modulation of the mucosal epithelial barrier played a critical role in the initiation and propagation of IBD. It seems that compromised intestinal barrier function is a specific feature of IBD [2]. A member of the C-X-C chemokine family, interleukin (IL)-8, was secreted excessively by a variety of cells at the site of inflammation, such as intestinal epithelial cells, in IBD [3]. IL-8 causes the excessive recruitment and transmigration of neutrophils into inflamed tissues following injury of the epithelium [4]. Lamina propria mononuclear cells (LPMCs) from patients with Crohn's disease spontaneously secreted tumor necrosis factor (TNF)- α and induced epithelial destruction [5]. Although it is well-known that the secretion of inflammatory cytokines like IL-8 and TNF- α may be an important part of the immune response, the dysregulation of these cytokines is implicated in the pathogenesis of IBD [6,7].

Fucoidans are defined as fucose-rich sulfated polysaccharides contained in brown seaweeds such as *Laminaria* sp. and *Undaria* sp. Fucoidan is mainly composed of fucose, galactose, and sulfate, with minor components of mannose, glucuronic acid, glucose, rhamose, arabinose, and xylose [8]. Fucoidan has immune modulatory activities including anti-inflammatory, anti-tumor, anti-angiogenic, and anti-adhesive properties [9]. The mode of action begins through regulating the function of immune cells such as macrophages, natural killer (NK) cells, and dendritic cells (DCs) [10-12]. Though the effect of fucoidan on murine colitis *in vivo* showed a decrease in disease activity indices such as occult, stool consistency, and body weight [13], the anti-inflammatory effects of fucoidan are not understood well at the cellular level.

From research into food factors with anti-inflammatory properties against intestinal inflammation, animal intestinal inflammation models that are induced by the administration of either dextran sodium sulfate (DSS) [14] or 2,4,6-trinitrobenzene sulfonic acid (TNBS) [15], and IL-7 transgenic mice [16], IL-2 knockout mice [17], and IL-10 knockout mice [18] have been used as IBD models [19]. The intake of resveratrol, curcumin, vitamin D, and probiotics resulted in the arrest of weight loss, increase in stool consistency, and improvement of mucosal appearance using these model mice [19]. However, the anti-inflammatory effect by these factors is still poorly understood at the cellular level. Therefore, a more precise *in vitro* assessment model of anti-inflammatory effects is required to elucidate this mechanism. In the present study, a gut inflammation

in vitro model was established, in which an intestinal epithelial cell line, Caco-2 cells, were placed in transwell at the apical side and a macrophage cell line, RAW264.7 cells, were placed at the basolateral side. When RAW264.7 cells were stimulated with lipopolysaccharide (LPS), IL-8 and TNF- α secretion increased, and transepithelial electrical resistance values (an index of intestinal epithelial barrier function) decreased. It was also observed that treatment with anti-TNF- α antibodies and budesonide, drugs for Crohn's disease patients in USA and Europe [20], suppressed the level of IL-8 mRNA expression in Caco-2 cells and TNF- α production from RAW264.7 cells. Moreover, this study examined the effect of fucoidan on the improvements of gut inflammation in this *in vitro* model.

Material and methods

Reagents: Dulbecco's Modified Eagle Medium (DMEM, glutamine, low glucose), lipopolysaccharide (LPS) from *E.coli* O127, and recombinant murine TNF- α were purchased from Wako Pure Chemical Industries (Osaka, Japan). MEM (Eagle's Minimum Essential Medium) was purchased from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan). RPMI 1640 medium and MEM non-essential amino acids (NEAA) were purchased from Gibco BRL (Grand Island, N.Y., USA). DMEM (glutamine, high glucose) and budesonide were obtained from Sigma (St Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Beit, Israel). Anti-mouse TNF- α rabbit polyclonal antibody (TNF- α Ab) was purchased from Calbiochem (Darmstadt, Germany). Purified fucoidan from *Laminaria japonica* Areschoug was a gift from Professor Takao Ojima, Hokkaido University.

Cell culture: Human intestinal epithelial cell line, Caco-2, cells were cultured in DMEM (glutamine, high glucose), supplemented with 1% MEM-NEAA, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% decompemented FBS (56 °C, 30 min). Murine macrophage cell line, RAW 264.7, cells were cultured in DMEM (glutamine, low glucose) supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Murine fibrosarcoma cell line, L929, cells were cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cell cultures were incubated in a humidified 5% CO₂

incubator at 37°C.

