The inhibitory effect of oral administration of lentinan on DSS-induced inflammation is exerted by the migration of T cells activated in the ileum to the colon

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Title page

Full title: The inhibitory effect of oral administration of lentinan on DSS-induced inflammation is exerted by the migration of T cells activated in the ileum to the colon

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23 **Declarations of interest:** None

24

Abstract

Oral administration of lentinan ameliorated dextran sulfate sodium (DSS)-induced colitis through Dectin-1 receptor on intestinal epithelial cells. However, it is unclear where lentinan affects in the intestine to prevent the inflammation. We found that the administration of lentinan has induced migration of $CD4^+$ cells from the ileum to the colon by using Kikume Green-Red (KikGR) mice in this study. This result suggests that the oral lentinan treatment could accelerate the migration of Th cells in lymphocyte from ileum into the colon during lentinan intake. Then, C57BL/6 mice were administered 2% DSS to induce colitis. The mice were administered lentinan daily via oral or rectal route before DSS administration. Its rectal administration also suppressed DSS-induced colitis, but its suppressive effects were lower compared to when orally administered, indicating that the biological responses to lentinan in the small intestine contributed to the anti-inflammatory effects. In normal mice (without DSS treatment), the expression of *Il12b* was significantly increased in the ileum by the oral administration of lentinan, but not by rectal one. On the other hand, no change was observed in the colon by either administration method. In addition, *Tbx21* was significantly increased in the ileum. These suggested that IL-12 was increased in the ileum and Th1 cells differentiated in dependence on it. Therefore, Th1 predominant

condition in the ileum could influence immunity in the colon and improve the colitis.

Key words:

DSS-induced colitis, inflammatory bowel disease, intestinal immunity, Kikume Green-Red (KikGR) mice, lentinan, *Lentinula edodes*

Highlights:

- The oral administration of lentinan induces a migration of $CD4^+$ cell to the colon
- The stimulation of lentinan in small intestine induces an anti-inflammatory effect
- A lentinan treatment increases IL-12 and induces Th1 differentiation in the ileum
- The immune response of the Th1 cells improves the colitis in a colon

Abbreviations:

BMDCs, Bone marrow-derived dendritic cells; CD, Crohn's disease; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; IL, Interleukin; INF-γ, Interferon-γ ; KikGR, Kikume Green-Red; RT-PCR, Reverse transcription-polymerase chain reaction; Th1, Type 1 helper T; Th17, T helper 17; TER, Transepithelial electrical resistance; TNF-α, Tumor necrosis factor-α; TNF receptor 1, Tumor necrosis factor receptor 1; UC, Ulcerative colitis.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory state in the intestinal tract that is related to abnormal immune responses. Although the mechanism of development of IBD is still unknown, the pathology has been well studied (Cummins et al., 2013). IBD is divided into Crohn's disease (CD) and ulcerative colitis (UC). CD involves chronic inflammation over a wide range from the small intestine to the large intestine and is categorized as Type 1 helper T (Th1)/T helper 17 (Th17)-type enteritis (Abraham and Cho, 2009; Strober and Fuss, 2011). On the other hand, UC is associated with limited chronic inflammation in the large intestine and is categorized as Th2-type enteritis (Strober and Fuss, 2011). Several papers have reported mouse models corresponding to these diseases; 2,4,6-trinitrobenzene sulfonic acid-induced colitis and IL-10 knockout mice for CD (Goettel et al., 2011; Zhang et al., 2015), and carrageenan-induced colitis and dextran sulfate sodium (DSS)-induced colitis for UC (Elson et al., 1995). Immunosuppressants including prednisone and tacrolimus (Landy et al., 2013; Hicks et al., 2015), and the anti-tumor necrosis factor (TNF)-α antibody infliximab (Marits et al., 2014) are used for therapeutic interventions against IBD. However, because of their side effects, several studies have recently explored the efficiencies of food factors, curcumin (Deguchi et al., 2007) and luteolin (Nishitani et al., 2013a), with

82 anti-colitis activity.

83 Lentinan is a β-1,3-1,6-glucan isolated from a hot water extract of *Lentinula edodes*
84 (Shiitake mushroom) (Sasaki and Takasuka, 1976) and possesses physiological
85 activities, such as antitumor properties (Wasser, 2002). Our previous study has shown
86 that daily oral administration of lentinan (100 μg/mouse) suppressed DSS-induced
87 colitis (Nishitani et al., 2013b). According to the *in vitro* intestinal inflammatory model
88 using a co-culture system with intestinal epithelial Caco-2 cells and macrophage
89 RAW264.7 cells (Tanoue et al., 2008), the treatment with lentinan to the apical side
90 suppressed the expression of IL-8 mRNA in Caco-2 cells, which were stimulated by
91 TNF-α secreted from LPS-treated RAW264.7 cells (Mizuno et al., 2009). It was also
92 reported that lentinan decreased TNF receptor 1 (TNFR1) expressed on the basolateral
93 side of Caco-2 cells, resulting in a reduction in susceptibility to TNF-α in Caco-2 cells
94 (Nishitani et al., 2013b). Furthermore, an oral administration of lentinan could stimulate
95 Dectin-1, a β-glucan receptor (Sakaguchi et al., 2018), to ameliorate the inflammatory
96 disease on intestinal epithelial cells. These reports support the hypothesis that lentinan
97 affected inflammatory sites in the colon through the small intestine, and suppressed
98 colitis. On the other hand, lentinan is reported to affect immune cells directly in an
99 immunomodulatory manner (Wasser, 2002). In the small intestine there exist many

100 Peyer's patches which contain several immunocompetent cells (Brayden et al., 2005).
101 These reports suggest that the anti-inflammatory effects of lentinan on colitis may be
102 involved with not only direct actions on the large intestine but also actions in the small
103 intestine (Nishitani et al., 2013b; Mizuno et al., 2009). To evaluate a utilization of oral
104 lentinan administration, in the present study, that effect on the DSS-induced colitis was
105 examined in comparison with the effect of rectal administration. Moreover, we utilized
106 KikGR, a photoconvertible protein, knock-in mice (Niwa et al., 1991; Tsutsui et al.,
107 2005) to trace Th cells migration from small intestine to large intestine in the DSS-
108 induced colitis. We here indicated that the suppressive effects of the oral administration
109 of lentinan on the DSS-induced colitis were mediated by the stimulation of Th immune
110 responses in a small intestine.
111

112 **2. Materials and Methods**

113 **2.1 Reagents**

114 Lentinan derived from Shiitake mushroom was kindly provided by Ajinomoto (Tokyo,
115 Japan). The authentic lentinan was a β-1,3-1,6-glucan with 400 kDa of molecular mass
116 (Minato et al., 2004). DSS (molecular weight; 36,000–50,000) was purchased from MP
117 Biomedicals (Santa Ana, CA), Dulbecco's modified Eagle's medium containing 4500

mg/ml glucose was purchased from Wako Pure Chemical Industries (Osaka, Japan). RPMI1640 medium and minimum essential media non-essential amino acids were purchased from Gibco BRL (Grand Island, NY). Recombinant mouse IL-4 and recombinant mouse GM-CSF were purchased from R&D system (Minneapolis, MN). Fetal bovine serum (FBS) was purchased from Biological Industries (Beit-Haemek, Israel). All other reagents were of the highest grade commercially available.

2.2 Mice

This study was approved by the Institutional Animal Care and Use Committee (Permission number: 28-10-04) and carried out in accordance with the Kobe University Animal Experimentation Regulations. Female 6-week-old C57BL/6NCrSlc mice were purchased from Japan SLC (Shizuoka, Japan). Mice were acclimated for 1 week with *ad libitum* access to a basal diet (DC-8; Clea Japan, Tokyo, Japan) and tap water in a temperature-controlled room at 23 ± 2°C and at 50 ± 10% humidity with a 14-h light and 10-h dark cycle at Kobe University Life-Science Laboratory, and then used for animal experiments.

