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Lentinan Exerts its Anti-Inflammatory Activity by Suppressing TNFR1 Transfer to the Surface of Intestinal Epithelial Cells through Dectin-1 in an *in vitro* and mice model

Kana Sakaguchi[1] , **Yasuhito Shirai**[1], **Toshiki Itoh**[2], **and Masashi Mizuno**[1*]

[1]*Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, 1-1, Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan*

[2]*Biosignal Research Center, Kobe University, 1-1, Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan*

[*]**Corresponding author:** Masashi Mizuno, Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, 1-1, Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan, Tel: +81-78-803-5835; E-mail: mizuno@kobe-u.ac.jp

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Abstract

It has been reported that lentinan, β-1,3; 1,6-glucan derived from *Lentinula edodes*, suppresses intestinal inflammation and ameliorates symptoms of colitis. However, the mechanism of intestinal anti-inflammatory activity of lentinan and how it is recognized by intestinal epithelial cells remains largely unclear. The receptor for lentinan on intestinal epithelial cells was identified using an *in vitro* gut inflammation model consisting of Caco-2 and RAW264.7 cells. Colitis was induced in 7 to 8 week-old wild-type (WT) or knockout (KO) mice by the free intake of water containing 2% dextran sulfate sodium (DSS) for 7 days. Lentinan was administered daily via intragastric administration. Tumor necrosis factor receptor 1 (TNFR1)-GFP complex was constructed to monitor its movement in Caco-2 cells using confocal and total internal fluorescence microscopy. The results indicated that lentinan suppressed DSS-induced body weight loss, shortening of colon length, histological score, and inflammatory cytokine mRNA expression in the inflamed tissues of WT mice, whereas these suppressive effects of lentinan were not observed in Dectin-1 KO mice. Furthermore, lentinan reduced TNFR1 expression in intestinal epithelial cells of WT mice but not those from Dectin-1 KO mice. Using TNFR1-GFP constructs, it was confirmed that lentinan reduced TNFR1 expression on Caco-2 cell membranes through Dectin-1 ligation. Our study revealed that lentinan suppressed intestinal inflammation by Dectin-1-mediated regulation of TNFR1 transfer to the surface of intestinal epithelial cells.

Keywords: Lentinan; Dectin-1; TNFR1; Anti-inflammation; Intestinal epithelial cells

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the intestine that is mainly composed of two types of diseases such as Crohn's disease (CD) and ulcerative colitis (UC). CD usually affects the whole intestinal wall and may potentially extend to any part of gastrointestinal tract, whereas UC mainly attacks the mucosal lining of the colon and rectum. Both of these are chronic diseases presenting with symptoms such as diarrhea, rectal bleeding, and abdominal pain, and repeat recurrence and amelioration [1,2]. Although IBD had been considered a condition mainly associated with developed countries, the prevalence of IBD is now rising in developing countries and it is considered as a global disease [1-3]. Although the pathogenesis of IBD remains unclear, it has been reported that intestinal immune cells and intestinal epithelial cells (IECs) play a critical role in the exacerbation of IBD [4]. In the inflamed intestinal tissues of IBD patients, pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, IL-6, IL-12, IL-17, and IL-23 are secreted from activated immune cells such as macrophages and dendritic cells, and these upregulate the inflammatory response [5,6]. In addition, the intestinal barrier composed of IECs is destroyed in IBD patients. This disruption leads to a flow of intestinal microorganisms into the basolateral side of the IECs, and these microorganisms stimulate immune cells. This process triggers excessive production of chemokines such as IL-8 by IECs, which recruit neutrophils into the inflamed sites [7]. The abnormal intestinal immune responses such as the excessive production of pro-inflammatory cytokines and chemokines, and the dysregulation of the intestinal barrier functions are important in the pathogenesis of IBD.

Lentinan, which has the basic unit β-1,6-branched β-1,3-glucohexaose (molecular weight; 300-500 kDa), possesses various biological activities, such as anti-tumor and immune regulatory effects [8,9]. Lentinan is able to activate innate effector cells such as macrophages and NK cells directly [8,10]. In contrast, lentinan exerted intestinal anti-inflammatory effects in dextran sulfate sodium (DSS)-induced colitis mice model, when the mice were administered lentinan orally [11]. However, the mechanism of intestinal anti-inflammatory activity of lentinan and how it is recognized by IECs have not been studied sufficiently.

In this study, the interaction between lentinan and the well-known β-glucan receptors, Dectin-1 and Toll-like receptor (TLR) 2 were investigated. Both of these have been identified as receptors for a yeast-derived β-1, 3-glucan, zymosan [12,13]. Dectin-1 is expressed in myeloid cells such as macrophages, neutrophils, dendritic cells, and IECs [14,15]. TLR2 is a member of the Toll-like receptor family, which recognizes peptidoglycans from gram-positive bacteria, lipoproteins, and yeast [16]. TLR2 is expressed in various cells including most immune cells and IECs [15,17]. Moreover, the relationship between the anti-inflammatory effect of lentinan and its receptors has been investigated using an *in vitro* gut inflammation model [18] and a DSS-induced colitis murine model using Dectin-1 KO mice. Although the

previous study reported that lentinan suppressed the expression of TNF receptor (TNFR) 1 on IECs and suppressed nuclear factor-kappa B (NF-κB) signal activation, production of chemokines, and exacerbation of inflammation [11], it is unknown how the expression of TNFR1 is decreased by lentinan. To investigate this mechanism, green fluorescence protein (GFP)-tagged TNFR1 was constructed, and the motion of TNFR1 was observed by fluorescence microscopy to visualize the decrease in TNFR1 on the surface of IECs and to reveal the effect of lentinan on the expression of TNFR1.

Materials and Methods

Cell culture

Human intestinal epithelial cell line, Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM containing 4.5 g/l glucose :high glucose with 2 mM glutamine, supplemented with 10% fetal bovine serum (FBS), 1% Eagle's minimum medium (MEM)-nonessential amino acids (NEAA), 100 μg/ml streptomycin, and 100 U/ml penicillin. Murine macrophage cell line, RAW264.7 cells were grown in DMEM (containing 1.0 g/l glucose: low glucose) with 2 mM glutamine, supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. Murine fibrosarcoma cell line L929 cells were cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cell cultures were incubated at 37°C in a 5% CO_2 incubator.

Mice

Dectin-1-deficient mice (Dectin-1 KO) of a C57BL/6NCr (wild type; WT) background were kindly provided by Professor Yoichiro Iwakura (Tokyo University of Science). C57BL/6NCr and Balb/cCr (WT) mice were purchased from SLC (Shizuoka, Japan). Mice were housed in an air-conditioned animal room at 23 ± 2°C, and acclimated for 7 days before experiments. Mice were fed on a laboratory diet and distilled water *ad libitum*. The care and use of the animals and experimental protocol were approved by the institutional Animal Care and the Use Committee and carried out in accordance with the Kobe University Animal Experimentation Regulations.

