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Bacterial β-(1,3)-glucan prevents DSS-induced IBD by restoring the reduced population of regulatory T cells

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ABSTRACT

Bacterial β-(1,3)-glucan has more advantages in terms of cost, yield and efficiency than that derived from mushrooms, plants, yeasts and fungi. We have previously developed a novel and high-yield β-(1,3)-glucan produced by *Agrobacterium* sp. R259. This study aimed to elucidate the functional mechanism and therapeutic efficacy of bacterial β-(1,3)-glucan in dextran sulfate sodium (DSS)-induced inflammatory bowel disease (IBD).Mice were orally pretreated with bacterial β-(1,3)-glucan at daily doses of 2.5 or 5 mg/kg for 2 weeks. After 6 days of DSS treatment, clinical assessment of IBD severity and expression of pro-inflammatory cytokines were evaluated. *In vivo* cell proliferation was examined by immunohistochemistry using Ki-67 and ER-TR7 antibodies. The frequency of regulatory T cells (Tregs) was analyzed by flow cytometry. Natural killer (NK) activity and IgA level were evaluated using NK cytotoxicity assay and ELISA.The deterioration of body weight gain, colonic architecture, disease score and histological score was recovered in DSS-induced IBD mice when pretreated with bacterial β-(1,3)-glucan. The recruitment of macrophages and the gene expression of proinflammatory cytokines, such as IL-1β, IL-6 and IL-17A/F, were markedly decreased in the colon of β-(1,3)-glucan-pretreated mice. β-(1,3)-Glucan induced the recovery of Tregs in terms of their frequency in DSS-induced IBD mice. Intriguingly, β-(1,3)-glucan reversed the functional defects of NK cells and excessive IgA production in DSS-induced IBD mice.We conclude that bacterial β-(1,3)-glucan prevented the progression of DSS-induced IBD by recovering the reduction of Tregs, functional defect of NK cells and excessive IgA production.

Introduction

Inflammatory bowel disease (IBD) is caused by intestinal inflammation or ulceration and is often referred to as Crohn's disease (CD) or ulcerative colitis (UC) (Baumgart and Sandborn, 2007; Hendrickson et al., 2002). While CD causes inflammation in any part of gastrointestinal tract from the mouth to the anus, UC only affects the colon and rectum (Baumgart and Sandborn, 2007; Hendrickson et al., 2002; Podolsky, 2002; Strober et al., 2002; Fiocchi, 1998; Huibregtse et al., 2007). IBD is characterized by pathologic symptoms, such as bloody diarrhea, intestinal motility disorder and colonic shortening (Baumgart and Sandborn, 2007; Hendrickson et al., 2002). Although environmental, genetic and immune factors are likely involved in the development of IBD, its pathogenesis remains unclear (Coombes et al., 2005; Kaser et al., 2010; Allez and Mayer, 2004; Singh et al., 2001). In experimental studies, DSS-induced IBD is a standard model that is widely used to test the efficacy of therapeutic approaches for IBD (Jurjus et al., 2004; Wirtz and Neurath, 2007).

Among cells with the ability to inhibit progression of IBD, Tregs play critical roles in the regulation of inflammatory responses by inhibiting the proliferation of inflammatory cells and the production of proinflammatory cytokines (Groux and Powrie, 1999; Pandolfi et al., 2009). CD8αα$^+$TCRαβ$^+$ Tregs, a newly described subset of CD8αα$^+$TCRαβ$^+$ Tregs, recognize T cell receptor-derived peptide in the context of class Ib major histocompatibility complex (MHC) molecule Qa-1. CD8αα$^+$TCRαβ$^+$ Tregs play an important role

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in controlling autoimmune disease including IBD by suppressing activated $CD4^+$ and $CD8^+$ T cells (Fanchiang et al., 2012; Smith and Kumar, 2008). In contrast, $CD4^+Foxp3^+$ Tregs recognize class II MHC and primarily suppress the priming of naïve or unstimulated $CD4^+$ and $CD8^+$ T cells. $CD4^+Foxp3^+$ Tregs were compromised in lymphoproliferative disorder, autoimmune disease and allergy (Fanchiang et al., 2012; Smith and Kumar, 2008). Moreover, Tregs suppress the effector functions of immune cells, such as natural killer (NK) cells and B cells (Terme et al., 2008; Ghiringhelli et al., 2005; Lim et al., 2005). NK cell – mediated lysis of human tumor cells is impaired by Tregs in cancer patients (Yadav et al., 2011). In secondary lymphoid organs, IgA production is reduced by Treg-mediated inhibition of B cell activation and Ig class switch recombination (Manzano et al., 1992). With regard to IBD, studies *in vivo* and *in vitro* have demonstrated that the incidence of IBD is accompanied by a reduction of NK cytotoxicity (Yadav et al., 2011; Manzano et al., 1992) and an augmentation of IgA production (Sandborn, 2004; Wang et al., 2011).