All KikGR mice (B6.Cg-Gt(ROSA)26Sor<tm1.1(CAG-kikGR)Kgwa> mice (Niwa et al., 1991; Tsutsui et al., 2005) used in this study were bred in specific pathogen-free

136 facilities at Meijo University. All experimental procedures were approved according to
137 the guideline by the Institutional Animal Care and Use committee of Meijo University
138 Faculty of Pharmacy and Faculty of Agriculture (Permission number: 2020PE15).
139 Colitis was induced by DSS administration in 10-12 weeks old KikGR mice. The mice
140 were given a daily administration of 100 μg lentinan dissolved in 50 μl of sterilized
141 water via oral route for 14 days from 7 days before the DSS administration. The mice
142 were administered *ad libitum* 2% (w/v) DSS in drinking water to induce colitis for 7
143 days (Day 0 to Day 7) and then given drinking water for 2 days (Day 8 and Day 9). On
144 Day 7 the photoconversion of cells in the ileum was performed under anesthesia with
145 1% isoflurane. Non-photoconverted regions were protected from light using aluminum
146 foil. The region of the intestine targeted for photoconversion was drawn out from the
147 abdominal cavity, and exposed to violet light (405 nm, 54 mW/cm^2) for 2 minutes from
148 the front and behind following laparotomy (Tomura et al., 2014). The photoconvertible
149 protein, KikGR, is irreversibly converted from green to red upon exposure to violet
150 light. Following photoconversion the abdominal wall was closed with bulldog forceps.
151 To keep exposed tissues moist during exposure to light, warmed PBS was applied to the
152 region of photoconversion. The mice were by 1% isoflurane induction on Day 9, and
153 the ileum and colon were removed. Preparation of cell suspensions from ileums and

colons were performed according to the method by Nakanishi et al. (2018). Briefly, the resected guts were washed in PBS, stirred in 1 μM EDTA buffer (pH 8.0) at 37 °C, and minced with scissors. The minced tissue was stirred for 80 min at 37 °C in collagenase (1.5 mg/m; Fuji Film-Wako, Japan) and dispase (1 U/ml; Fuji Film-Wako) containing RPMI-1640 medium. Cell suspensions were filtered with 40 μm cell strainer. Fluorochrome-conjugated antibodies were obtained from BioLegend (San Diego, CA). For flow cytometric analysis, cells were washed with Dulbecco's PBS containing 2% FBS. Next, cells were incubated with Mouse BD Fc Block™ (BD Pharmingen) to block Fc binding, then stained with Allophycocyanin (APC) mAbs (for CD4; BioLegend, 100515) or FITC-labeled (for CD25; BioLegend, 102005) mAbs for 15 min at 4°C. For intracelleur staining, the cells were fixed and permeabilized by using Mouse Foxp3 Buffer set (BD Pharmingen), and then stained with Brilliant Violet 421-conjugated anti-FoxP3 antibody (BioLegend, 126419). Stained samples were acquired using LSRFortessa™ X-20 Cell Analyzer (BD Bioscience). KikGR- green and red signals were detected using 530/30 and 610/20 bandpass filters, respectively. Flow cytometry data were analyzed using Flowjo software (BD Biosciences).

2.3 Dextran sulfate sodium-induced colitis

172 Mice (7 weeks old) were administered *ad libitum* 2% (w/v) DSS in drinking water to
173 induce colitis for 7 days (Day 0 to Day 7) and then given drinking water for 2 days (Day
174 8 and Day 9). For administration with lentinan, the mice were anesthetized using
175 isoflurane and given a daily administration of 100 μg lentinan dissolved in 50 μl of
176 sterilized water via an oral or rectal route with a 3 cm flexible feeding needle from 7
177 days before the DSS administration. Mice were euthanized by 1% isoflurane induction
178 on Day 9, and the ileum and colon were removed. In an experiment without DSS-
179 induced colitis, mice were given a daily administration of 100 μg lentinan for 16 days
180 using same methods, and then the ileum and colon were removed from mice euthanized
181 by 1% isoflurane after this time.
182
183 **2.4 Cell culture**
184 Human intestinal epithelial Caco-2 cells were maintained in Dulbecco's modified
185 Eagle's medium containing 4500 mg/ml glucose supplemented with 10% inactivated
186 FBS, 1% minimum essential media non-essential amino acids, 100 U/ml penicillin, and
187 100 mg/ml streptomycin. Cells were seeded in 100 mm dishes at 5.0×10^5 cells/dish or
188 24-well Transwell insert culture plates (0.33 cm^2, 0.4 μm pore size) at 4.0×10^4
189 cells/well. The integrity of the Caco-2 monolayer on the Transwell insert, *i.e.*, the

190 formation of tight junctions and barrier function, were confirmed by the measurement of
191 transepithelial electrical resistance (TER). For TER measurement, the cell monolayers
192 were washed with Hank's balanced salts solution composed of 137 mM NaCl, 5.36 mM
193 KCl, 1.67 mM $CaCl_2$, 1 mM $MgCl_2$, 1.03 mM $MgSO_4$, 0.44 mM KH_2PO_4, and 0.34 mM
194 Na_2HPO_4, pH 7.4 at 37°C for 30 min, followed by renewal with fresh Hank's balanced
195 salts solution. The resistance value (Ω) was measured using a Millicell-ERS instrument
196 (Millipore; Billerica, MA), and TER ($\Omega \cdot cm^2$) was calculated as the value (Ω) × culture
197 area (0.33 cm^2). The monolayers whose TER was more than 400 $\Omega \cdot cm^2$ were used in
198 this study.
199
200 **2.5 Preparation of bone marrow-derived dendritic cells**
201 Preparation of bone marrow-derived dendritic cells (BMDCs) was performed by the
202 method of Inaba et al. (1992) and Kang & Lim (2012) with a slight modification. The
203 thighbone and shinbone were removed from female C57BL/6NCrSlc mice (8 weeks
204 old), immersed in 70% ethanol for 5 min, and washed with PBS. All mice were
205 anesthetized and euthanized as above. The bone marrow was removed from the bones
206 with RPMI1640 medium containing 10% inactivated FBS, 2 mM L-glutamine, 100
207 U/ml penicillin, and 100 mg/ml streptomycin and loosened by pipetting. The cells were

208 filtered through a 70 μm cell strainer and collected by centrifugation at 300 *g* for 3 min
209 followed by treatment with hemolytic buffer (BD Bioscience; Franklin Lakes, NJ) and
210 washing with RPMI1640. For differentiation toward BMDCs, the cells were suspended
211 in RPMI1640 containing 10 ng/ml GM-CSF, 10 ng/ml IL-4, and 5 μM 2-
212 mercaptoethanol and seeded in 100 mm culture dishes at 3.0×10^7 cells/dish. Fresh
213 medium was supplied at intervals of 2 days. After 6 days, $CD11c^+$ BMDCs were
214 isolated using RoboSep (Stemcell Technologies; Vancouver, Canada) using an EasySep
215 mouse CD11c Positive Selection Kit (Stemcell Technologies) in accordance with the
216 manufacturer's protocol. BMDCs were seeded in 24-well plates at 7.0×10^5 cells/well.
217 Some of the wells were used for a co-culture system with Caco-2 cells seeded on a
218 Transwell insert and treated with 100 μg lentinan in 200 μl RPMI1640 medium. The
219 remaining wells were directly treated with 100 μg lentinan in 1 ml RPMI1640 medium.
220 The dose of lentinan was chosen according to the previous report (Nishitani et al.,
221 2013a). That amount did not any cytotoxicity for several cells such as Caco-2,
222 Raw264.7, and mouse intestinal epithelial cells. BMDCs were harvested for the
223 extraction of total RNA 6 h after each treatment.
224

225 **2.6 Real-time reverse transcription-polymerase chain reaction (RT-PCR)**

226 The expression of mRNAs was measured quantitatively using a real-time reverse
227 transcription-polymerase chain reaction (real-time RT-PCR) with an Applied
228 Biosystems 7500 Fast (Applied Biosystems; Foster City, CA) and related reagent kits in
229 accordance with the manufacturer's protocol. Total RNA was extracted from mouse
230 ileum and colon using an RNAqueous Kit (Applied Biosystems) and Plant RNA
231 Isolation Aid (Ambion; Austin, TX), respectively. The extraction of total RNA from
232 BMDCs was performed with Sepasol-RNA I Super G (Nacalai Tesque; Kyoto, Japan).
233 cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit
234 (Applied Biosystems) with a thermal cycler (Gene Amp PCR System 9700, Applied
235 Biosystems) programmed at 25°C for 10 min, at 37°C for 120 min, and then at 85°C for
236 5 min. Real-time PCR was performed using Taqman Fast Advanced Master Mix
237 (Applied Biosystems) as the PCR enzyme solution and TaqMan Gene Expression
238 Assays Inventoried (Applied Biosystems) as the PCR primer set as follows;
239 Mm00607939_s1 for mouse *Actb*, Mm01168134_m1 for mouse *Ifng*, Mm01336189_m1
240 for mouse *Il1b*, Mm99999068_m1 for mouse *Tnf*, Mm00446190_m1 for mouse *Il6*,
241 Mm00445259_m1 for mouse *Il4*, Mm00439614_m1 for mouse *Il10*, Mm00434174_m1
242 for mouse *Il12b*, Hs99999905_m1 for human *GAPDH*, and Hs01042313_m1 for human
243 *TNF receptor 1*. After activation of uracil-*N* glycosylase at 50°C for 2 min and DNA

polymerase at 95°C for 20 sec, PCR was performed using the following protocol; denaturation program, 90°C for 3 sec, and annealing and elongation program, 60°C for 30 sec, repeated 40 times. The data were analyzed using the comparative CT method and normalized with housekeeping genes *Actb* and *GAPDH* as endogenous controls for mouse and human, respectively.