Transepithelial electrical resistance (TER) measurement: The integrity of the Caco-2 monolayer was determined by measuring the TER value. Tight junctions serve as barriers to paracellular diffusion and TER reflects the tightness of the junctions between epithelial cells [21]. The monolayer cells were gently rinsed with Hank's Balanced Salts Solution (HBSS: 137 mM NaCl, 5.36 mM KCl, 1.67 mM CaCl₂, 1 mM MgCl₂, 1.03 mM MgSO₄, 0.44 mM KH₂PO₄, and 0.34 mM Na₂HPO₄) for 30 min. TER value was measured using a Millicell-ERS instrument (Millipore, Eschborn, Germany).

Co-culture system: Caco-2 cells were seeded at 3.75×10^5 cells/well onto Transwell insert plates (4.67 cm², 0.4 µm pore size, Corning CoStar Corp., Cambridge, MA). The cell culture medium was changed every 3 day until the cells were fully differentiated (TER value > 1200 Ω·cm²), and the cells were used in passages number 48-62. RAW264.7 cells were seeded at 8.5×10^6 cells/well into the 6-well tissue culture plate and incubated overnight to completely adhere to the well, and the cells were used in passages number 10-30. After replacing all media with RPMI1640, the Transwell insert on which Caco-2 cells had been cultured were added into multiple plate wells preloaded with RAW264.7 cells as shown in Fig. 1. In an experiment to evaluate the anti-inflammatory effect of fucoidan, 1.5 ml of fucoidan (500 µg/ml) was applied the apical side for 3 h, and then LPS was added to the basolateral side in this model. After an additional incubation of 3 h, culture supernatants from the basolateral side were

collected for TNF- α measurement. The cultured cells were harvested for total RNA isolation and applied to subsequent RT-PCR.

TNF- α content measurement: TNF- α contents were quantified with a cytotoxicity assay with L929 cells (actinomycin D-treated murine fibroblast cell line) using murine rTNF- α as the standard as described by Takada et al [22].

RNA isolation and RT-PCR: Total RNA was isolated from Caco-2 cells using Sepasol RNA I super (Nakarai Tesque, Inc. Kyoto, Japan) with High salt precipitation solution (Nippon Gene Co., Ltd. Tokyo, Japan) according to manufacturer's protocol. The reverse transcription of the RNA was performed using Prime Script Reverse TranscriptaseTM obtained from Takara (Shiga, Japan) according to manufacturer's protocol. RT generated cDNA was amplified using ExpandTM High Fidelity PCR System from Roche Diagnostics (Tokyo, Japan) according to manufacturer's protocol. After an initial incubation at 94°C for 15 min, PCR was performed with 20 cycles for GAPDH and 30 cycles for IL-8 consisting of denaturation (95°C, 15 s), annealing (63°C, 20 s), and extension (72°C, 30 s), followed by an extension at 72°C, 7 min. The oligonucleotide primers used were as follows [23]: IL-8 (forward) 5'-TGGCTCTCTTGGCAGCCTTC-3' (reverse) 5'-TGCACCCAGTTTTCTTGTTGGG-3' (238 bp); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward) 5'-TGAACGGGAAGCTCACTGG-3' (reverse) 5'-TCCACCACCCTGTTGCTGTA-3' (307 bp). PCR products were resolved on a 2.3%

agarose gel and stained with ethidium bromide. To semiquantify the induction of IL-8 mRNA expression in Caco-2 cells, the IL-8 mRNA expression levels were normalized relative to the expression of GAPDH mRNA and IL-8/GAPDH ratios were calculated using densitometric analysis by Image J software. The fold increase in IL-8 mRNA expression was calculated relative to the control.

Statistical analyses: Statistical comparisons were performed using Student's t-test. Statistical significance was defined as $P < 0.05$. Data are presented as means \pm standard error ($M \pm SE$).

Results and discussion

Establishment of a gut inflammation model using Caco-2 cells and RAW264.7 cells stimulated with LPS: An abnormal immunological response of intestinal immune cells to enteric microflora and any food substances is a critical factor driving IBD in genetically susceptible hosts [24, 25]. It has been reported that the inflamed tissue in IBD patients contains a large number of immune cells producing pro-inflammatory cytokines in excess [26]. IL-8 was secreted excessively by a variety of cells at the site of inflammation such as intestinal epithelial cells in IBD [4]. LPMCs from patients with Crohn's disease spontaneously secreted TNF- α [5], and then newly produced TNF- α induced epithelial barrier destruction [27], which culminated in the reduction of TER values [28]. These parameters (IL-8, TNF- α , and TER) are characteristic of the pathology of IBD. As shown in Fig. 1, a co-culture model using a combination of intestinal epithelial Caco-2 cells and macrophage RAW264.7 cells was developed as an intestine model *in vitro*. LPS was applied to the basolateral compartment to imitate gut inflammation. The TER value of Caco-2 cells showed no changes for 24 h and then started to decrease at 36 h after stimulation of RAW264.7 cells with LPS (Fig. 2A). However, no drastic change was recognized without LPS stimulation (data not shown). This result suggested that the monolayer of Caco-2 cells was damaged by LPS treatment in the co-culture model. Similar trends were observed in other *in vitro* gut inflammation models [29] and in IBD patients [27]. As shown in Fig. 2B, the mRNA expression level