Reagents

DMEM mixed with 2 mM glutamine containing 1.0 g/l glucose (low glucose), DMEM mixed with 2 mM glutamine containing 4.5 g/l glucose (high glucose), LPS (lipopolysaccharide) from *E. coli* O127, and recombinant murine TNF-α (rmTNF-α) were purchased from Wako Pure Chemical Industries (Osaka, Japan). MEM was purchased from Nissui Pharmaceutical (Tokyo, Japan). RPMI 1640 medium, MEM-NEAA, and Alexa 594-conjugated anti-mouse IgG secondary antibody were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Budesonide was obtained from Sigma-Aldrich (St Louis, MO, USA). FBS was purchased from Daiichi Kagaku (Tokyo, Japan). Anti-β-actin Ab, anti-human Dectin-1 antibody, anti-human TNFR1 antibody, and normal goat IgG control (isotype control) were obtained from R&D Systems (Minneapolis, MN, USA). Anti-human TLR2 Ab was obtained from Hycult Biotech (Uden, Netherlands). Anti-goat IgG (H+L) HRP conjugated secondary antibody was purchased from Bethyl Laboratories (Montgomery, TX, USA). Lentinan from *Lentinula edodes*, a dietary β- 1,3;1,6-glucan used in this study, was a gift from Ajinomoto. (Tokyo, Japan). All chemicals and reagents were of standard grade and guaranteed products.

Transepithelial electrical resistance measurement

For the assembly of the co-culture system, Caco-2 cells were seeded at 5.0×10^4 cells on the transwell insert (0.4 μm pore size; Corning Costar Co, Cambridge, MA, USA). The monolayer of cells was gently rinsed with Hank's balanced salts solution (HBSS; 137 mM NaCl, 5.36 mM KCl, 1.26 mM $CaCl_2$, 0.55 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 2.92 mM NaH_2PO_4) for 30 min in a CO_2 incubator. The integrity of the Caco-2 monolayer was evaluated by measuring transepithelial electrical resistance (TER) using a Millicell-ERS equipment (Millipore, Eschborn, Germany).

Co-culture system

A Caco-2/RAW264.7 cells co-culture system was used as described previously [18]. When the Caco-2 monolayer had fully differentiated (TER value >400 $\Omega.cm^2$), it was subjected to the following experiment. RAW264.7 cells were seeded at 4.5×10^5 cells/well in 12-well tissue culture plates and incubated overnight to fully adhere to the well. For the estimation of candidates that recognized lentinan, anti-Dectin-1 antibody at a final concentration of 20 μg/ml or anti-TLR2 antibody at a final concentration of 5 μg/ml were used to pretreat with Caco-2 cells at 37°C in a CO_2 incubator. After 30 min, the Caco-2 cells were washed three times with PBS to remove excess antibodies. All media were replaced with RPMI 1640, and the transwell inserts of Caco-2 cells were added into multiple-plate wells preloaded with RAW264.7 cells. Lentinan (500 μg/ml) was applied to the apical side. After 3 h, RAW 264.7 cells at the basolateral side were stimulated with 5 ng/ml LPS at 37°C in a CO_2 incubator for an additional 3 h. The culture supernatants in the basolateral side were collected for the measurement of TNF-α. The treated Caco-2 cells were harvested for total RNA isolation and applied to subsequent real-time polymerase chain reaction (PCR) assays.

RNA interference

Dectin-1 shRNA sc-63276-SH (Santa Cruz, Dallas, TX, USA) and control shRNA sc-108060 (Santa Cruz, CA) were transfected into Caco-2 cells by electroporation using a Neon™ Transfection System Kit (10 μl) and Neon™ Transfection System (Thermo Fisher Scientific). Caco-2 cells were prepared until sub-confluent 70-90% in a cell culture dish (10 cm^2; BD Biosciences, Franklin Lakes, NJ, USA) for use in further transfection. Caco-2 cells were collected by trypsinization and 1.0×10^5 cells in suspension were prepared in Resuspension Buffer R (Thermo Fisher Scientific). This cell suspension was mixed with 0.5 μg of Dectin-1 or control shRNA. Ten microliters of this mixture was aspirated into a Neon™ Tip inserted a Neon™ pipette and this was placed into the Neon™ pipette station and subjected to a voltage of 1000 V for 40 ms. After the exposure to electric pulses, the cells were incubated at 37°C in culture medium without penicillin and streptomycin. Stably transfected cells were selected by addition of 10 μg/ml puromycin into the culture medium. For the assessment of RNAi, shRNA transfected Caco-2 cells were cultured in a 12-well culture plate (Corning Costar Co) and 10 cm^2 dish (BD Biosciences).

TNF-α content measurement

TNF-α contents were quantified by a cytolytic assay with L929 cells using recombinant mouse TNF-α as the standard as described

previously [19]. In brief, L929 cells (100 ml of 3.5×10^5 cells/ml) were plated in 96-well microplates in MEM culture medium and cultured for 24 h in a 5% CO_2 incubator at 37°C. All media were replaced with RPMI 1640 medium containing actinomycin D (4 μg/ml) and each sample was added to the wells. The microplate was incubated for 20 h in the CO_2 incubator and then the supernatants were removed completely. Cells viability was determined by staining with 0.05% crystal violet in 10% ethanol/12% formaldehyde. The stained cells were washed with water and dissolved in ethanol-PBS (1:1, v/v). The absorbance of each well was measured using an SH-1000 Lab microplate reader (Corona Electric, Ibaraki, Japan) at 570 nm for analysis wavelength and 630 nm as the reference wavelength.

DSS-induced colitis

Colitis was induced in 7 to 8 week-old WT or Dectin-1 KO mice by the free intake of drinking water containing 2% (w/v) DSS (molecular weight; 36–50 kDa) for 7 days. On day 7 (initial DSS treatment as day 0), drinking water containing 2% (w/v) DSS was stopped and switched to distilled water for 2 more days. Mice were sacrificed on day 9. Lentinan (100 μg/mouse) or vehicle was administered daily via intragastric administration, starting 7 days before DSS treatment and continuing until sacrifice. Body weights were monitored and recorded daily, and the colon length was measured as a parameter for colitis severity. Subsequently, the colons were lavaged with PBS and transversely divided into several parts to use in histological analysis or RNA extraction.

Histological analysis

Mouse colons divided transversally were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 8μm thick sections and stained with hematoxylin and eosin (H&E). The degree of inflammation and epithelial damage on microscopic H&E stained sections of the colon was calculated as histological scores for four parameters: (i) severity of inflammation (0: no inflammation, 1: mild, 2: moderate, and 3: severe); (ii) thickness of inflammatory infiltration (0: no inflammation, 1: mild, 2: moderate, and 3: severe); epithelial damage score consisting of the (iii) character (0: intact epithelium, 1: disruption of architectural structure, 2: erosion, and 3: ulceration) and (iv) extent of lesions (0: no lesions, 1: punctuate, 2: multifocal, and 3: diffuse). The histological score was a combined score of (i) severity of inflammation and (ii) thickness of inflammatory infiltration, and the epithelial damage score consisted of (iii) character and (iv) extent of lesions.