2.7 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed using TaKaRa Ex Taq Hot Start Version (Takara-Bio; Shiga, Japan) in accordance with the manufacturer's protocol with corresponding primers (Invitrogen; Carlsbad, CA): *Tbx21*, 5'-GGATGTTTGTGGATGTGGTCTTG-3' for forward and 5'-GGAACAGGATACTGGTTGGATAG-3' for reverse; and *Hprt*, 5'-TCCCAGCGTCGTGATTAG-3' for forward and 5'-GTCAAGGGCATATCCAACAAC-3' for reverse. PCR was conducted for 37 and 24 cycles for *Tbx21* and *Hprt*, respectively, following an initial denaturation for 2 min at 98°C, and each cycle consisted of denaturation (10 sec at 98°C), annealing (30 s at 59°C for *Tbx21* and at 57°C for *Hprt*), and extension (72 sec at 72°C), followed by an additional extension for 30 sec at 72°C. The reaction products were electrophoresed in a

2% agarose gel with TBE buffer (pH 8.2, 89 mM Tris, 89 mM boric acid, and 2 mM EDTA), and cDNA fragments (*Tbx21*, 547 bp and *Hprt*, 568 bp) were stained with 1 μg/ml ethidium bromide and visualized by UV irradiation. The bands were quantified using a Java-based image processing program, Image J. The expression of *Tbx21* was normalized with a housekeeping gene, *Hprt*, and the values were calculated as relative expression to vehicle control.

2.8 Statistical analysis

A one-way ANOVA was performed followed by the Tukey's multiple comparisons test in the KikGR experiments. Statistical significance was defined as $P<0.05$. The level of significance was stated in the figure legends. Data are expressed as the mean ± SE. Statistical analysis between two groups was performed using Student's t-test. Figs 4 and 5 were analyzed statistically using Dunnett's test, compared with DSS-treated mice. Statistical differences in changes in body weight in a DSS-induced colitis model were evaluated by analysis of variance (ANOVA) and the Tukey–Kramer test to determine differences between groups.

3. Results

3.1 Lymphocyte proliferation and migration from the ileum to the colon in the DSS-induced colitis KikGR mice

In order to quantify the effect of lentinan on recruiting lymphocytes in the gut of DSS-induced colitis mice, we analyzed the profiles of KikGR-red cells in the ileum and the colon. The ileum was exposed to violet light to induce photoconversion of the KikGR protein from green to red (KikGR-Red) (Fig. S1). As shown in Fig. 1A, it was shown that the red cells which was photoconverted in the ileum could proliferate and be recruited into the colon in the mice with the colitis by oral administration of lentinan. The 4.09 ± 0.16 % of KikGR-red cells increased to 5.60 ± 0.15 % by the lentinan treatment in the ileum of DSS-induced colitis mice. In addition, in the colon, it increased to 13.07 ± 0.03 %. These results could suggest that oral lentinan treatment induced a proliferation of lymphocytes in an ileum of a mouse, and then the cells were recruited into the colon during developing DSS-induced colitis. Moreover, we examined the level of $CD4^+$ cells (pan Th cells) in the lymphocytes recruited into a colon of DSS-induced colitis mice. As shown in Fig. 1B and C, the recruiting cells into the colon from the ileum was augmented by the lentinan treatment. The KikGR-red $CD4^+$ cells increased to 15.63 ± 0.07 % (with DSS plus lentinan treatment) as compared to the control group (7.57 ± 0.48 %) in the colon of the mice (Fig. 1C). Furthermore, Treg

298 cells ($CD4^+CD25^+FoxP3^+$ cells) also migrated into the colon of the colitis mice by oral
299 lentinan treatment. It significantly increased from 2.54 ± 0.22 to 5.55 ± 0.19 % ($p <$
300 0.05) (Fig. 1D). These results suggested that the recruit of Th and Treg cells from an
301 ileum into a colon was also induced by the lentinan oral treatment in the mice with
302 DSS-induced colitis.
303
304 **3.2 Effects of oral or rectal administration of lentinan on DSS-induced colitis in**
305 **mice**
306 To investigate whether lentinan affects not only the large intestine but also the small
307 intestine, suppressive effects on DSS-induced colitis were compared between two
308 administration routes of lentinan, oral and rectal. Treatment with 2% DSS began to
309 decrease body weight on Day 5, and significantly decreased body weight to 83.1±1.0%
310 on Day 9 compared with that on Day 0 (Fig 2). On the other hand, both oral and rectal
311 administration of lentinan suppressed the weight loss to 92.6±2.2% and 87.5±3.4% of
312 the weight on Day 0, respectively (Fig. 2). The suppressive effect of rectal
313 administration was obviously smaller than that of oral administration on Day 9
314 (p<0.05). These results promised that the effects on the small intestine contributed to the
315 anti-colitis activity more than that on the large intestine.

316

317 **3.3 Expression of cytokines in the colon of mice with DSS-induced colitis**

318 It was reported the expression levels of IFN-γ, IL-1β, TNF-α, IL-6, IL-4, and IL-10
319 mRNAs increased in the colon in studies on DSS-induced colitis (Elson et al., 1995;
320 Nishitani et al., 2013b; Yan et al., 2009; Adisakwattana et al., 2013). The effects of
321 lentinan administration on these mRNA expression levels were investigated using real-
322 time RT-PCR on Day 9. DSS-treatment increased the expression levels of *Ifng*, *Il1b*,
323 *Tnf*, *Il6*, *Il4*, and *Il10*. Both oral and rectal administration of lentinan tended to suppress
324 the expression levels but not significantly (Fig. 3). The oral administration of lentinan
325 suppressed *Ifng*, *Il1b*, *Tnf*, *Il6*, *Il4*, and *Il10* expression levels induced by DSS-treatment
326 to 37% (p=0.20), 14% (p=0.15), 58% (p=0.12), 21% (p=0.57), 34% (p=0.06), and 25%
327 (p=0.32), respectively. On the other hand, rectal administration of lentinan slightly
328 suppressed the expression levels of *Ifng*, *Il1b*, *Tnf*, and *Il4* to 70% (p=0.64), 43%
329 (p=0.37), 69% (p=0.27), and 62% (p=0.29), respectively, but those of *Il6* and *Il10* were
330 unaffected. These results indicated that the suppressive effects of oral administration of
331 lentinan were stronger than those of rectal administration, *i.e.*, it may be important for
332 the anti-colitis activity of lentinan to affect not only the large intestine but also the small
333 intestine.

334

335 **3.4 Expression of Th1 and Th2 cytokines in the ileum of mice with DSS-induced**

336 **colitis**

337 It was reported that Th1 and Th2 cytokines are associated with the development of

338 DSS-induced colitis (Dieleman et al., 1998). The expression levels of INF-γ and IL-4

339 mRNAs in the ileum were investigated as representative Th1 and Th2 cytokines,

340 respectively. Rectal administration of lentinan hardly affected the expression levels of

341 *Ifng* and *Il4* in the ileum of DSS-treated mice (Fig. 4). On the other hand, oral

342 administration of lentinan tended to upregulate *Ifng* expression (p=0.06) and

343 downregulate *Il4* expression (p=0.19). These results suggested that lentinan may

344 enhance Th1-type immune responses in the ileum of mice with DSS-induced colitis.

345

346 **3.5 Effects of oral and rectal administration of lentinan on *Ifng* in the ileum and**

347 **colon of mice without DSS-induced colitis**

348 According to results using mice with DSS-induced colitis, oral administration of

349 lentinan increased the mRNA levels of the Th1 cytokine *Ifng*. Because the effect might

350 be caused secondarily by suppressive effects on DSS-induced colitis, we investigated

351 the effects of lentinan on Th1-type immune responses in mice without DSS-induced

352 colitis. Intragastric administration of lentinan for 16 days significantly increased the
353 expression of *Ifng* in the ileum, but rectal administration significantly decreased it (Fig.
354 5A). Of note, oral administration tended to also increase *Ifng* expression in the colon,
355 whereas rectal administration did not (Fig. 5B). These results indicated that orally
356 administered lentinan enhanced Th1-type immune response in the ileum, and these
357 changes in immune responses might affect colorectal immunity.
358
359 **3.6 Effects of lentinan on *Tbx21* and *Il12b* expression in the ileum of mice without**
360 **DSS-induced colitis**
361 Th1 cells, one of the helper T cell subsets, are well known as IFN-γ producing cells
362 (Chen and Liu, 2009). To investigate if the increase in *Ifng* expression, as shown in Fig
363 6, was due to an increase in Th1 cells, *Tbx21* expression was analyzed as a marker gene
364 of Th1 cells by semi-quantitative RT-PCR. The oral administration of lentinan for 16
365 days increased *Tbx21* expression in the ileum of mice without DSS-induced colitis.
366 Because Th0 cells are known to require IL-12 for differentiation into Th1 cells (Zhu and
367 Paul, 2010), the expression of IL-12 p40 mRNA (*Il12b*) in the ileum was investigated
368 by real-time RT-PCR. Oral administration of lentinan increased *Il12b* expression in the
369 ileum of mice without DSS-induced colitis, but rectal administration did not (Fig. 7A).