of IL-8 in the co-culture system with LPS stimulation maintained the basal levels at 1 h, then increased rapidly to approximately 5.5 times the initial level at 3 h, kept the same level to 6 h, and thereafter decreased to 3-fold at 12 h. Satsu et al. [29] also showed the high IL-8 mRNA expression level from intestinal epithelial cells in their *in vitro* gut inflammation model. TNF- α production in the co-culture model rapidly increased at 1 h after LPS stimulation, and thereafter remained at almost the same level during protracted incubation. Mizuno et al. [30] also reported the same finding of TNF- α production in RAW264.7 cells that was stimulated by purified polysaccharide from *Agaricus blansiliensis*. Since the levels of IL-8 mRNA expression and TNF- α production peaked at 3 h after LPS stimulation, the incubation time was fixed at 3 h after LPS stimulation in the subsequent experiments.

Responses of the Caco-2/RAW264.7 co-culture system towards known stimulators:

It is well-known that LPS treatment of RAW264.7 cells enhances TNF- α production via TLR4 signaling [31]. When the Caco-2/RAW264.7 co-culture model was stimulated with 100 ng/ml LPS to the basolateral side, approximately 90 ng/ml TNF- α was produced into the basolateral compartment (Fig. 3B) but not into the apical compartment. It has been reported that TNF- α induces IL-8 expression in virtually all types of cells including intestinal epithelial cells [32], and as indicated in Fig. 2C, IL-8 mRNA expression was slower at 2 h than TNF- α production. Therefore, it was

postulated that TNF- α secretion is necessary to up-regulate the IL-8 mRNA expression level in Caco-2 cells in this model. To prove this hypothesis, TNF- α Ab, which are used as a therapeutic drug for Crohn's disease [33], was applied simultaneously with LPS into the basolateral compartment in the Caco-2/RAW264.7 co-culture system. As expected, IL-8 mRNA expression was completely suppressed by TNF- α Ab addition (Fig. 3A). Moreover, TNF- α production was not detected (Fig. 3B). Budesonide is also employed as an oral medicine for Crohn's disease patients [20], and its effect is due to inhibition of the function of nuclear factor κ B and consequently the transcription of pro-inflammatory genes such as TNF- α [34]. To further ascertain that this co-culture system was accurate enough as a gut inflammation model, the effect of budesonide on TNF- α production and IL-8 mRNA expression was examined in the Caco-2/ RAW264.7 co-culture system. Budesonide (1 μ M) treatment of the apical compartment significantly down-regulated the IL-8 mRNA expression level (Fig. 3C) and reduced TNF- α production compared with the control (Fig. 3D). Pahl et al. [35] also reported the inhibitory effect of budesonide on TNF- α production from monocytes. Moreover, the direct simultaneous application of budesonide and LPS to RAW264.7 cells inhibited the mRNA expression level and production of TNF- α (data not shown). Thus, the down-regulating effect of budesonide on IL-8 mRNA expression in Caco-2 cells was due to the suppression of TNF- α production from RAW264.7 cells stimulated with LPS. This result indicated that the inhibitory effect by budesonide on the Caco-2/RAW264.7

co-culture system stimulated with LPS showed a similar anti-inflammatory effect in Crohn's disease patients. Together with the results in Figs. 2 and 3, it was demonstrated that gut inflammation like IBD can be imitated by the co-culture system consisting Caco-2 cells and RAW264.7 cells with LPS treatment.

Anti-inflammatory effect of fucoidan on IL-8 mRNA expression in the gut inflammation model of Caco-2 cells and LPS activated RAW264.7 cells: Satsu et al. [29] reported that activated macrophages induced the disruption of the intestinal epithelial monolayer using an intestine model *in vitro*. However, there are few studies on the establishment of gut inflammation models *in vitro* to search for anti-inflammatory agents like drugs and food substances. To test whether this Caco-2 / RAW264.7 gut inflammation model is applicable for searching for anti-inflammatory factors in foods, the anti-inflammatory effect of fucoidan, a food factor from brown algae with various biological activities [9], was assessed. Fucoidan (750 µg) was added to the apical compartment of the Caco-2/RAW264.7 co-culture system for 3 h, and thereafter LPS was added to the basolateral side at a final concentration of 100 pg/ml and the cells were incubated for an additional 3 h. As shown in Fig. 4A, treatment with fucoidan resulted in down-regulation of IL-8 mRNA expression in Caco-2 cells. Fucoidan also reduced IL-8 production from Caco-2 cells in the basolateral compartment (data not shown). Moreover, fucoidan decreased TNF-α production from RAW264.7 cells compared with the control (Fig. 4B). These results suggested the