Isolation of intestinal epithelial cells

WT and Dectin-1 KO Mice (C57BL/6, Female or Male, 9 to 10 week old) were euthanized after oral administration of lentinan for 1 week, and the colons were removed and rinsed in calcium and magnesium-free HBSS for 5 min at 4°C in accordance with the procedures described by Kim et al. [20] and Su et al. [21]. The tissue was slit longitudinally and cut into each 1 cm pieces, and placed into calcium- and magnesium-free HBSS containing 5 mM EDTA and incubated at 37°C for 30 min with shaking. The colon epithelium was isolated with needles and monitored with the use of a stereo microscope SMZ 645. The isolated epithelium was kept in ice-cold HBSS until RNA extraction.

RNA isolation and reverse transcription

Total RNA was isolated from colon sections using RNAqueous with a Plant RNA Isolation Aid (Thermo Fisher Scientific) in accordance with the manufacturer's protocols. Additionally, total RNA was isolated in the case of Caco-2 cells using Sepasol RNA I super (Nacalai Tesque, Kyoto, Japan) in accordance with the manufacturer's protocols. The reverse transcription of the RNA for quantitative PCR was performed using a High-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). The reverse transcription reactions were performed in a thermal cycler (Gene Amp® PCR System 9700, Thermo Fisher Scientific) at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. In the assessment of RNAi and the construction of TNFR1-GFP, reverse transcription was conducted using ReverTra Ace-α® (TOYOBO, Osaka, Japan) and the reverse transcription reactions were performed in a thermal cycler at 30°C for 10 min, 42°C for 60 min and 99°C for 5 min.

Quantitative PCR

Quantitative PCR was conducted using a 7500 Fast Real Time PCR system and Taqman Fast universal PCR master mix kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. TaqMan probes were used in real-time PCR, and the product numbers were as follows; mouse TNF-α assay ID: Mm99999068_ m1, mouse IFN-γ assay ID: Mm99999071_m1, mouse IL1-β assay ID: Mm99999061_m1, mouse, mouse IL-6 assay ID: Mm99999064_m1, mouse IL-10 assay ID: Mm01288386_m1, mouse TNFR1 assay ID: Mm 00441815_m1, Human IL-8 assay ID: Hs00174103_m1Human CLEC7A (Dectin-1) assay ID: Hs 01902549_s1. Mouse β-actin assay ID: Mm00607939_s1 or human GAPDH Assay ID: Hs99999905_m1 was used as an endogenous control (Thermo Fisher Scientific). For all panels, the bars represent the ratio of the target gene to endogenous gene expression, as determined by the relative quantification method (ΔΔCT).

Western blotting analysis

Protein was extracted with radioimmunoprecipitation buffer (25 mM Tris-HCl, pH 7.6; 150 mM NaCl; 1% NP-40; 0.1% SDS; 1 % sodium deoxycholic acid; 500 mM DTT; 20 μg/ml leupeptin). The protein concentration was assessed using a BCA™ Protein Assay Kit (Pierce, Rockford, IL). The samples (30 μg) were mixed with sample buffer (125 mM Tris-HCl, pH 6.8; 4% SDS; 10% 2-mercaptoethanol; 0.2 % bromophenol blue; and 20 % glycerol) in a 1:1 ratio, boiled at 95°C for 3 min, and separated on a 12 % polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with Blocking One (Nacalai Tesque) for 1 h and incubated with the primary antibody against Dectin-1 (1:1000) overnight at 4°C. The membrane was incubated with HRP-conjugated secondary antibody against goat IgG-heavy and light chain (1:5000) for Dectin-1 primary antibody or against mouse IgG (1:5000) for β-actin primary antibody at room temperature for 30 min. The blot of Dectin-1 and β-actin was detected by Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan) in accordance with the recommended procedure, and then the membrane was exposed to X-Ray Film (Fujifilm, Tokyo, Japan) in a film cassette.

Cloning of TNFR1 expression vectors

The cDNA of TNFR1 in Caco-2 cells was amplified by PCR reaction using KOD FX Neo (TOYOBO). The reactions were performed in a thermal cycler at 94°C for 2 min, 40 cycles of 98°C for 10 sec, 58°C for 30 sec, and 68°C for 1 min. The primers included the following;

forward, 5'-AAGAGCTCATGGGCCTCTCCACC-', reverse, 5'-AAGGTACCTCTGAGAAGACTGGG-'. The PCR products were cloned into the Kpn I and Sac I sites of the vector pUC118 (Takara Bio, Shiga, Japan). TNFR1 WT and TNFR1 lacking intracellular domain ($TNFR1^{\Delta ID}$) were generated by PCR from a pUC118 TNFR1 construct and cloned in frame in pEGFP-N3 vector (Takara Bio). For the PCR reaction of $TNFR1^{\Delta ID}$, the forward primer was same as TNFR1 WT with reverse primer was the following; 5'-CCGGTACCATACATTAAACCAATGAAGAG-'.

Confocal microscopy

Caco-2 cells (1×10^5) were seeded into 3 cm glass-based dishes (Matsunami Glass, Osaka, Japan). pEGFP vectors containing the various TNFR1 constructs were transfected using Lipofectamine 3000 (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. The cells expressing TNFR1-GFP were treated with lentinan (500 μg/ml) for 1 h, and then fixed in 4% paraformaldehyde. The cells were blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories, PA, USA) in PBS for 1 h. They were incubated with anti-human TNFR1 antibody (0.25 μg/ml) for 1 h, followed by incubation with Alexa 594-conjugated secondary antibodies for 30 min. These samples were observed using a confocal microscope (LSM510; Carl Zeiss, Oberkochen, Germany). Anti-Dectin-1 antibody and isotype antibody were preincubated for 30 min prior to lentinan treatment.

Total internal fluorescence microscopy

Caco-2 cells (3×10^5) were seeded into 3 cm glass-based dishes (AGC Techno Glass, Shizuoka, Japan). Cells expressing $TNFR1^{\Delta ID}$-GFP constructs were prepared as described for confocal microscopy. The culture medium was changed to Ringer's solution (165 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5 mM HEPES-NaOH, 10 mM Glucose, pH 7.4) or lentinan solution (500 μg/ml in Ringer's solution) and the cells were incubated at 37°C for 1 h. The images were acquired every 2 sec for 15 min with a total internal reflection fluorescence (TIRF) microscope using an Olympus IX-71 inverted microscope (Olympus, Tokyo, Japan) with ion lasers (Melles Griot) through an objective lens (×100 oil immersion objective, numerical aperture =1.45; Olympus) and with a Cascade II cooled charge-coupled device camera (Photometrics). For the inhibition of endocytosis, cells expressing $TNFR1^{\Delta ID}$-GFP were pretreated with dancylcadaverine at a final concentration of 100 μM for 30 min before lentinan treatment.

Statistical analysis

Each result was expressed as the mean ± standard error (M ± SE). Statistical significance between any two groups was analyzed using the Student's t-test. Statistical significance between more than two groups was analyzed by Dunnett's test, two-way ANOVA test, and Tukey-Kramer test. Statistical significance was defined as $p \leq 0.05$ or 0.01.