370 On the other hand, both oral and rectal administrations hardly affected *Il12b* expression
371 in the colon (Fig. 7B). These results indicated that orally administered lentinan may
372 promote IL-12 production in the ileum, resulting in the differentiation of Th0 cells into
373 Th1 cells producing INF-γ in the small intestine.
374
375 **3.7 Effects of lentinan on IL-12 p40 mRNA expression in BMDCs**
376 Dendritic cells are known to produce IL-12 and to play an important role in the
377 differentiation of helper T cells (Heufler et al., 1996). To investigate the involvement of
378 dendritic cells in the increase in *Il12b*, BMDCs were treated with 100 μg lentinan
379 through a monolayer of Caco-2 cells in a Caco-2/BMDC co-culture system, in which
380 Caco-2 cells and BMDCs were placed on the apical and basolateral sites, respectively. A
381 preliminary experiment demonstrated that lentinan decreased *tnfr1* expression in Caco-2
382 cells in the co-culture system after 6 h of treatment (Fig. 8A), consistent with our
383 previous study (Nishitani et al., 2013b). Treatment of Caco-2 cells of the co-culture
384 system with lentinan hardly changed *Il12b* expression in BMDCs (Fig. 8B). On the
385 other hand, the direct treatment of BMDCs with lentinan significantly increased *Il12b*
386 expression (Fig. 8C). These results indicated that lentinan directly stimulated dendritic
387 cells followed by the induction of IL-12 production, but not through intestinal epithelial

388 cells.

389

390 **4. Discussion**

391 Lentinan, a β-glucan derived from *Lentinula edodes*, is well-known to possess several
392 pharmacological effects (Wasser, 2002; Ina et al., 2013). The present study examined
393 which mechanism was mediated in the anti-colitis activity of lentinan, the direct
394 stimulation of large intestine or the indirect stimulation of small intestine. Our previous
395 study reported that oral administration of lentinan suppressed DSS-induced colitis in
396 mice (Nishitani et al., 2013b). Therefore, it was hypothesized that lentinan could
397 stimulate a cell signaling in the small intestine, and then activated cells there were
398 recruited into the large intestine and could play a pivotal role in preventing a colitis
399 development. A quantification of endogenous Th cells migration through gut is
400 fundamental of understanding the dynamics of prevention mechanism for colitis. To
401 track the cell migration from ileum to colon without irritation, in the present study we
402 used KikGR mice which had been generated by Tomura et al. (2014) and analyzed a
403 transition of the cells in gut by using FACS analyzer. We here showed that oral lentinan
404 treatment induced a $CD4^+$ (Th) cell migration from the ileum to the colon during
405 developing DSS-induced colitis (Fig. 1B, C). Tomura et al. (2010) have also reported

406 that $CD4^+$ cells could migrate from mesenteric lymph nodes to a site of inflammatory
407 lesion, and those cells could facilitate a prevention of the inflammatory disease. Our
408 current results, furthermore, suggest that $CD4^+CD25^+FoxP3^+$ (Treg) cells were recruited
409 into the colon of mice with DSS-induced colitis by the lentinan treatment (Fig. 1D).
410 Indeed, even rectal administration of lentinan significantly suppressed body weight loss
411 associated with colitis in the mouse model. However, oral administration of lentinan
412 showed more suppressive effects on body weight loss and the mRNA expression levels
413 of cytokines compared with rectal administration (Figs. 2 and 3). These results indicated
414 that lentinan exerted anti-colitis effects mainly through a stimulation in the small
415 intestine.
416 It was unknown which immune response is associated with the anti-inflammatory
417 effects on DSS-induced colitis. Our study indicated that an increase in *Infg* expression
418 was observed in mice administered lentinan by an oral route but not by a rectal route
419 (Fig. 4). In addition, the administration of lentinan significantly increased *Ifng*
420 expression in the ileum of DSS-untreated mice (Fig. 5A). Oral administration of
421 lentinan also showed a tendency to decrease *Il4* expression in the ileum of DSS-treated
422 mice. IFN-γ and IL-4 are well known as Th1 and Th2 cytokines, respectively (Zheng
423 and Flavell, 1998; Szabo et al., 2000). IFN-γ is produced and secreted from $CD4^+$ T

424 cells as well as natural killer cells and γδT cells and $CD8^+$ T cells (Chen and Liu, 2009).
425 Furthermore, a marker gene for a Th1 cell, *Tbx21*, increased in the ileum of mice
426 administered orally with lentinan (Fig. 6). Stevceva et al. (Stevceva et al., 2001) showed
427 that DSS-induced colitis is ameliorated in IL-4 deficient mice compared with wild-type
428 mice. The present study demonstrated that lentinan exerted anti-inflammatory effects in
429 the large intestine accompanied with the induction of Th1 immune responses in the
430 small intestine. Those suggested that the enhancement of Th1 immune responses in the
431 ileum might be related to the suppression of Th2 immune responses in the colon,
432 resulting in the suppression of colitis. Furthermore, it was reported that $CD4^+$ T cells
433 differentiated in Peyer's patches and mesenteric lymph nodes migrated to the colon
434 (Nagai et al., 2007). Weng et al. (2007) reported that Th2 cells migrated from the small
435 intestine to the colon. Taken together, oral administration of lentinan might cause the
436 differentiation of Th1 cells capable of producing INF-γ in the ileum, and Th1 cells
437 might migrate from the small intestine to the large intestine, resulting in increases in
438 *Infg* in the colon. These findings suggested that the glucan increased Th1 cells, one of
439 the $CD4^+$ T cell subsets among IFN-γ-producing cells. Regarding a $CD4^+$ T cell (naïve
440 T cells/Th0 cells) differentiation as a result of several stimuli, IL-12 also induces
441 differentiation into Th1 cells (Chen and Liu, 2008; Zhu and Paul, 2010). Therefore, we

442 hypothesized as follows: 1) lentinan could promote IL-12 production from some cell
443 types, resulting in differentiation into Th1 cells, if not 2) lentinan directly stimulated T
444 cells and then induced their differentiation. IL-12 is a hetero-complex of p35 and p40
445 subunits. Although p35 mRNA (*Il12a*) is constantly expressed in ubiquitous cells, p40
446 mRNA (*Il12b*) is specifically expressed in IL-12-producing cells (Trinchieri, 2003).
447 Unlike *Ifng* expression, *Il12b* expression increased in the ileum but not in the colon.
448 Oral administration of lentinan for 16 days increased *Il12b* expression in the ileum (Fig.
449 7A). These results suggested that lentinan did not induce the migration of certain types
450 of cells such as a dendritic cell producing IL-12 from the ileum to the colon, supporting
451 the hypothesis that Th1 cells differentiated in the ileum and migrated to the colon. This
452 idea may be applied to the treatment of not only colitis but also other immune-related
453 diseases such as allergy, which is caused by an excessive immune response with Th2
454 cells (Maggi, 1998). Th1 cells induced by lentinan in the small intestine may migrate to
455 an allergic lesion and regulate the Th1 and Th2 balance, leading to the improvement of
456 allergy.
457 Kadowaki (2007) reported that the properties of dendritic cells were altered by
458 recognized bacterial components, resulting in changes in the subtypes of helper T cells.
459 Nagai et al. (2007) suggested that *Helicobacter pylori* induced IL-12 production from