possibility that the oral administration of fucoidan exerted an anti-inflammatory effect in the intestine through the inhibition of excessive IL-8 secretion by suppressing the production of TNF- α from intestinal immune cells. It was ascertained that fucoidan could not penetrate the Caco-2 monolayer as fucoidan was not detected in the basolateral compartment by ELISA using an anti-fucoidan monoclonal antibody (data not shown). These results suggest that the anti-inflammatory effect exerted by fucoidan is not direct but through intestinal epithelial Caco-2 cells. Further study will be needed to investigate the mechanism of inflammation suppression by fucoidan. These results indicated that Caco-2/RAW264.7 cells stimulated with LPS developed as a gut inflammation model was applicable for the screening of anti-inflammatory factors against intestinal inflammation.

In conclusion, this study established a gut inflammation *in vitro* model using Caco-2 cells and LPS stimulated-RAW264.7 cells, and indicated that this co-culture model could imitate gut inflammation as seen in an IBD intestine. With this model, apical treatment of fucoidan down-regulated IL-8 mRNA expression in Caco-2 cells through the inhibition of TNF- α secretion from LPS stimulated-RAW264.7 cells.

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Figure legends

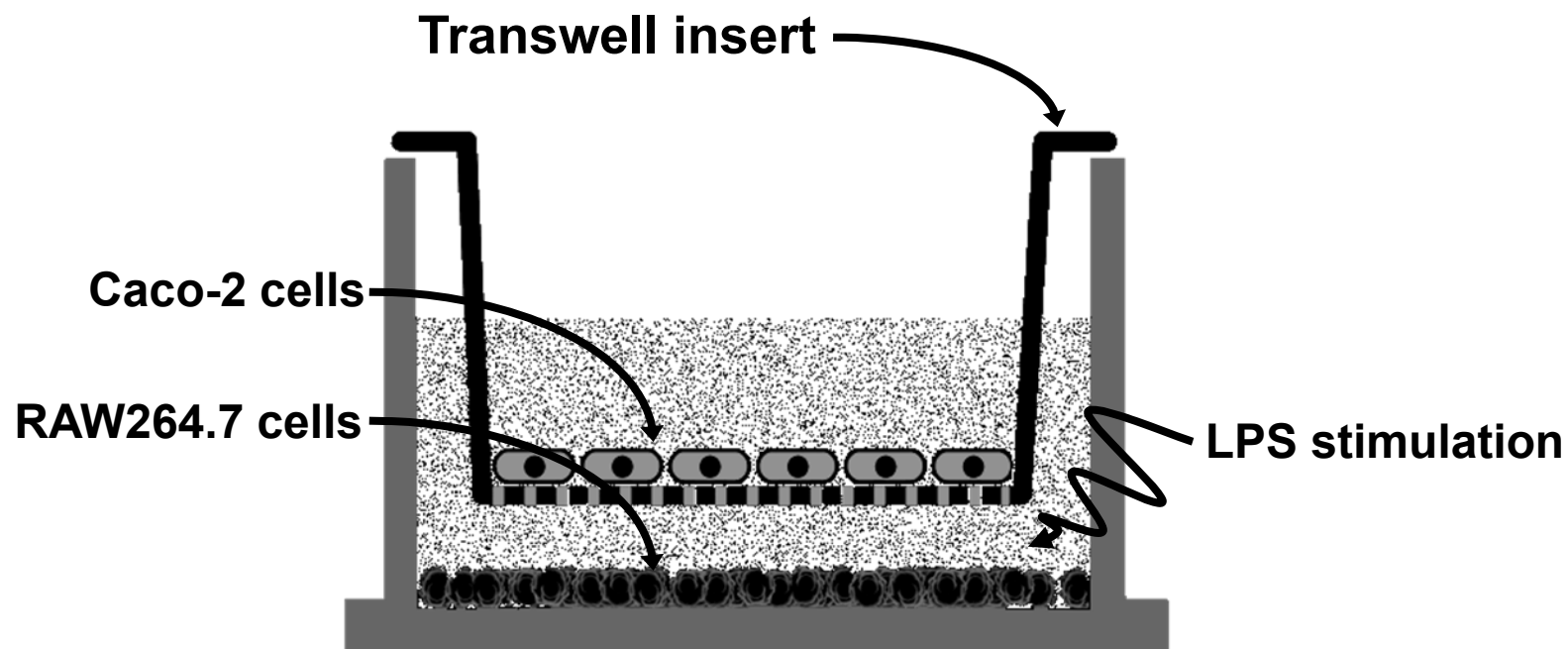
Fig. 1. Co-culture system constructed with Caco-2 cells and RAW264.7 cells.