Results

Suppressive effect of lentinan is disrupted by an anti-Dectin-1 antibody, but not by an anti-TLR2 antibody

To identify the receptor that recognized lentinan and induces the anti-inflammatory properties in a co-culture system composed with Caco-2 and RAW264.7 cells [18], anti-Dectin-1 or anti-TLR2 antibodies were applied to Caco-2 cells prior to lentinan treatment of the cells. As shown in Figure 1, neutralization by anti-Dectin-1 antibodies restored IL-8 mRNA expression level significantly in Caco-2 cells to the same level as cells stimulated with LPS, but anti-TLR2 antibodies did not. On the contrary, TNF-α production by RAW 264.7 cells did not show any differences with any of the treatments tested except for with budesonide as a positive control, suggesting that they did not affect RAW264.7 cells (Figure 1).

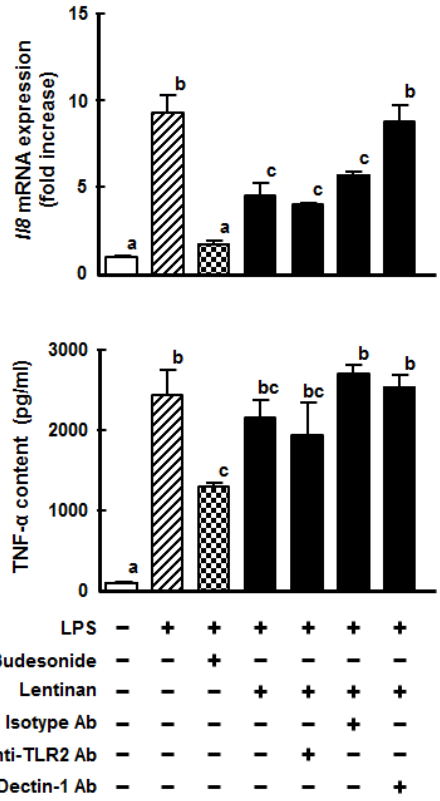

Figure 1: Suppressive effect of lentinan is disrupted by an anti-Dectin-1 antibody, but not by an anti-TLR2 antibody. Caco-2 cells were pretreated with an anti-Dectin-1 or anti-TLR2 antibodies for 30 min. Lentinan (500 μg/ml) was added to the apical sides in the Caco-2/RAW264.7 co-culture model, followed by 3 h incubation. Then, LPS was added to the basolateral sides at a final concentration of 10 ng/ml followed by 3 h incubation. The level of IL-8 mRNA expression in Caco-2 cells and TNF-α release from RAW264.7 cells were measured by quantitative PCR and or cytolytic assay using L929 cells. Values represent the means ± SE (n=3). Different superscript letters indicate statistical the significance between groups ($P \leq 0.05$).

Dectin-1 shRNA transfection abolished the suppressive effect of lentinan on IL-8 mRNA levels

Because Dectin-1 was predicted as one of the receptors that result in decreased IL-8 mRNA expression with lentinan treatment (Figure 1), Dectin-1 shRNA transfected Caco-2 cells were prepared to confirm the participation of Dectin-1. The level of Dectin-1 mRNA and Dectin-1 protein was detected by a quantitative PCR and western blotting,

indicating approximately 40% and 50% reductions in Dectin-1 shRNA transfected Caco-2 cells compared with the control (data not shown). As shown in Figure 2, TNF-α production from LPS-stimulated RAW264.7 cells in control shRNA or Dectin-1 shRNA transfected Caco-2 cell co-culture systems was not influenced by lentinan treatment. Budesonide reduced TNF-α production in both systems, suggesting the RAW264.7 cells and transfected Caco-2 cells were functioning in a normal manner. Lentinan inhibited IL-8 mRNA expression in a control co-culture system, but not in Dectin-1 shRNA transfected Caco-2 cells, demonstrating that lentinan was recognized by Dectin-1 in Caco-2 cells.

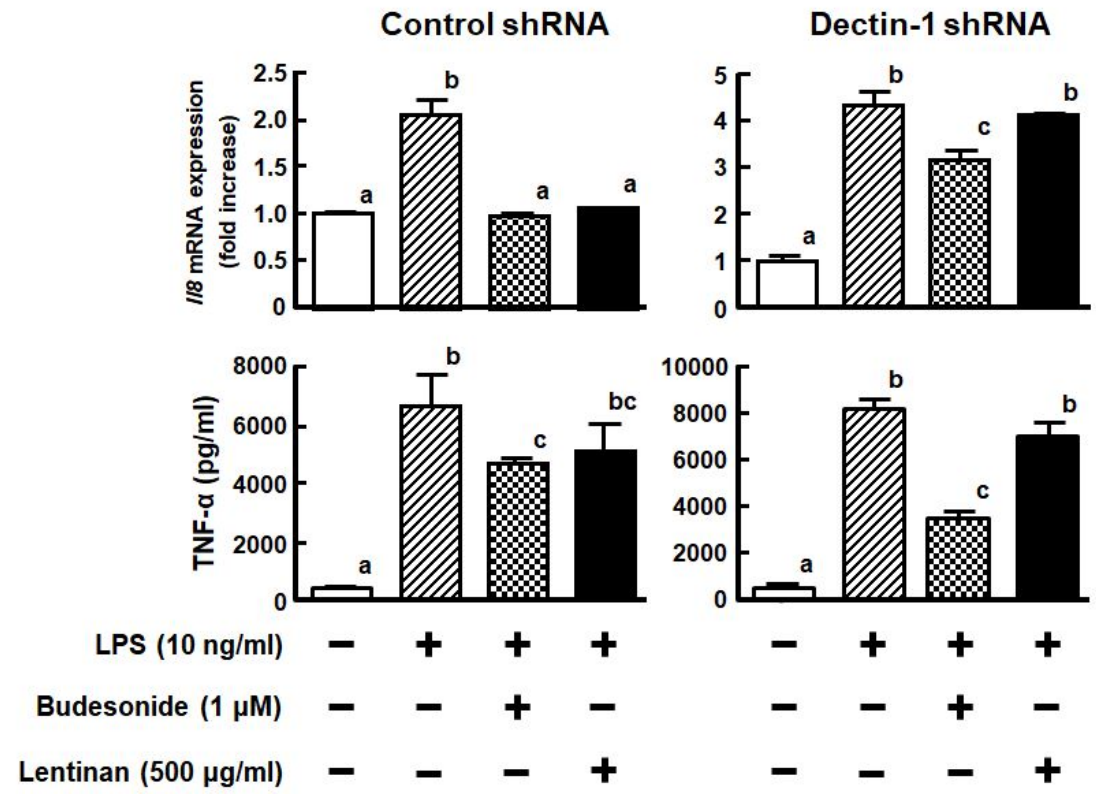

Figure 2: Dectin-1 shRNA transfection abolished the suppressive effect of lentinan on IL-8 mRNA levels. Dectin-1 shRNA or control shRNA was transfected into Caco-2 cells, as described in the Materials and Methods. Lentinan (500 μg/ml) was added to the apical sides in the shRNA transfected Caco-2/RAW264.7 co-culture model. TNF-α release from RAW264.7 cells and IL-8 mRNA expression levels in Caco-2 cells were measured. Value represent the means ± SE (n=3). Different superscript letters indicate the statistical significance between groups ($P \leq 0.05$).