460 dendritic cells in Peyer’s patches, resulting in the induction of Th1 cells. Thus, dendritic
461 cells are also well known to play a critical role in the differentiation of helper T cell
462 subsets. Batbayar et al. (2012) showed that β-glucans, despite their large size, can be
463 absorbed through M cells into Peyer's patches or directly through binding to the tips
464 projected into digestive tract lumen from the dendritic cells in Peyer’s patches. A
465 previous study using a co-culture system with Caco-2 cells and RAW264.7 cells
466 indicated that lentinan decreased TNF receptor 1 (TNFR1) on the basolateral side of
467 Caco-2 cells via Dectin-1 on the apical side, resulting in nonsusceptibility to the
468 inflammatory cytokine TNF-α secreted from RAW264.7 cells stimulated by
469 lipopolysaccharides (Nishitani et al., 2013b; Sakaguchi et al., 2018). The present study
470 demonstrated that treatment with lentinan through the Caco-2 cells hardly altered *Il12b*
471 expression in BMDCs in the co-cultured system (Fig. 8B). On the other hand, direct
472 treatment with lentinan increased *Il12b* expression in BMDCs (Fig. 8C), supporting the
473 possibility that lentinan stimulated dendritic cells in Peyer’s patches directly and
474 promoted IL-12 production from these cells. That might be the first step in the
475 mechanism by which the oral administration of lentinan could prevent the gut
476 inflammatory disease. However, it is still unknown whether lentinan is absorbed into
477 Peyer’s patches *in vivo*. Further studies will be needed to elucidate the mechanism by

478 which lentinan regulates the suppressive effect of dendritic cells on DSS-induced colitis.
479 The present results indicate the possibility that colitis might be controlled from the
480 small intestine by an intake of functional food factors such as lentinan.
481 In addition to the anti-inflammatory effects of lentinan on the colon by Th1-type
482 immune responses, we have suggested that Treg cells were recruited into the colon
483 during the curing of the colitis by the lentinan oral administration (data not shown). This
484 finding might suggest that a regulation of immunological environment, such as Th
485 balance, in the small intestine affects the colon, resulting in suppressive effects on
486 colitis.
487

488 **5. Conclusion**

489 In our study, the oral treatment of lentinan could be more effective in an inhibition of
490 the colitis than a rectal treatment. The administration of lentinan induced migration of
491 $CD4^+$ Th cells from the ileum to the colon in the DSS-induced colitis mice. Moreover,
492 the oral administration showed additive suppressive effects on body weight loss and the
493 mRNA expressions of cytokines in the mice ileum with DSS-induced colitis. Especially,
494 the expression levels of *Il12b* and *Tbx21* increased remarkably in the ileum, suggesting
495 an IL-12-dependent differentiation of Th1 cells in the ileum. Therefore, we considered

that DSS-induced colitis was inhibited by the Th1 cells, which were differentiated by IL-12 secreted from dendritic cells. Those cells might rather regulate a Th balance in the colitis than exacerbate the inflammation. Taken together, our current study suggests that the oral administration of lentinan regulates the balanced Th1 immune responses mainly in the ileum, and then exerts the suppressive effects on a colitis (Fig. 9).

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Author contributions:

Keigo Oura: Methodology, Validation, Formal analysis, Investigation. Ken-ichiro Minato: Methodology, Validation, Formal analysis, Investigation, Writing-Original Draft. Takashi Hashimoto: Validation, Writing-Original Draft. Masashi Mizuno: Conceptualization, Resources, Writing-review and editing, Supervision, Project administration.

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670

671 **Figure legends**

672 **Fig. 1 Migratory of CD4$^+$ cells from ileum to colon in the DSS-induced colitis**

673 **KikGR mice**

674 The mice were administered *ad libitum* 2% (w/v) DSS in drinking water to induce

675 colitis for 7 days (Day 0 to Day 7) and then given drinking water for 2 days (Day 8 and

676 Day 9). From 7 days before Day 0, the mice were daily administered 100 μl of lentinan

677 (200 μg/mouse) or sterilized water as a vehicle via an oral route for 14 days. On day 8,

678 the region of the intestine was exposed to violet light (405 nm, 54 mW/cm^2) for 2

679 minutes from the front and behind following laparotomy. The mice were killed on day

680 10. The cells were incubated with Mouse BD Fc Block^{TM} (BD Pharmingen) to block Fc

681 binding, then stained with Allophycocyanin (APC) (for CD4) mAb. (A) The level of

682 KikGR-Red cells (%) in lymphocyte of the ileum and the colon in the mice. The level of

683 CD4$^+$ cells in the ileum (B), and the colon of the mice (C). Moreover, the cells were

684 stained with FITC-labeled (for CD25) mAbs, and then with Brilliant Violet 421-

685 conjugated anti-FoxP3 antibody (D). Data are expressed as mean ± SE (n=3). For

686 statistical analysis, a one-way ANOVA followed by the Tukey multiple comparisons test

687 was used; there are significant differences between different letters ($p < 0.05$).

688

689 **Fig. 2 Effects of oral or rectal administration of lentinan on DSS-induced colitis**

690 **in mice**

691 Mice (C57BL/6NCrSlc) were administered *ad libitum* 2% (w/v) DSS in drinking water

692 to induce colitis for 7 days (Day 0 to Day 7) and then given drinking water for 2 days

693 (Day 8 and Day 9). From 7 days before Day 0, the mice were daily administered 50 μl

694 of lentinan (100 μg/mouse) or sterilized water as a vehicle via an oral or rectal route.

695 The body weight of mice was measured daily during the experiment and expressed as a

696 percentage of that on Day 0. Data are expressed as mean ± SE (n=4). *, $P < 0.05$; **, P

697 < 0.01; ***, $P < 0.001$, DSS (+), lentinan by a rectal route group (▨) or DSS (+),

698 lentinan by an oral route group (■) *versus* DSS (+), vehicle group (□). #, $P < 0.05$

699 DSS (+), lentinan by a rectal route group (▨) *versus* DSS (+), lentinan by an oral route

700 group (■).

701

702 **Fig. 3 Effects of lentinan administration on cytokine expression levels in the**

703 **colon**

704 Mice were sacrificed on Day 9. Total RNA was extracted from the colon, and *Ifng*, *Il1b*,

705 *Tnf*, *Il6*, *Il4*, and, *Il10* expression levels were determined by real-time RT-PCR, as

706 described in the *Materials and Methods*. The values except for *Il4* were calculated as

relative expression levels to untreated controls without DSS or lentinan. The values for *Il4* were calculated as the relative expression levels to mice treated with DSS but not lentinan. Data are expressed as mean ± SE (*n*=4).

Fig. 4 Effects of lentinan administration on cytokine expression levels in the ilium

The total RNA was extracted from the ilium of the same mice as analyzed in Fig. 3, and *Ifng*, and *Il4* expression levels were determined by real-time RT-PCR, as described in the *Materials and Methods*. The values were calculated as relative expression levels to those in untreated controls with DSS or lentinan. Data are expressed as mean ± SE (*n*=4).

Fig. 5 Different effects of oral and rectal administration of lentinan on *Ifng* expression in normal mice

Mice were daily administered lentinan (100 μg/mouse) for 16 days or sterilized water as a vehicle without DSS treatment via oral or rectal routes. Total RNA was extracted from the ilium (A) and colon (B) of mice, and *Ifng* expression levels were analyzed by real-time RT-PCR, as described in the *Materials and Methods*. The values were calculated as

relative expression levels to the vehicle control. Data are expressed as mean ± SE (n=4). *, $P < 0.05$; **, $P < 0.01$ *versus* vehicle control group.

Fig. 6 Effects of lentinan administration on *Tbx21* expression in the ilium

Tbx21 expression levels in the ilium of the same mice as used in Fig. 5 were analyzed by semi-quantitative RT-PCR as described in the *Materials and Methods*. The values were calculated as relative expression levels to the vehicle control. Data are expressed as mean ± SE (n=4). *, $P < 0.05$ *versus* vehicle control group.

Fig. 7 Effects of lentinan administration on *Il12b* expression in the ilium and colon

Il12b expression levels in the ilium (A) and colon (B) of the same mice as used in Fig. 5 were analyzed by real-time RT-PCR, as described in the *Materials and Methods*. The values were calculated as relative expression levels to the vehicle control. Data are expressed as mean ± SE (n=4). *, $P < 0.05$ *versus* vehicle control group.

Fig. 8 Effects of lentinan on *Il-12b* expression in BMDCs *in vitro*

Caco-2 cells in a co-culture system of Caco-2 cells and BMDCs were treated with 100

743 μg lentinan, as described in the *Materials and Methods*. The expression levels of *TNF*
744 *receptor1* in Caco-2 cells (A) and *Il12b* in BMDCs (B) were analyzed by real-time RT-
745 PCR 6 h after treatment. The values were calculated as relative expression levels to the
746 vehicle control. Data are expressed as mean ± SE (n=3). (C) BMDCs were cultured as a
747 monolayer and received by direct treatment with 100 μg lentinan. *Il12b* expression
748 levels in BMDCs were analyzed by real-time RT-PCR 6 h after treatments. The values
749 were calculated as relative expression levels to the vehicle control. Data are expressed
750 as mean ± SE (n=3). *, $P < 0.05$; **, $P < 0.01$ *versus* vehicle control.
751
752 **Fig. 9. The oral administration of lentinan induces the Th1 immune responses**
753 **mainly in the ileum, and then exerts the suppressive effects on a colitis**
754 In this study, it was ascertained that oral administration of lentinan enhanced Th1-type
755 immune responses mainly at ileum and then accelerate the migration of $CD4^+$ cells in
756 lymphocyte from ileum into the colon in which colitis was induced by DSS treatment.
757 These findings suggested that alternation of the immunological environment in the
758 small intestine affects the colon, resulting in suppressive effects on colitis. The present
759 results indicate the possibility that colitis might be controlled from the small intestine by
760 food intake, such as lentinan.