Drugs used for the treatment of IBD include antibiotics, immunosuppressive drugs, anti-inflammatory drugs and antibody-based therapeutics (Hendrickson et al., 2002). Although numerous studies have aimed to develop side effect-free treatment for IBD, many of these drugs cause adverse side effects, including osteoporosis, neurotoxicity and gastrointestinal intolerance (Hendrickson et al., 2002). To circumvent these side effects, several studies have attempted to develop an IBD therapy using a variety of natural products (Debnath et al., 2013; Hur et al., 2012; Fan et al., 2013). The curcumin extracted from the rhizome of *Curcuma longa* decreases the disease activity index, histological colitis score, cellular infiltration and epithelial disruption in DSS-induced colitis mice (Deguchi et al., 2007). Anthocyanins extracted from blueberries inhibit the levels of nitric oxide (NO), IL-12, TNF-α and IFN-γ in trinitrobenzene sulfonic acid (TNBS)-induced IBD mice (Wu et al., 2011). Other natural products, including probiotics, vitamin C/E and n-3 fatty acids, have been reported to improve symptoms of IBD (Haddad et al., 2005). However, these findings are inconsistent, and the underlying mechanisms remain unclear.

β-(1,3)-Glucans are a heterogeneous group of polysaccharides derived from bacteria, fungi, yeast and mushrooms, primarily in the form of β-(1,3)/(1,6)-glucan, and are found in oats and barleys as β-(1,3)/(1,4)-glucan (Mantovani et al., 2008). While β-(1,3)/(1,6)-glucan has immunostimulatory properties and antitumor activities, β-(1,3)/(1,4)-glucan reduces blood sugar and cholesterol levels (Mantovani et al., 2008). The significance of the immunopharmacological activities of β-glucans varies depending on their sources and structures, including molecular weight, degree of branching and conformation (Kim et al., 2006; Brown and Gordon, 2005). Previous studies have reported that β-(1,3)-glucan exhibits various immunomodulatory properties, including humoral and cellular immunity, and thereby protects against tumor development and infection by pathogens (Mantovani et al., 2008; Kim et al., 2006; Brown and Gordon, 2005). β-(1,3)-glucan derived from *Geastrum saccatum* mushrooms induces anti-inflammatory responses by inhibiting nitric oxide synthase (NOS) and cyclooxygenase (COX) in croton oil-induced ear edema mice (Guerra Dore et al., 2007). Recently, β-(1,3)-glucan extracted from *Pleurotus pulmonarius* mushrooms revealed anti-inflammatory activity by suppressing expression of pro-inflammatory cytokines, such as IL-1 and TNF-α, in DSS-induced colitis mice (Lavi et al., 2010). In particular, bacterial β-(1,3)-glucan has more advantages in terms of cost, yield and efficiency than that derived from mushrooms, plants, yeasts and fungi (Kim et al., 2003). However, the precise mechanisms by which bacterial β-(1,3)-glucan inhibits inflammatory responses in IBD have not yet been elucidated.

We have previously identified a novel and high-yield β-(1,3)-glucan produced by *Agrobacterium* sp. R259, which may stimulate immune responses by increasing IFN-γ production in peripheral blood mononuclear cells (Kim et al., 2003). In the present study, to investigate the preventive or therapeutic effects of bacterial β-(1,3)-glucan in DSS-induced IBD, mice were preadministered β-(1,3)-glucan before DSS treatment, and the clinical signs of IBD were evaluated. To date, the mechanisms by which β-(1,3)-glucan is involved in the suppression of DSS-induced IBD through the regulation of Tregs and immune cell homeostasis has not yet been investigated.

Materials and methods

Mice

Seven-week-old male C57BL/6 wild-type (WT) mice were purchased from the Damool Animal Breeding company (Daejeon, Korea). All of the mice were maintained under specific pathogen-free conditions, and all of the animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care Committee of Chonnam National University.

Antibodies

Flow cytometry analysis, enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry were carried out using the following antibodies: horse radish peroxidase (HRP)-conjugated anti-IgA (Southern Biotechnology, Birmingham, AL), anti-IgG (Bethyl, Montgomery, TX), anti-Ki67, anti-ER-TR7 (Abcam, Cambridge, UK), FITC anti-Mac-1, PE anti-Gr-1, FITC anti-CD4, PE anti-FoxP3, APC anti-CD8α and PE anti-TCRαβ (BD bioscience, San Diego, CA).