Dectin-1 knockout abolished lentinan suppressive effects in a DSS-induced colitis murine model

Nishitani et al. [11] reported that lentinan was effective at improving symptoms, macroscopic and histological scores, and decreased mRNA levels of the pro-inflammatory cytokines in DSS-induced colitis mice. Here, the effects of lentinan on DSS-induced colitis were studied using Dectin-1 KO mice (Figure 3A). In WT mice, lentinan (100 μg/mouse) significantly suppressed body weight loss by about 10% on Days 7, 8, and 9 (Figure 3B). Shortening of the colon length was also significantly improved by lentinan treatment compared with the DSS-treated group (Figure 3C). On the other hand, in Dectin-1 KO mice, neither body weight loss (Figure 3B) nor shortening of colon length (Figure 3C) were rescued by lentinan administration. As shown in Table 1 and Figure 3D, the DSS-treated group induced a significant increase in histological score compared with the non-colitis groups (control) in both WT and KO mice. The total colitis score of the lentinan treated group (6.5 ± 0.25) was significantly improved compared with the DSS-treated group (10.0 ± 0.2) in WT mice. Oral administration of lentinan inhibited the increase in histological score, especially for the two parameters of damage degree and lesion size, compared with the DSS-treated group in WT mice. However, in Dectin-1 KO mice, lentinan did not inhibit the increase in histological scores for any parameters.

	Wild type		Dectin-1 KO	
	DSS	Lentinan	DSS	Lentinan
Inflammation				
Severity	2.75 ± 0.22	1.25 ± 0.22**	2.25 ± 0.22	2.75 ± 0.22
Thickness	2.50 ± 0.26	1.75 ± 0.22	2.5 ± 0.26	2.5 ± 0.26
Epidermal damage				
Character	2.0 ± 0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0
Extent	2.75 ± 0.22	1.50 ± 0.26*	2.75 ± 0.22	2.75 ± 0.22
Total	10 ± 0.29	6.5 ± 0.25**	9.5 ± 0.25	10.25 ± 0.24

Severity of inflammation, thickness of inflammatory cell infiltration, character and extent of epithelial damage were scored as described in Materials and Methods. Values represent the means ± SE (n=4). *, p≤0.05, **, p≤0.01 versus each DSS treated group.

Table 1: Histological score of DSS-induced colitis.

Lentinan suppressed expression of pro-inflammatory cytokines in a DSS-induced colitis model of WT and Dectin-1 KO mice

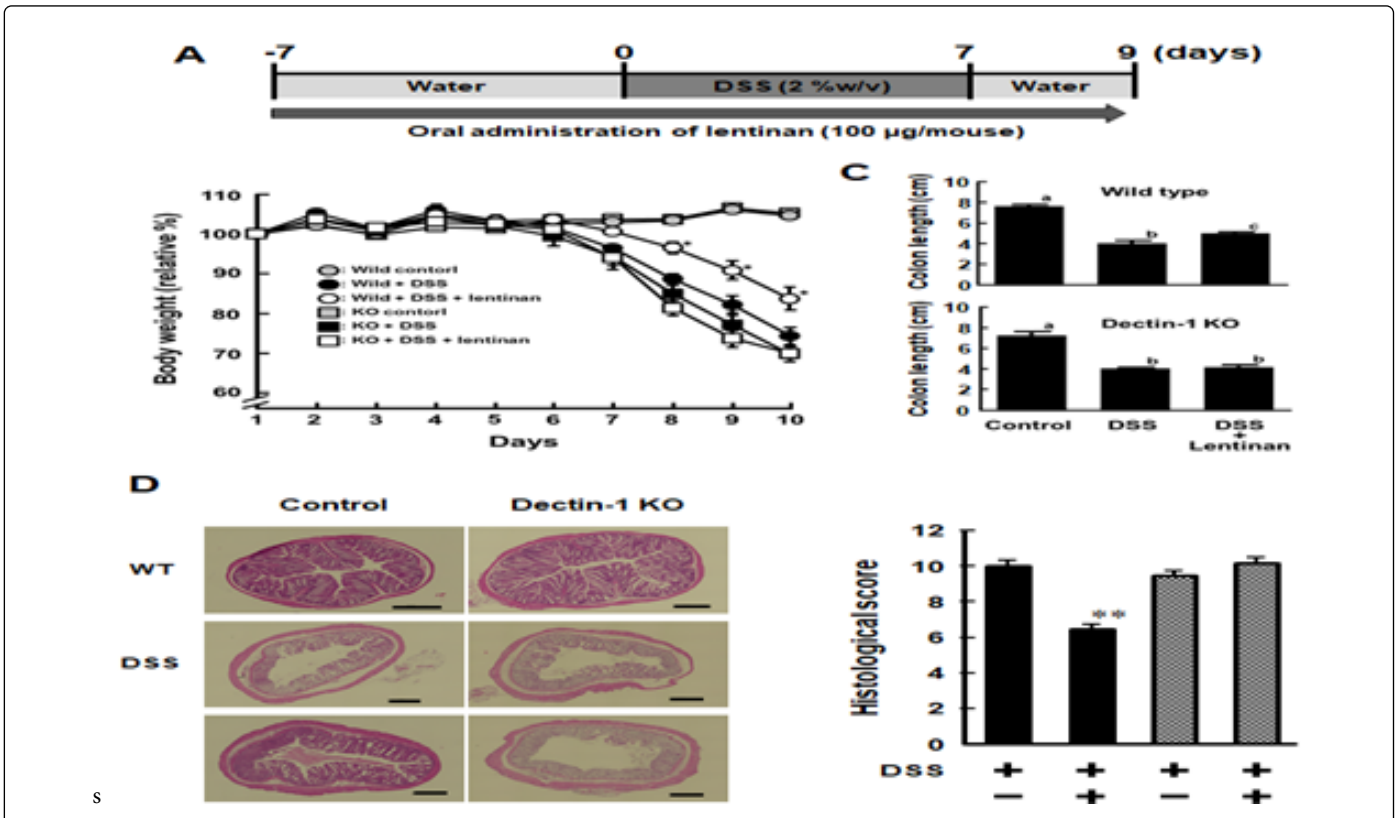

Figure 3: Dectin-1 knockout abolished lentinan suppressive effects in a DSS-induced colitis murine model. (A) Schedule of the DSS-induced colitis experiment. Oral administration of lentinan (100 μg/mouse/day) was started 7 days before DSS drinking and continued until the last day of the experiment. (B) The body weight of WT and Dectin-1 KO mice was measured each day. Values are presented as % of starting body weight. The colon lengths of WT and Dectin-1 KO mice were measured (C) and tissue sections were stained with H&E (D). Value represent the means ± SE (n=4). Different superscript letters indicate the statistical significance between groups (P ≤ 0.05). **P ≤ 0.01 *versus* control group.