Transwell inserts on which Caco-2 cells had been cultured were inserted into multiple plate wells containing RAW264.7 cells. To imitate the gut inflammation, LPS was added to the basolateral compartment of this co-culture system for various incubation times.

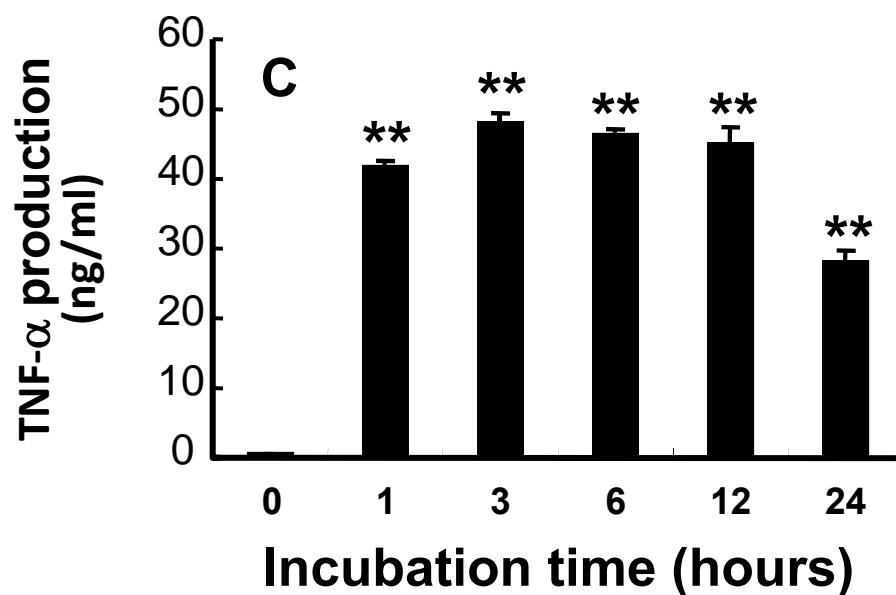
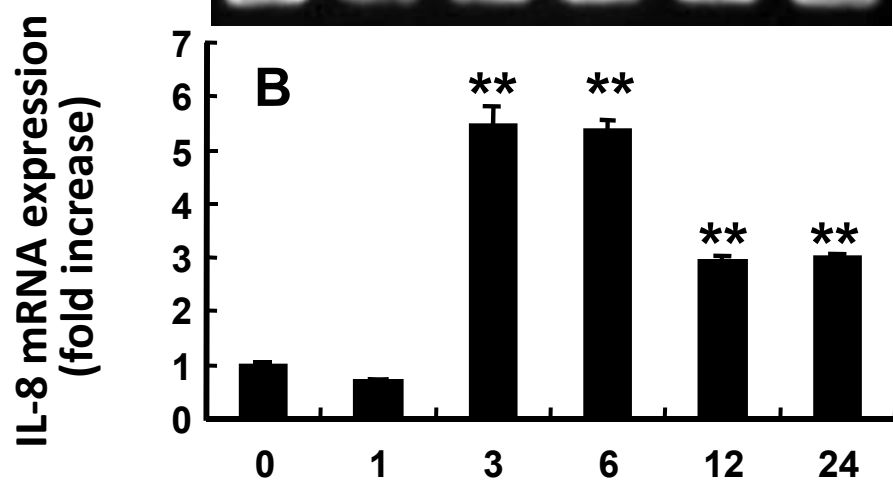
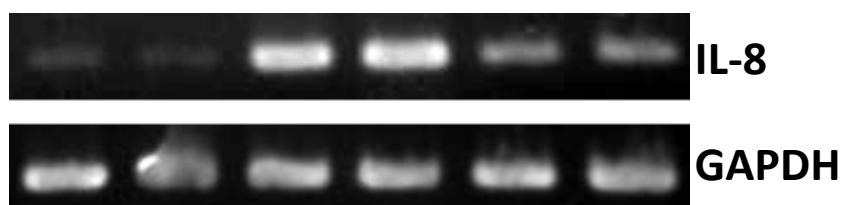
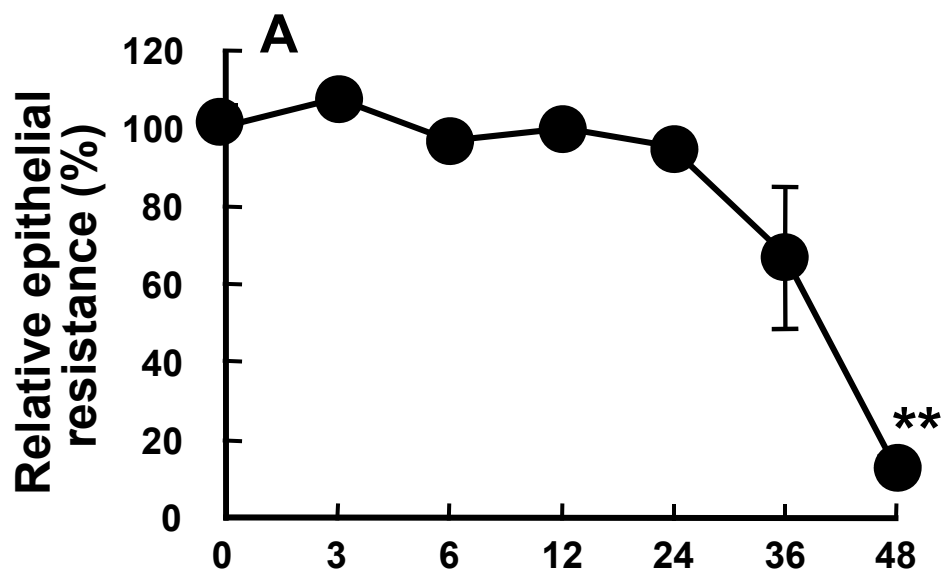
Fig. 2. Time course of TER value, IL-8 mRNA expression and TNF- α production in the Caco-2/RAW264.7 co-culture model. (A) Caco-2 cells were incubated with 100 ng/ml LPS stimulated-RAW264.7 cells. The TER value was measured using a Millicell-ERS instrument at the indicated time points and is given as a percentage of that at the start point of the analysis. (B) Caco-2 cells were incubated with 100 ng/ml LPS stimulated-RAW264.7 cells for various incubation times. After incubation, total RNA from Caco-2 cells was extracted and RT-PCR was performed as described in Material and methods. (C) Caco-2 cells were incubated with 100 ng/ml LPS stimulated-RAW264.7 cells for various incubation times. TNF- α production in the basolateral compartment was determined by a L929 cytotoxicity assay as described in Material and methods. Values represent the means \pm SE ($n=3$). $**P < 0.01$.