Bacterial β-(1,3)-glucan

Bacterial β-glucan was purified by Naturence Co., Ltd. (Gongju City, Korea) after the fermentation of *Agrobacterium* sp. R259 (Korean Culture Type Collection, Daejeon, Korea; KCTC 10197BP). Fermentation was carried out in a 300 L fermenter (KoBiotech Inc., Incheon, Korea). A suitably diluted fermented broth was centrifuged at 8000 × *g* at 4 °C for 30 min. The pellet, consisting of cells and β-glucan, was washed with 0.01 M HCl and harvested by centrifugation. The β-glucan was kept soluble by the addition of 0.5 M NaOH over a 1 h period. Cells were separated by centrifugation at 8000 × *g* for 30 min. The β-glucan present in the supernatant phase was precipitated under acidic conditions by the addition of an appropriate volume of 2.0 M HCl. Both cells and β-glucan were washed and spray-dried to a constant weight. The purified β-glucan was used in this study. The estimated molecular size of the β-glucan was approximately 300 kDa. Purity of the β-glucan was above 85.0%, as determined by HPLC analysis. Based on the analysis of monosaccharides, the purified β-glucan was found to consist exclusively of glucose. The infrared (IR) spectroscopy showed an absorption band at 890 cm^{-1} but not at 840 cm^{-1}, indicating that no α-configuration existed. According to the NMR spectrum, β-glucan had linear (1→3)-linkages. The endotoxin level of bacterial β-(1,3)-glucan was determined using a Limulus Amebocyte Lysate (LAL) test kit (Pyrogent Plus, Lonza, Walkersville, MD) and the level was less than 0.07 EU/ml.

DSS-induced IBD mice, Treg-depleted mice and administration of β-(1,3)-glucan

Experimental IBD was induced in the mice as described previously (Farooq et al., 2009). Briefly, mice were treated with dextran sulfate sodium (DSS) (2.5% w/v, 36,000–50,000 kDa; MP Biomedicals, Solon, OH, USA) dissolved in drinking water. To deplete Tregs *in vivo*, mice were intraperitoneally injected with 200 μg of anti-CD25

monoclonal antibody (clone PC61) every other day for 2 weeks (Ishikawa et al., 2013). Bacterial β-(1,3)-glucan was orally preadministered at daily doses of 2.5 or 5 mg/kg for 2 weeks prior to the DSS treatment in WT and Treg-depleted mice. After 6 days of DSS treatment, mice were maintained on drinking water for another 5 days.

Clinical assessment of DSS-induced IBD

Clinical assessment of IBD severity was determined as described previously (Ito et al., 2006). Briefly, body weight loss, stool consistency (normal stool, loose stool and diarrhea) and gross blood in feces were recorded daily. The colon length was measured between the ileocecal junction and the rectum. The disease activity index (DAI) was calculated by scoring average body weight, stool consistency and intestinal bleeding (normal stool, red-colored stool, gross blood).

Isolation of primary colon cells

The isolation of primary colon cells was performed as described previously (Dupaul-Chicoine et al., 2010). In brief, whole colon tissue was removed and dissected away from any attached connective tissues. The tissues were cut longitudinally into 0.5–1 cm lengths and washed gently with washing solution (RPMI-1640 supplemented with 2% FBS) (GIBCO, Big cabin, OK, USA). They were transferred to 50 ml conical tubes containing 5 ml of RPMI-1640 plus 1 mg/ml type IV collagenase (Sigma, St. Louis, MO, USA) and incubated in a shaking incubator at 250 rpm for 15 min. They were filtered through a 100 mesh (150 micron) screen and centrifuged at 500 × *g* for 5 min at 4 °C. These steps were repeated five more times and the pellets were resuspended in RPMI-1640 medium supplemented with 10% FBS. The isolated primary colon cells were used for flow cytometry analysis.

Isolation of primary mesenteric lymph node (MLN) cells

Isolation of primary MLN cells was performed as described previously (Lessard et al., 2009). Briefly, total MLN cells were isolated by crushing MLN tissues and then filtering them through a 70 μm cell strainer. After centrifugation, the cells were resuspended in RPMI-1640 medium supplemented with 10% FBS and used for flow cytometry analysis.

Flow cytometry analysis

Cells were stained with individual antibodies for 10 min on ice in staining buffer (PBS containing 3% FBS and 0.1% NaN_3) and analyzed by FACS Calibur (BD) using the Cell Quest software.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the colon, spleen and MLN was extracted by using TRIZOL® reagent (MRC, Cincinnati, OH, USA), according to the manufacturer's instruction. The cDNA reaction was performed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). Reverse transcribed cDNA samples were added to a PCR mixture containing 10× PCR buffer, 0.25 mM dNTP, 0.5 U of Taq DNA polymerase and 10 pmol of primers for each gene. The primer sequences are listed in Supplementary Table 1. All PCR mixtures were denatured at 95 °C for 1 min; they were then cycled 27 times for GAPDH and 30 times for all other reactions at 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min, followed by an additional extension step at 72 °C for 10 min. PCR products were electrophoresed and visualized by ethidium bromide staining.