761

762 **Figure S1. Expression of KikGR-Green and KikGR-Red in the murine lymphocytes**

763 **before and after photoconversion.**

764 Upon exposure violet light for 2 minutes, cells were converted from green (KikGR-

765 Green) to red (KikGR-Red).

766

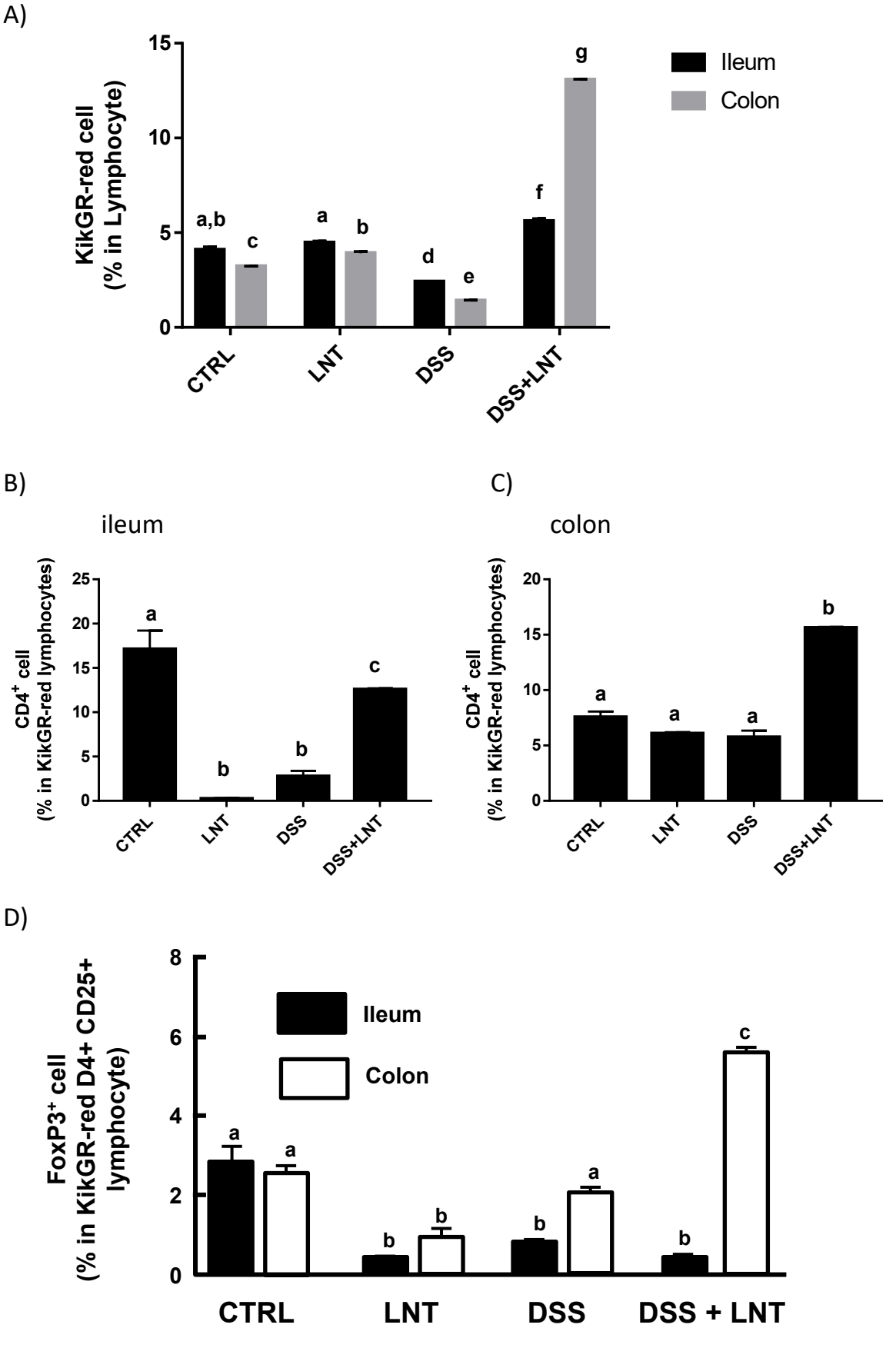
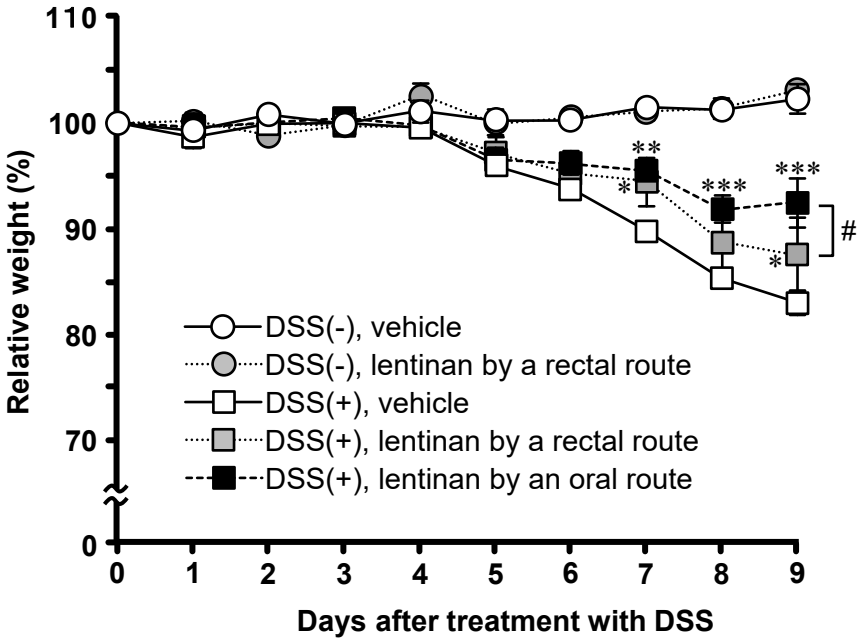
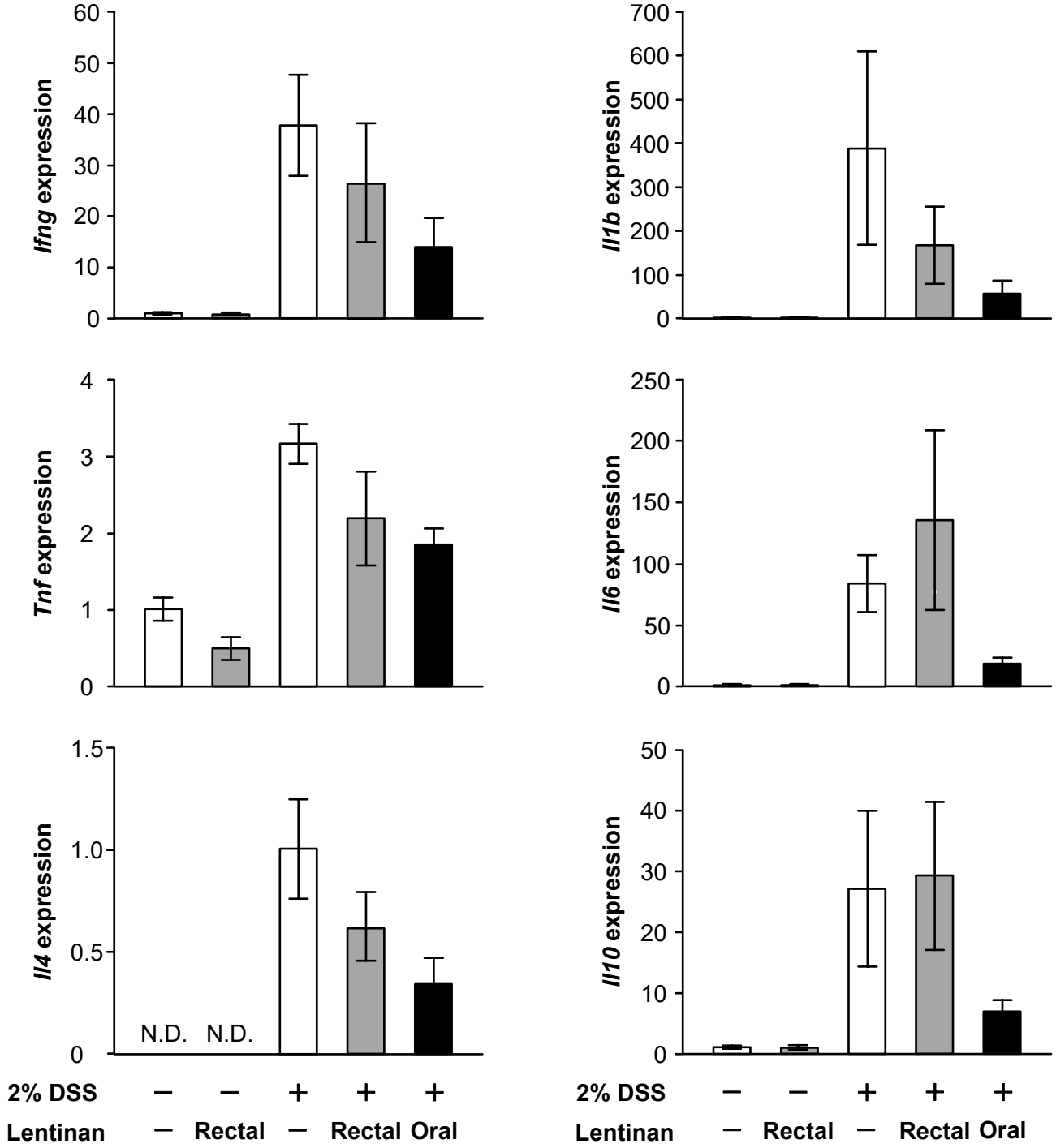