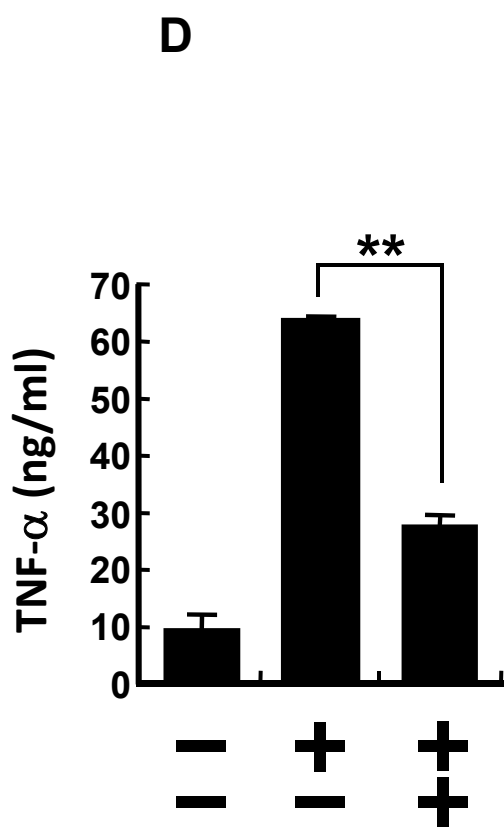
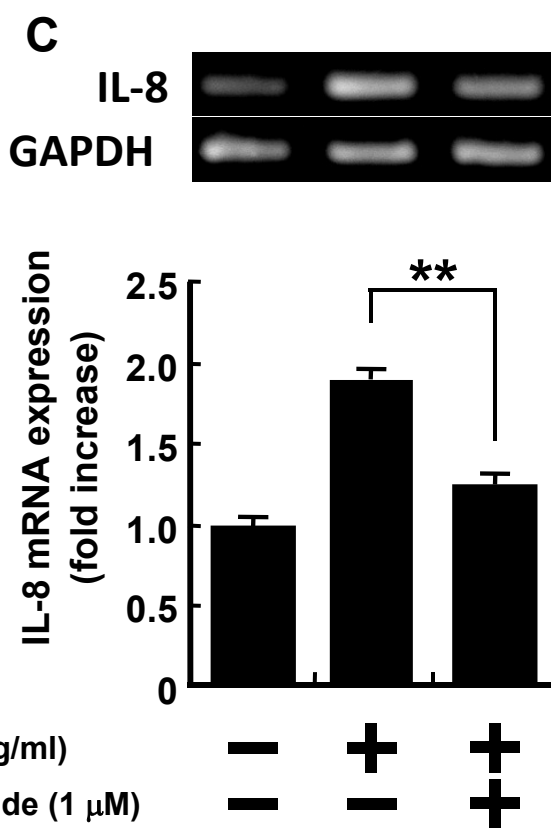
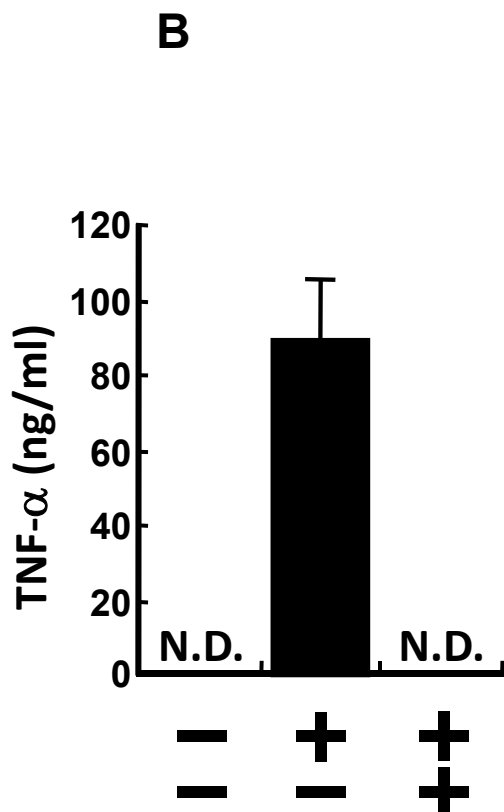
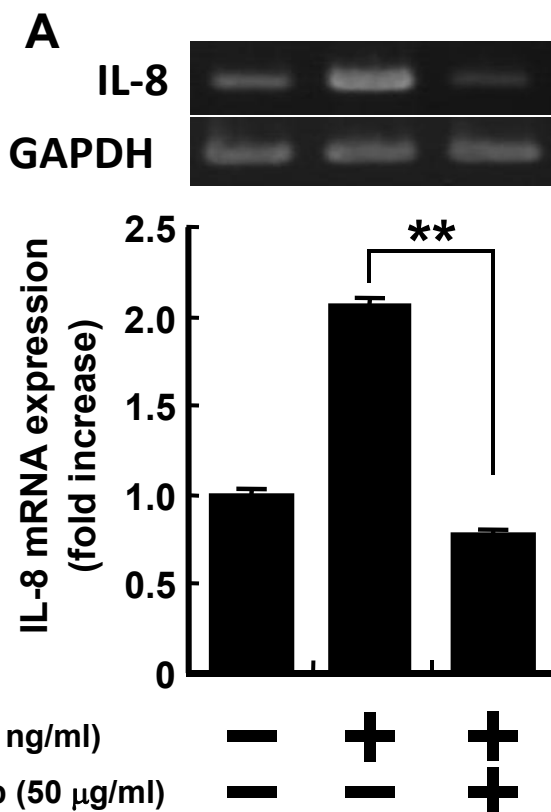
Fig. 3. The inhibitory effect of TNF- α Ab and budesonide on IL-8 mRNA expression and TNF- α production in the Caco-2/RAW264.7 co-culture model. Caco-2 cells were incubated with 100 ng/ml LPS stimulated-RAW264.7 cells in the presence of TNF- α Ab (50 μ g/ml) in the basolateral compartment for 3 h. IL-8 mRNA expression in Caco-2 cells was detected by RT-PCR (A). TNF- α production was determined by a L929 cytotoxicity assay as described in Material and methods (B). Caco-2 cells were incubated with 1 ng/ml LPS stimulated-RAW264.7 cells in the presence of budesonide (1 μ M) in the apical compartment for 3 h. IL-8 mRNA expression in Caco-2 cells was detected by RT-PCR (C). TNF- α production was determined by a L929 cytotoxicity assay as described in Material and methods (D). Values represent the means \pm SE ($n=3$). ** $P < 0.01$.