Natural killer (NK) cytotoxicity assay

NK cytotoxicity was measured using cytotox96 lactate dehydrogenase (LDH)-release assay kit (Promega), according to the manufacturer's instruction. Briefly, NK cells from splenocytes were stimulated with recombinant murine IL-2 for 24 h and then mixed with MC38 mouse colon cancer target cells (1×10^4) at different effector-to-target (E:T) ratios, ranging from 400:1 to 50:1. After 4 h, 50 μl of culture supernatants were collected from each well and analyzed for LDH activity using a chromogenic substrate. Absorbance was measured at 490 nm using an ELISA reader (Bio-Rad, Hercules, CA, USA) and the percentage of specific lysis were determined by the following formula: (experimental effector spontaneous release − experimental target spontaneous release)/(target maximum release − target spontaneous release) × 100.

IgA ELISA

Fecal extracts were collected for IgA ELISA as described previously (Davis et al., 1998). Briefly, 96-well plates (Nunc maxisorb, Roskilde, Denmark) were coated with the fecal extracts and then blocked for 2 h at room temperature. After washing, HRP-conjugated IgA antibody was added within 1 h and then developed with tetramethylbenzidine as substrate. Absorbance was measured at 450 nm on an automated plate reader (SoftMax pro v5, Molecular Devices, Sunnyvale, CA, USA) after stopping the reaction with 10% sulfuric acid.

Histological analysis and scoring

The colonic tissues were fixed in 10% formaldehyde (Sigma) and then embedded in frozen OCT compound (Leica, Bannockburn, IL, USA). The tissue sections were stained with hematoxylin and eosin (H&E) (Sigma). The histological scores were determined using methods described previously (Ito et al., 2006).

Immunohistochemistry

Immunohistochemistry analysis was performed using an immunohistochemistry kit, according to the manufacturer's instruction (Bethyl). Briefly, OCT-embedded slides were incubated with HRP-conjugated antibodies for Ki67 and ER-TR7 overnight at room temperature. The slides were stained using DAB substrate and hematoxylin solution.

Statistical analysis

For the statistical analyses of the data, *P* values were calculated using a two-way analysis of variance (ANOVA) or Student's *t* test. The results were considered statistically significant when *P* values were < 0.05.

Results

Prevention of clinical signs by bacterial β-(1,3)-glucan in DSS-induced IBD mice

The toxicity of bacterial β-(1,3)-glucan has not been reported in both human and rodent studies (Spicer et al., 1999). In our preliminary study, the most potent immunostimulatory effects of β-(1,3)-glucan were observed in the colon of WT mice when orally administered with 2.5 or 5 mg/kg (data not shown). Based