It was reported that DSS-induced colitis was characterized by a predominantly Th1-mediated inflammatory response [22]. Moreover, lentinan reduced Th1-type cytokine expression in the DSS-induced colitis model [11]. To determine the effect of lentinan on Th1 cytokine

production in Dectin-1 KO mice, their mRNA levels were measured by real-time PCR. As shown in Figure 4, mRNA expression levels of TNF-α, IFN- γ, and IL-1β were significantly increased in both DSS-treated WT and Dectin-1 KO mice compared with in DSS-untreated mice. IL-6 mRNA expression was significantly increased in DSS-treated mice only in WT mice but not in KO mice. The oral administration of lentinan suppressed the expression of TNF-α, IL-1β, and IL-6 in WT mice. IFN-γ mRNA expression showed a tendency to decrease in response to lentinan treatment, although the difference was not significant. On the other hand, all cytokine mRNA expression levels showed no drastic differences between the DSS-treated group and those administered with lentinan in Dectin-1 KO mice. These results indicated that the anti-inflammatory effect of lentinan in DSS-induced colitis was mediated by the Dectin-1 receptor.

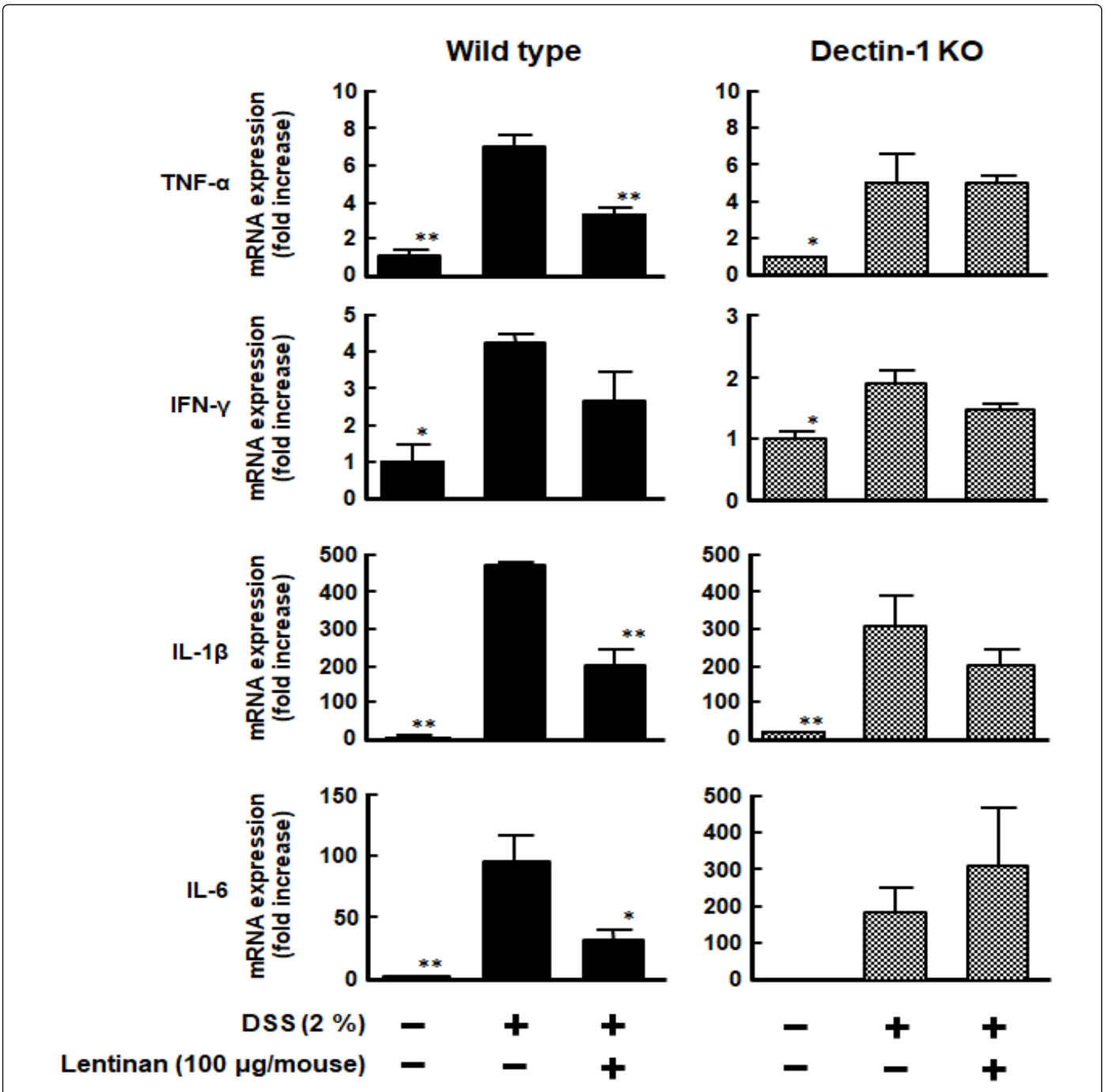

Figure 4: Lentinan suppressed expression of pro-inflammatory cytokines in a DSS-induced colitis model of WT and Dectin-1 KO mice. TNF-α, IFN-γ, IL-1β, and IL-6 mRNA expression levels in the colon tissues of WT and Dectin-1 KO mice in a DSS-induced colitis model were assessed by quantitative PCR. Value represent the means ± SE (n=3). *P ≤ 0.05, **P ≤ 0.01 *versus* DSS-treated group.

Lentinan reduced TNFR1 mRNA expression levels in WT and Dectin-1 KO mouse intestines

It was reported that lentinan treatment significantly decreased TNFR1 mRNA expression in mouse IECs [11]. Nenci et al. [23] demonstrated that TNFR1 inactivation inhibited intestinal inflammation, and TNFR1 signaling was crucial for the induction of colitis. To investigate whether the suppressive effect of lentinan for TNFR1 expression was mediated by Dectin-1, the levels of TNFR1 mRNA expression in intestines of WT or Dectin-1 KO mice orally administered lentinan (100 μg/mouse/day) for 1 week were measured. As shown in Figure 5, TNFR1 mRNA expression was suppressed in both the colon and ilium in WT mice but not in Dectin-1 KO mice. These results revealed that lentinan suppressed TNFR1 expression through Dectin-1.