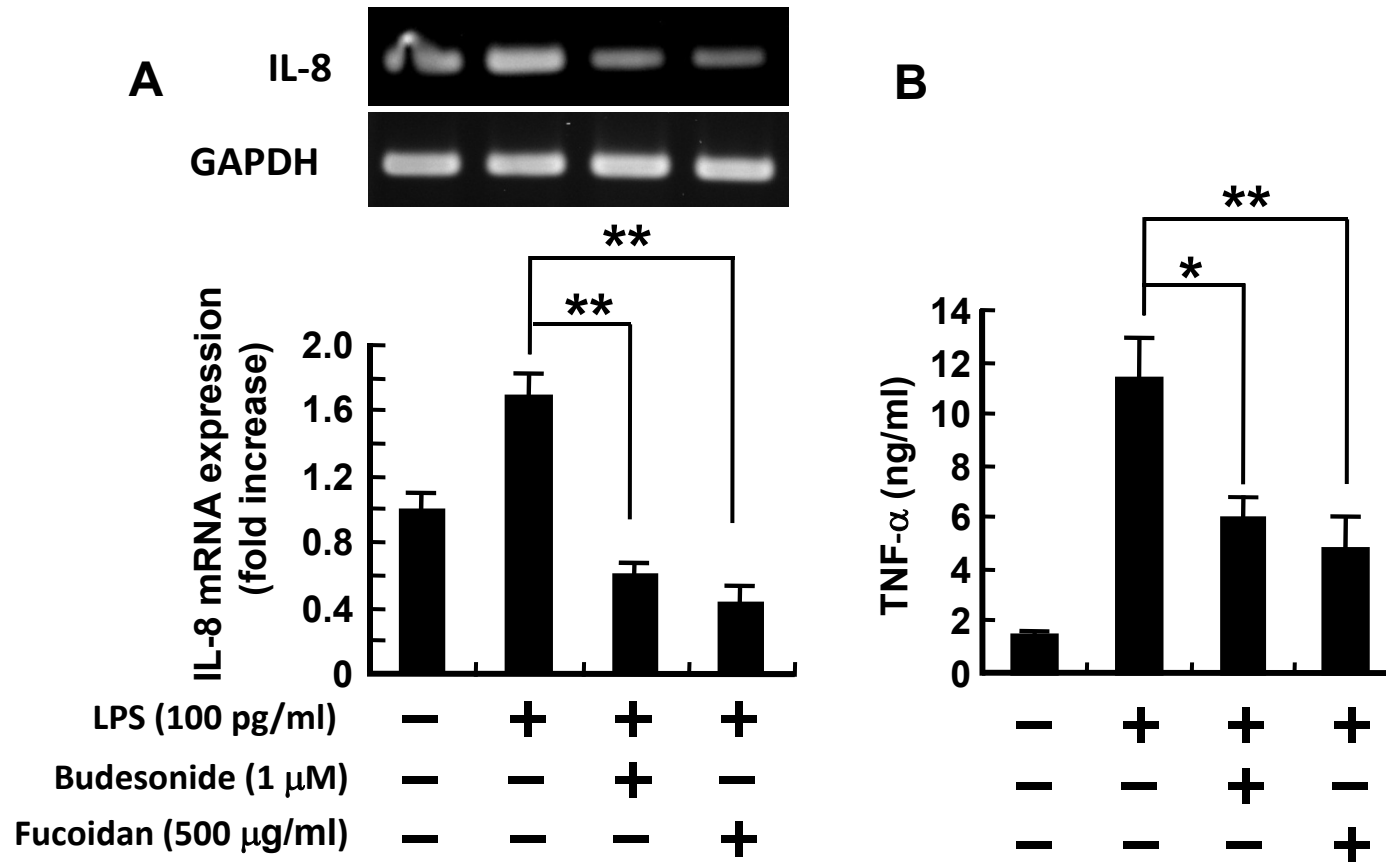
Fig. 4. The suppressive effect of fucoidan on IL-8 mRNA expression and TNF- α production in the Caco-2/RAW264.7 co-culture model. Fucoidan (500 μ g/ml) was added into the apical compartment in Caco-2/RAW264.7 co-culture model for 3 h. Subsequently, LPS was added to basolateral compartment up to the final concentration of 100 pg/ml, followed by incubation for an additional 3 h. (A) IL-8 mRNA expression in Caco-2 cells was detected by RT-PCR. (B) TNF- α production was determined by a L929 cytotoxicity assay as described in Material and methods. Values represent the means \pm SE ($n=3$). ** $P < 0.01$. * $P < 0.05$.



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Fig. 1. Tanoue *et al.*







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Fig. 4. Tanoue *et al.*