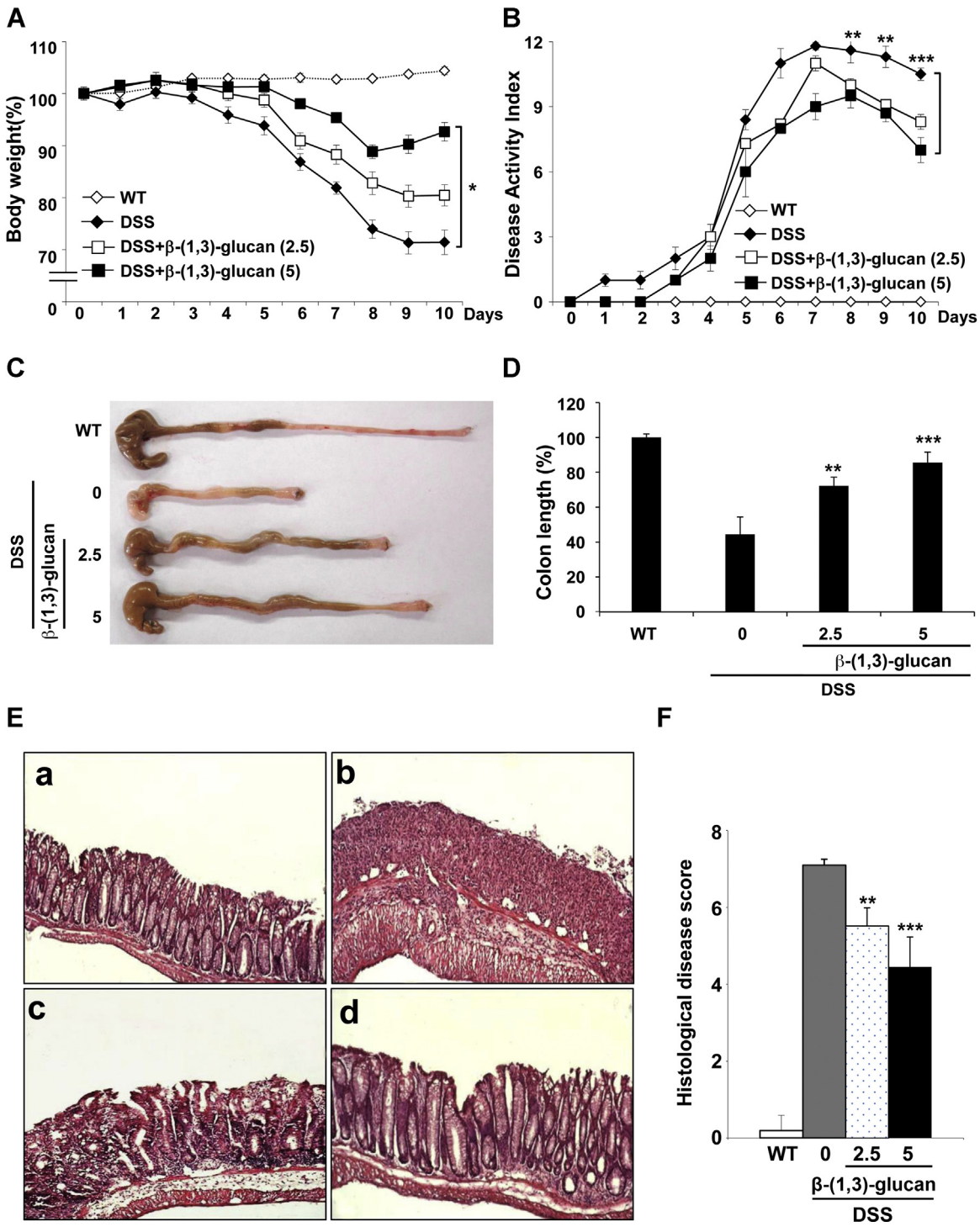

Fig. 1. Bacterial β-(1,3)-glucan prevented clinical signs of DSS-induced IBD. (A) Changes in body weights were measured from wild-type mice (WT), DSS-only treated mice (DSS) and mice pretreated with 2.5 or 5 mg/kg bacterial β-(1,3)-glucan before DSS treatment (DSS + β-(1,3)-glucan). Data are represented as a percentage of body weight relative to initial body weight. (B) The clinical DAI was calculated as described in the Materials and Methods. (C) Macroscopic appearance of the colon tissues. (D) Colon length was measured on day 11 after DSS treatment. (E) Representative microscopic views (200×) of H&E-stained colon tissues from WT mice (a), DSS-only treated mice (b) and mice pretreated with 2.5 or (c) 5 mg/kg (d) of β-(1,3)-glucan before DSS treatment. (F) The histological scores were calculated as described in the Materials and Methods. Data are shown as the mean ± SEM and representative of five independent experiments ($n = 10$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

on these data, doses of 2.5 and 5 mg/kg β-(1,3)-glucan were chosen for the present study. To determine whether β-(1,3)-glucan prevents DSS-induced IBD, we investigated the clinical signs of IBD in DSS-treated mice after a 2-week preadministration of the β-(1,3)-glucan. The severe body weight loss associated with IBD was observed in DSS-only treated mice compared to WT controls (23.7 ± 0.7 g in WT *vs.* 17 ± 0.8 g in DSS-only treated mice) (Fig. 1A). Body weight loss was much less in mice pretreated with β-(1,3)-glucan than in DSS-only treated mice (17 ± 0.8 g in DSS-only treated mice; 19 ± 1.1 g in DSS + 2.5 mg/kg β-(1,3)-glucan-pretreated mice; 22 ± 1.7 g in DSS + 5 mg/kg β-(1,3)-glucan-pretreated mice) (Fig. 1A). The disease activity index (DAI) score was also significantly lowered by preadministration of β-(1,3)-glucan before DSS treatment compared to DSS-only treated