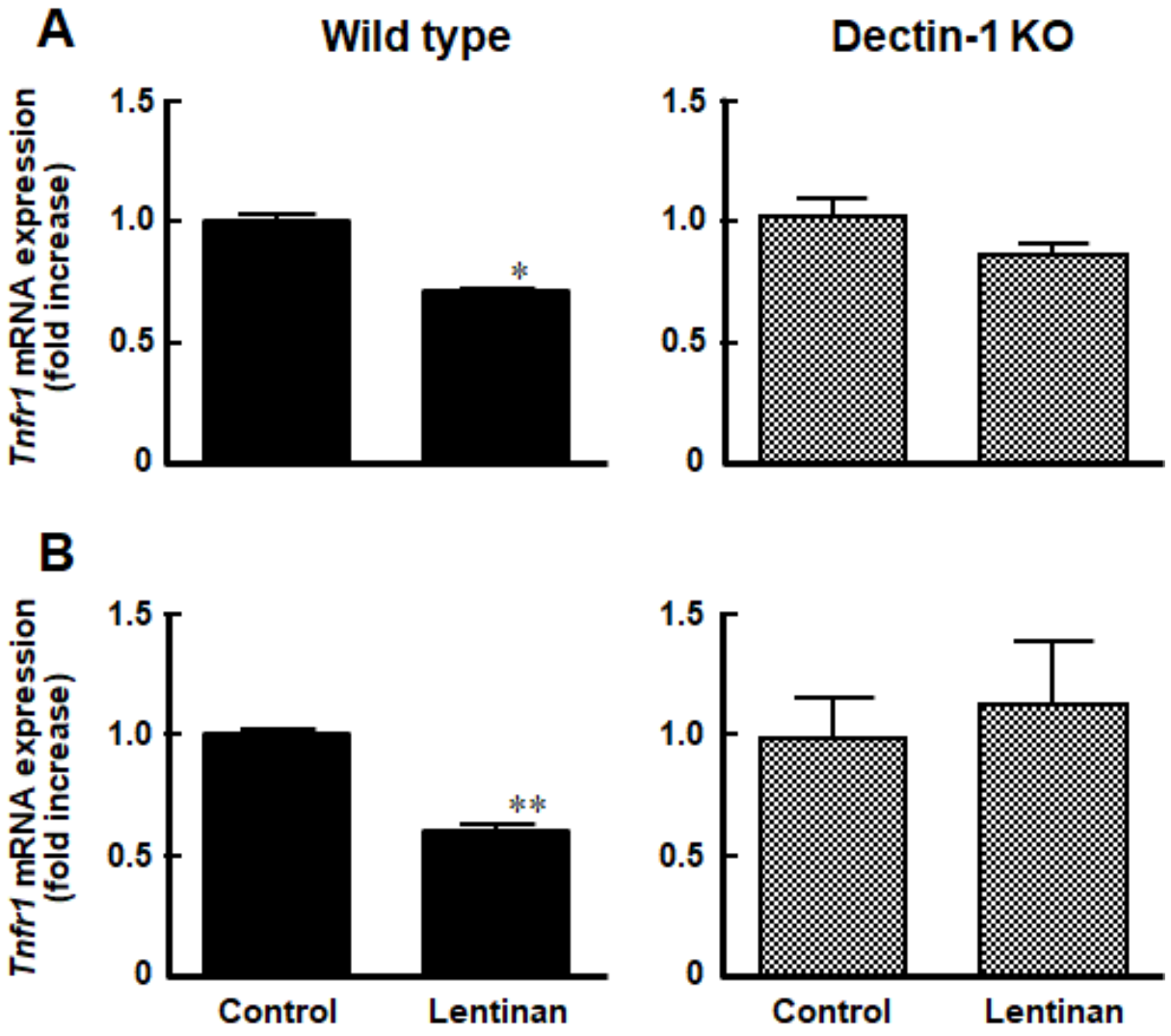

Figure 5: Lentinan reduced TNFR1 mRNA expression levels in WT and Dectin-1 KO mouse intestines. TNFR1 mRNA expression levels in small intestinal (A) or colonic (B) epithelial cells in WT or Dectin-1 KO mice were measured by quantitative PCR. Value represent the means ± SE (n=3). *P ≤ 0.05, **P ≤ 0.01 *versus* control group.

Lentinan inhibited TNFR1 expression on cell surface through Dectin-1

Nishitani et al. [11] reported that TNFR1 endocytosis from the surface of IECs contributed to a decline in TNFR1. To confirm the effect of lentinan on the cell surface expression of TNFR1, TNFR1-GFP plasmids were constructed and transfected into Caco-2 cells, and TNFR1-GFP on the surface of the plasma membrane was immunostained with a primary antibody for TNFR1 and an Alexa 594 Fluor secondary antibody without permeabilization. For this experiment, TNFR1 with a truncated the intracellular domain and GFP fusion protein ($TNFR1^{\Delta ID}$-GFP) were used because full-length TNFR1 and GFP fusion protein aggregated and was not expressed on the cell membrane. As shown in Figure 6, the expression of $TNFR1^{\Delta ID}$-GFP on the cell membrane was suppressed by lentinan treatment.

However, the suppressive effect of lentinan was abolished when the cells were neutralized with an anti-Dectin-1 antibody. It indicated that lentinan reduced the expression of TNFR1 on the cell membrane through Dectin-1.

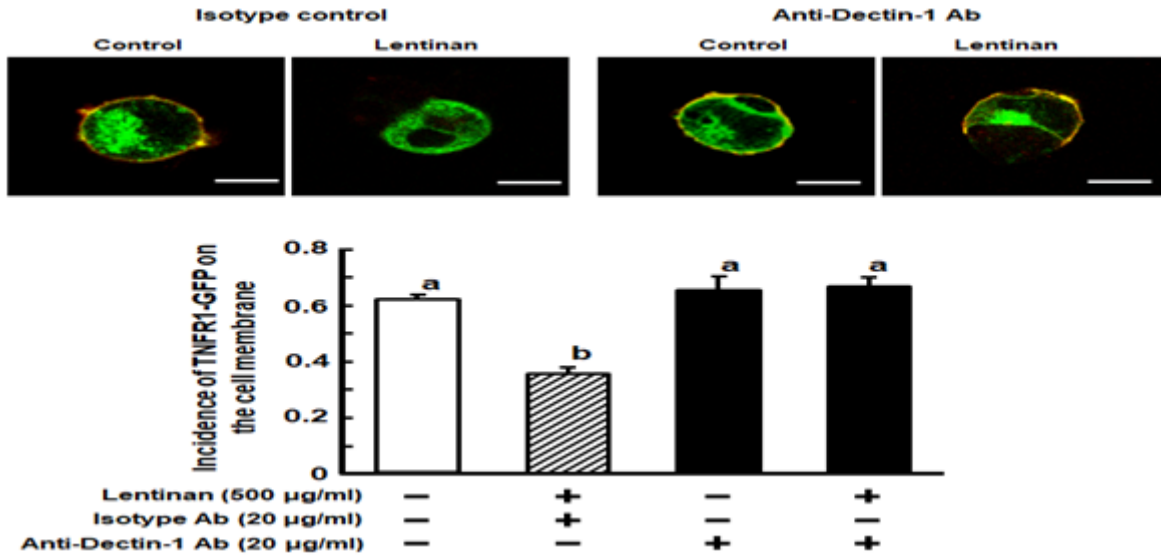

Figure 6: Lentinan inhibited TNFR1 expression on cell surface through Dectin-1. TNFR1ΔID-GFP was transfected into Caco-2 cells and the surface of the cells was immunostained with anti-TNFR1 and Alexa 594-conjugated antibodies, as described in the Materials and Methods. TNFR1ΔID-GFP expression on cell membrane was assessed by observation using confocal microscopy. Value represent the means ± SE (n=3). Different superscript letters after the values indicate statistical significance between groups ($P \leq 0.05$). Scale bar is 20 µm.