A)
KikGR-red cell (% in Lymphocyte)
Ileum
Colon
a,b
c
a
b
d
e
f
g
CTRL
LNT
DSS
DSS+LNT
B)
ileum
CD4+ cell (% in KikGR-red lymphocytes)
C)
colon
D)
FoxP3+ cell (% in KikGR-red D4+ CD25+ lymphocyte)
Up

Fig. 1


110
100
90
80
70
0
Relative weight (%)
0
1
2
3
4
5
6
7
8
9
Days after treatment with DSS
DSS(-), vehicle
DSS(-), lentinan by a rectal route
DSS(+), vehicle
DSS(+), lentinan by a rectal route
DSS(+), lentinan by an oral route
**
*
***
***
*
#

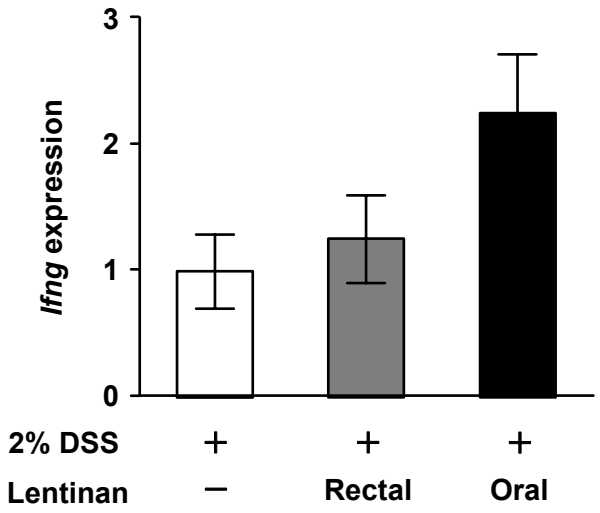
Up

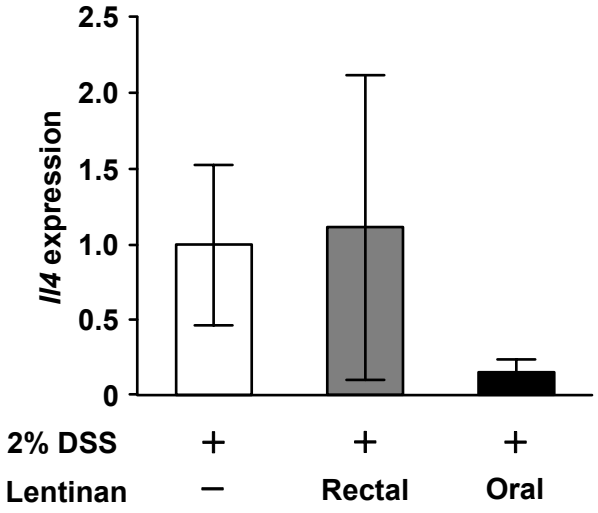
Fig. 2


Ifng expression
60
50
40
30
20
10
0
Il1b expression
700
600
500
400
300
200
100
0
Tnf expression
4
3
2
1
0
Il6 expression
250
200
150
100
50
0
Il4 expression
1.5
1.0
0.5
0
N.D. N.D.
Il10 expression
50
40
30
20
10
0
2% DSS − − + + +
Lentinan − Rectal − Rectal Oral
2% DSS − − + + +
Lentinan − Rectal − Rectal Oral