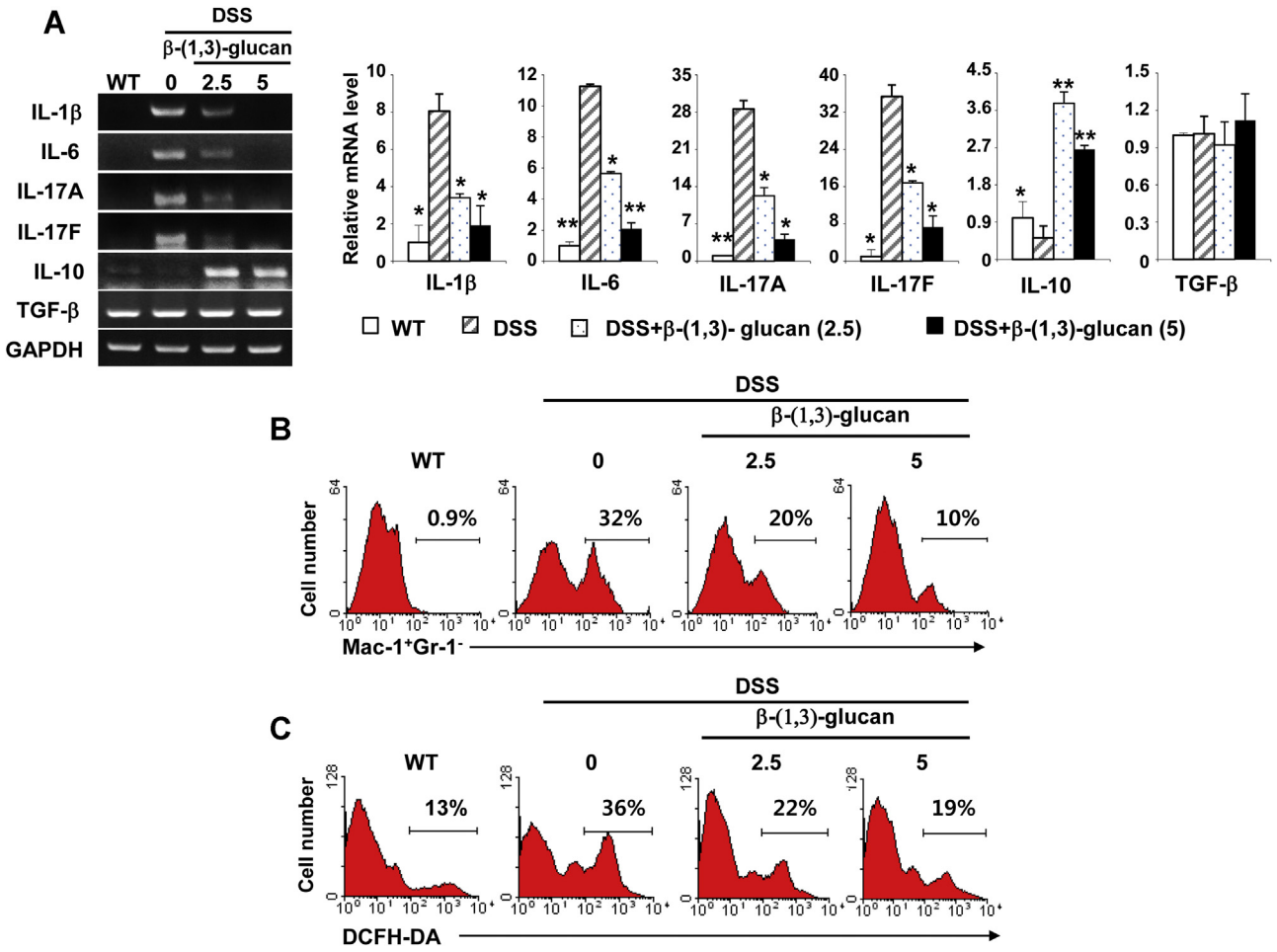

Fig. 2. Bacterial β-(1,3)-glucan suppressed gene expression of inflammatory cytokines and production of ROS in DSS-induced IBD. (A) Gene expression of cytokines was determined by RT-PCR in WT mice, DSS-only treated mice (DSS) and mice pretreated with 2.5 or 5 mg/kg of bacterial β-(1,3)-glucan before DSS treatment (DSS + β-(1,3)-glucan). RT-PCR analysis (left) and the relative expression ratio compared with WT controls (right). (B) Total primary colon cells were stained for cell surface expression of Mac-1 and Gr-1, and the percentages of Mac-1$^+$Gr-1$^-$ cells were measured by flow cytometry. (C) The ROS levels were analyzed by flow cytometry after staining the total primary colon cells with DCFH-DA. Data are shown as the mean ± SEM and representative of five independent experiments ($n = 10$). *, $P < 0.05$; **, $P < 0.01$.

mice (10.5 ± 0.2 in DSS; 8.3 ± 0.4 in DSS + 2.5 mg/kg β-(1,3)-glucan; 7.0 ± 0.5 in DSS + 5 mg/kg β-(1,3)-glucan) (Fig. 1B). A reduction of colon length serves as a major macroscopic marker for tissue injury caused by colon inflammation (McNamee et al., 2011). The colon length of DSS-only treated mice was remarkably reduced compared to WT controls, but the DSS-induced colon shortening was also reduced by preadministration of β-(1,3)-glucan in a dose-dependent manner (9.0 ± 0.2 cm in WT; 4.0 ± 0.4 cm in DSS; 6.5 ± 0.3 cm in DSS + 2.5 mg/kg β-(1,3)-glucan; 7.7 ± 0.4 cm in DSS + 5 mg/kg β-(1,3)-glucan) (Fig. 1C). The mean colon length in DSS-only treated mice was shortened by approximately 55.6% of WT controls, which was reduced to 27.8% and 14.4% by preadministration of 2.5 and 5 mg/kg β-(1,3)-glucan, respectively (100 ± 2.0% in WT; 44.4 ± 6% in DSS; 72.2 ± 4.7% in DSS + 2.5 mg/kg β-(1,3)-glucan; 85.6 ± 5.2% in DSS + 5 mg/kg β-(1,3)-glucan) (Fig. 1D). Severe histological disease, including disruption of epithelial cell lining, muscle layer thickness and infiltration of inflammatory cells, was found in the colonic tissue of DSS-only treated mice compared to WT controls (Fig. 1Ea and Eb); however, the severity of disease was decreased in β-(1,3)-glucan-pretreated mice compared to DSS-only treated mice (Fig. 1Ec and Ed). Consequently, the histological disease score of mice pretreated with β-(1,3)-glucan was significantly lower than that of DSS-only treated mice (7.1 ± 0.15 in DSS; 5.5 ± 0.37 in DSS + 2.5 mg/kg β-(1,3)-glucan; 4.4 ± 0.43 in DSS + 5 mg/kg β-(1,3)-glucan) (Fig. 1F). These results suggest that β-(1,3)-glucan is capable of preventing the clinical manifestations of DSS-induced IBD.