Lentinan-induced decrease of TNFR1 surface expression is independent of endocytosis

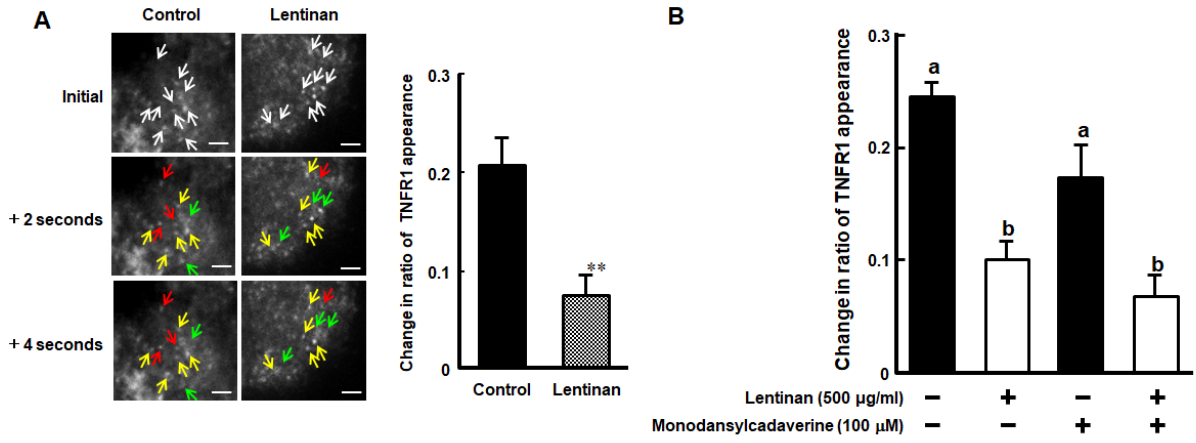

Figure 7: Lentinan-induced decrease of TNFR1 surface expression is independent of endocytosis. (A) TNFR1$^{\Delta ID}$-GFP was transfected into Caco-2 cells and the cells were treated with lentinan (500 µg/ml), as described in the Materials and Methods. TNFR1 expression on the cell membrane was assessed by observation using TIRF microscopy. Red arrows- recruited TNFR1, yellow arrows-maintained TNFR1, and green arrows-tapering TNFR1. The change in ratio of TNFR1 appearance was calculated using the following equation; (the number of recruiting TNFR1–the number of tapering TNFR1)/the number of total TNFR1. (B) Cells transfected TNFR1$^{\Delta ID}$-GFP were pretreated with monodansylcadaverine for 30 min and then treated with lentinan for 1 h. Values represent the means ± SE (n=3). **$P \leq 0.01$ versus control group. Different superscript letters indicate statistical significance values between groups ($P \leq 0.05$). Scale bar is 2 µm.

It was demonstrated that lentinan reduced the surface levels of TNFR1 in Caco-2 cells (Figure 6). To address the possibility that lentinan tapered TNFR1 on the plasma membrane, TNFR1$^{\Delta ID}$-GFP was expressed in Caco-2 cells and the movement of TNFR1$^{\Delta ID}$-GFP

molecules was observed under total internal reflection fluorescence (TIRF) microscopy (Figure 7). From the fluorescence changes over time, the number of tapering TNFR1 on the cell surface excessed that of TNFR1 recruited by lentinan treatment (Figure 7A), which was consistent with the confocal microscopy results (Figure 6). In particularly, TNFR1 recruitment decreased with lentinan treatment, but the number of tapering TNFR1 was almost constant. Nishitani et al. [11] reported that the suppressive effect of lentinan on epithelial TNFR1 expression after receptor endocytosis may be one of the key mechanisms for its anti-inflammatory activity in IECs. To confirm whether lentinan evoked the endocytosis of TNFR1, monodancylcadaverine which inhibits clathrin-dependent endocytosis [24] was applied to pretreat Caco-2 cells with $TNFR1^{\Delta ID}$-GFP prior lentinan treatment. As shown in Figure 7B, no drastic change demonstrated in the cell surface was evident after lentinan treatment. These results suggested that lentinan did not promote TNFR1 endocytosis, but instead it inhibited the transfer of TNFR1 to the cell membrane.

Discussion

Dectin-1 and TLR2 are well known receptors of β-glucan and expressed on IECs [25]. These receptors are known to synergize with each other, but there are cases where a specific β-glucan is recognized by just one or the other of the two receptors. For example, it has been reported that Maitake D-fraction (MD-fraction), a purified soluble β-1,3-branched β-1,6-glucan obtained from *Grifola frondosa* (an oriental edible mushroom), directly induced the maturation of dendritic cells through Dectin-1 but not TLR2 [26]. On the other hand, zymosan present in *Saccharomyces cerevisiae* as a cell wall component was recognized simultaneously by Dectin-1 and TLR2 [12,27]. In this study, as shown in Figure 1, it was revealed that anti-TLR2 antibody treatment did not affect the suppression of IL-8 mRNA expression in Caco-2 cells induced by lentinan in a co-culture system, but an anti-Dectin-1 antibody inhibited it. The experiment using silencing of Dectin-1 in Caco-2 cells also demonstrated that the inhibition of IL-8 mRNA expression by lentinan was cancelled completely in Dectin-1 shRNA transfection (Figure 2). These results suggested that lentinan was recognized through Dectin-1, not TLR2, on the surface of IECs and inhibited the increase in IL-8 mRNA expression.

The intestinal anti-inflammatory activities of lentinan *in vivo* were investigated using a DSS-induced colitis model in Dectin-1 KO mice. The deficiency of Dectin-1 did not affect the development of DSS-induced colitis, consistent with previous reports [28]. Lentinan inhibited the symptoms of DSS-induced colitis and pro-inflammatory cytokine production in WT mice, whereas lentinan did not suppress them in Dectin-1 KO mice (Figure 3 and 4). These results demonstrated that lentinan suppressed gut inflammation through Dectin-1.

The mechanism of how lentinan suppressed intestinal inflammation has been demonstrated as follows: 1) lentinan suppressed TNFR1 expression on the cell surface of IECs; 2) translocation of NF-kB into the nucleus was decreased; and 3) the expression of inflammatory chemokines such as IL-8 was suppressed [11]. Therefore, this study investigated how lentinan induced the down-regulation of TNFR1 expression. An *in vitro* assay using TNFR1-GFP transfection indicated that lentinan was recognized through Dectin-1 in Caco-2 cells and decreased TNFR1 level on the cell membrane (Figure 6). Furthermore, the observation of motion of TNFR1 molecules on the cell surface suggested that lentinan did not induce the endocytosis of TNFR1, but instead it suppressed the transfer of TNFR1 to the cell membrane (Figure 7), which was unlike the previous study [11]. TNFR1 is normally primarily localized within the Golgi storage pools and subsequently part of the TNFR1 pool is trafficked to the cell surface [29]. TNFR1 maintained in the Golgi-pool was degraded in the cytoplasm when it shifted from the Golgi to cytoplasm. The data in this study suggested that lentinan suppressed TNFR1 transfer to cell surface and promoted its degradation.

Conclusion

Our study determined that the receptor for lentinan is Dectin-1, and that this recognition induced the regulation of TNFR1 transfer to the cell membranes in IECs. Finally, the decrease in TNFR1 level on the cell surface contributed to the expression of anti-inflammatory activity by lentinan.

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