Up

Fig. 3

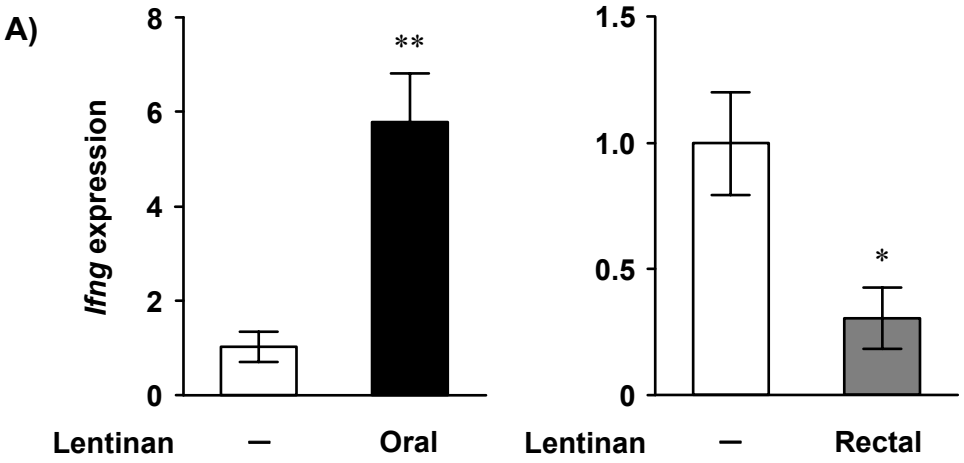

3
2
1
0
Ifng expression
2% DSS + + +
Lentinan − Rectal Oral


2.5
2.0
1.5
1.0
0.5
0
Il4 expression
2% DSS + + +
Lentinan − Rectal Oral

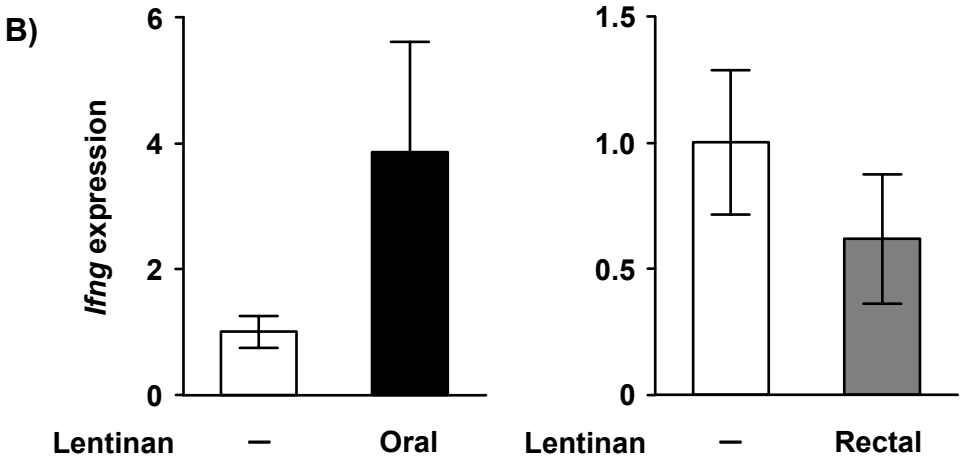
Up

Fig. 4

Ileum

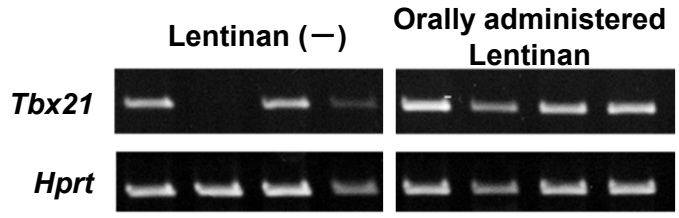


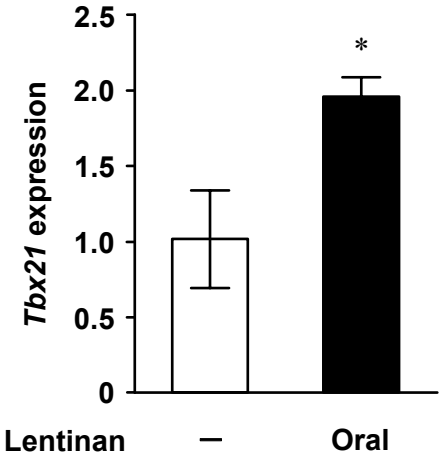
Colon



Up

Fig. 5

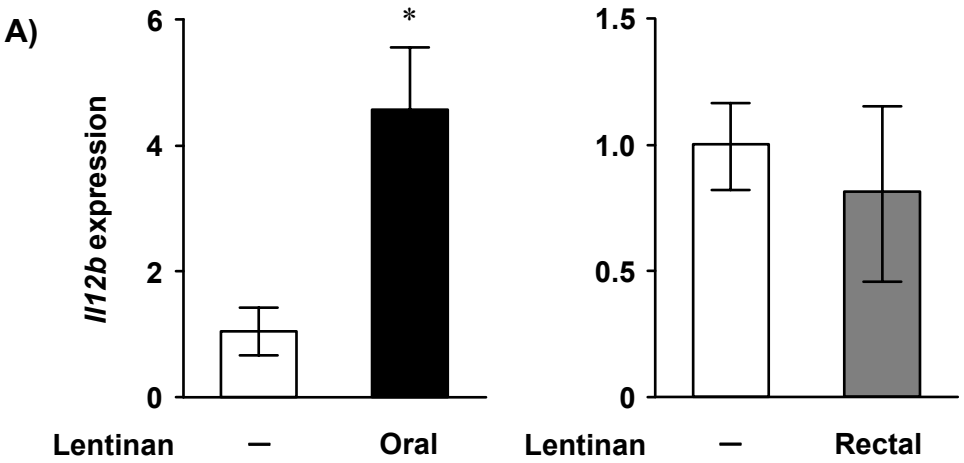

Lentinan (−)
Orally administered Lentinan
Tbx21
Hprt


2.5
2.0
1.5
1.0
0.5
0
Tbx21 expression
*
Lentinan
−
Oral

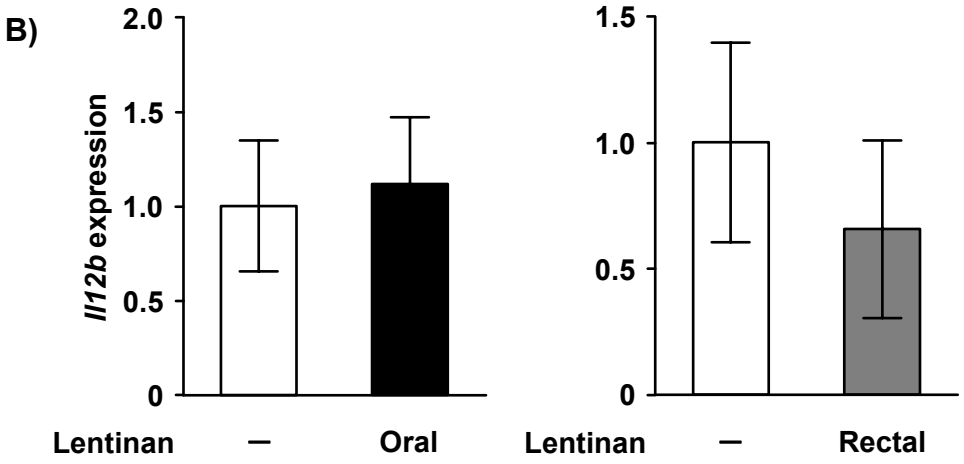
Up

Fig. 6

Ileum

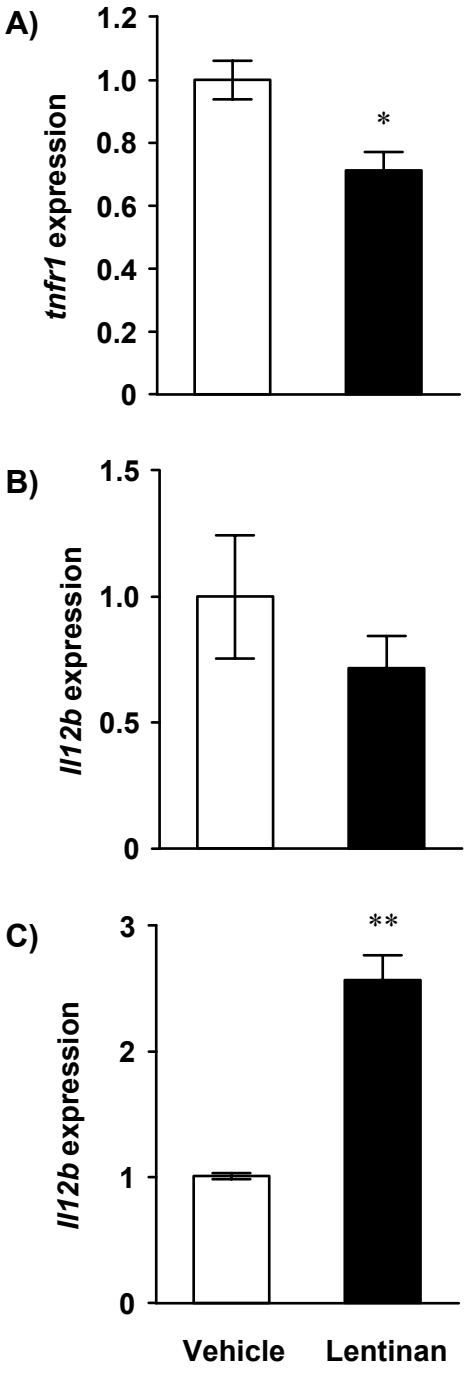


Colon



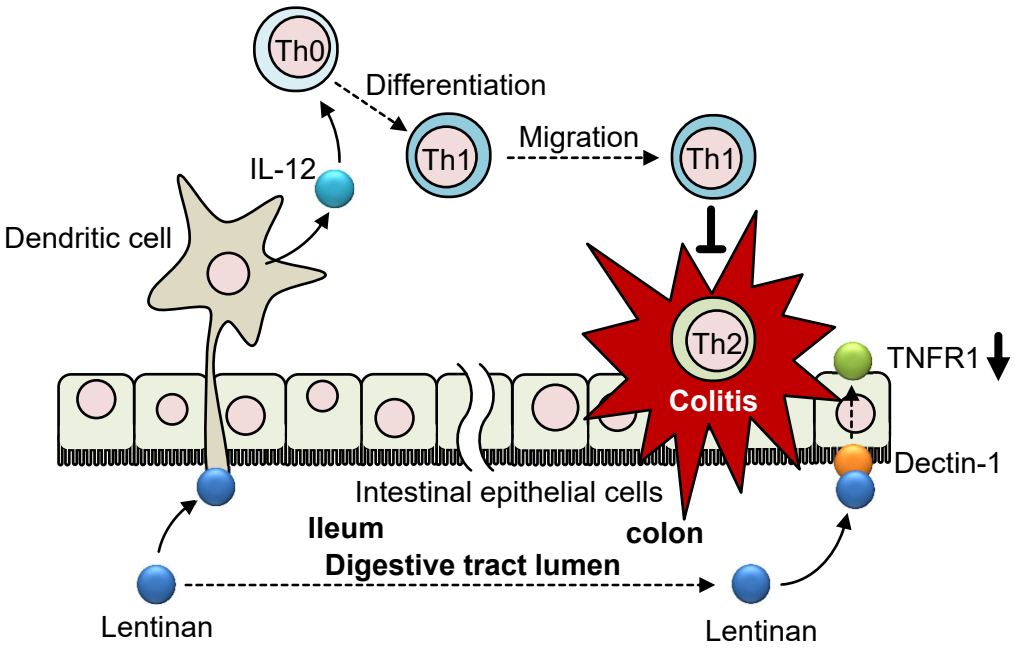
Up

Fig. 7


A)
1.2
1.0
0.8
0.6
0.4
0.2
0
tnfr1 expression
*
B)
1.5
1.0
0.5
0
Il12b expression
C)
3
2
1
0
Il12b expression
**
Vehicle
Lentinan

Up

Fig. 8


Th0
Differentiation
Th1
Migration
Th1
IL-12
Dendritic cell
Th2
Colitis
TNFR1
Dectin-1
Intestinal epithelial cells
Ileum
colon
Digestive tract lumen
Lentinan
Lentinan

Up

Fig. 9