Inhibition of pro-inflammatory cytokine gene expression and ROS production by bacterial β-(1,3)-glucan pretreatment of DSS-induced IBD mice

An increased production of pro-inflammatory cytokines is induced by the infiltration of mononuclear cells into the colon, which contributes to the development of IBD (Neurath et al., 1998; Jobin and Sartor, 2000). We found that the gene expression of pro-inflammatory cytokines, such as IL-1β, IL-6 and IL-17A/F, was increased in the colon tissue of DSS-only treated mice but was inhibited by preadministration of β-(1,3)-glucan in a dose-dependent manner (Fig. 2A). In contrast, gene expression of the anti-inflammatory cytokine IL-10 was markedly increased in mice pretreated with β-(1,3)-glucan compared to DSS-only treated mice, but no difference was observed in TGF-β gene expression. Reactive oxygen species (ROS) produced by infiltrating macrophages leads to intestinal mucosal damage (Namba et al., 2009). As shown in Fig. 2B, a remarkable infiltration of Mac-1$^+$Gr-1$^-$ macrophages was found in the colon of DSS-only treated mice; however, it was inhibited by preadministration of β-(1,3)-glucan in a dose-dependent manner. Consistently, DSS-induced ROS production (DCFH-DA$^+$ cells) was also reduced in β-(1,3)-glucan-pretreated mice (Fig. 2C). These data

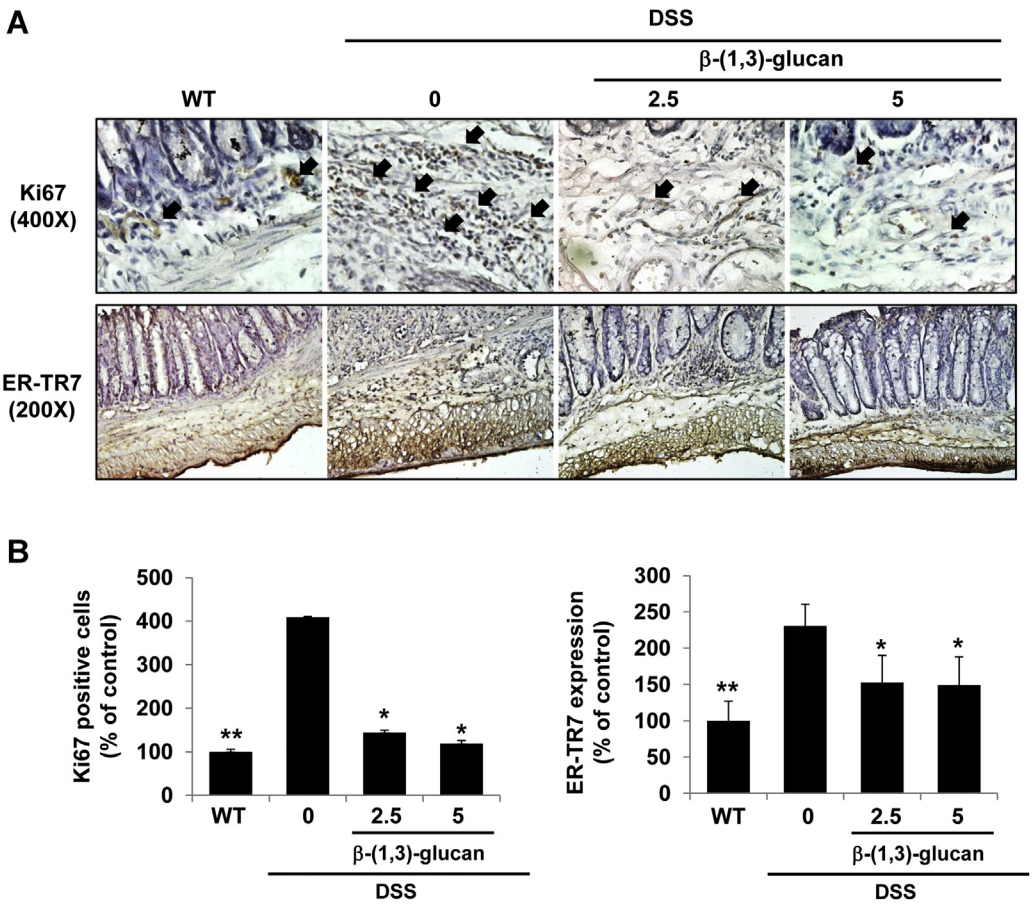

Fig. 3. Bacterial β-(1,3)-glucan inhibited the proliferation of colonic epithelial cells and fibroblasts in DSS-induced IBD. (A) Expression levels of Ki67 and ER-TR7 were evaluated by immunohistochemistry in the colon tissue from WT mice, DSS-only treated mice (DSS) and mice pretreated with 2.5 or 5 mg/kg of bacterial β-(1,3)-glucan before DSS treatment (DSS + β-(1,3)-glucan). Black arrows indicate the Ki67 positive cells. (B) The percentages of relative Ki67 and ER-TR expression were estimated by counting of Ki67 positive cells and measuring the density of ER-TR7 positive areas, respectively. Data are shown as the mean ± SEM and representative of five independent experiments ($n = 10$). *, $P < 0.05$; **, $P < 0.01$.

suggest that β-(1,3)-glucan pretreatment plays an important role in the anti-inflammatory response in DSS-induced IBD through the inhibition of pro-inflammatory cytokine gene expression and ROS production.

Reduced proliferation of epithelial cells and fibroblasts by bacterial β-(1,3)-glucan pretreatment of DSS-induced IBD mice

The proliferation of epithelial cells and fibroblasts is associated with human intestinal inflammation (Jeffers et al., 2002). To investigate the effect of β-(1,3)-glucan on the *in vivo* proliferation of colon epithelial cells and fibroblasts induced by DSS treatment, we performed immunohistochemistry using Ki-67 and ER-TR7 antibodies; these markers are specific for cellular proliferation and fibroblasts, respectively. The expression of Ki-67 and ER-TR7 was increased in the colon of DSS-only treated mice compared to WT controls but was reduced in β-(1,3)-glucan-pretreated mice (Fig. 3A). The mean numbers of cells positive for Ki-67 and ER-TR7 were also significantly diminished in β-(1,3)-glucan-pretreated mice compare to DSS-only treated mice (Fig. 3B). These results implied that β-(1,3)-glucan suppressed the proliferation of epithelial cells and fibroblasts in DSS-induced IBD mice.

Increased prevalence of Tregs by bacterial β-(1,3)-glucan pretreatment of DSS-induced IBD mice

Recent studies have demonstrated that Tregs play an important role in the suppression of the inflammatory response, and patients with IBD have relatively reduced numbers of Tregs in the colon (Groux and Powrie, 1999; Boden and Snapper, 2008). We found that the percentages of $CD8\alpha\alpha^{+}TCR\alpha\beta^{+}$ were much lower in the mesenteric lymph nodes (MLN) and colon of DSS-only treated mice than in those of WT controls (Fig. 4A). The lower percentages of $CD8\alpha\alpha^{+}TCR\alpha\beta^{+}$ Tregs induced by DSS treatment were restored in β-(1,3)-glucan-pretreated mice. Moreover, the absolute numbers of $CD8\alpha\alpha^{+}TCR\alpha\beta^{+}$ Tregs were also reduced in the MLN and colon of DSS-only treated mice but were significantly increased in β-(1,3)-glucan-pretreated mice (Fig. 4B). Similarly, while the MLN and colon of DSS-only treated mice had reduced percentages of $CD4^{+}FoxP3^{+}$, those of β-(1,3)-glucan-pretreated mice showed a dose-dependent restoration of the Treg populations (Fig. 4C). Mice pretreated with β-(1,3)-glucan exhibited higher absolute numbers of $CD4^{+}FoxP3^{+}$ Tregs in the MLN and colon compared to the DSS-only treated mice (Fig. 4D). These data indicated that β-(1,3)-glucan may be capable of suppressing the inflammatory response in DSS-induced IBD, at least in part, by the restoration of Treg populations.

Lack of preventive effect of bacterial β-(1,3)-glucan on DSS-induced IBD in Treg-depleted mice

To verify the preventive effect of β-(1,3)-glucan by enhancing Tregs populations in DSS-induced IBD, we assessed disease progression in Treg-depleted mice. The percentages and absolute numbers of $CD4^{+}FoxP3^{+}$ and $CD8\alpha\alpha^{+}TCR\alpha\beta^{+}$ Tregs were

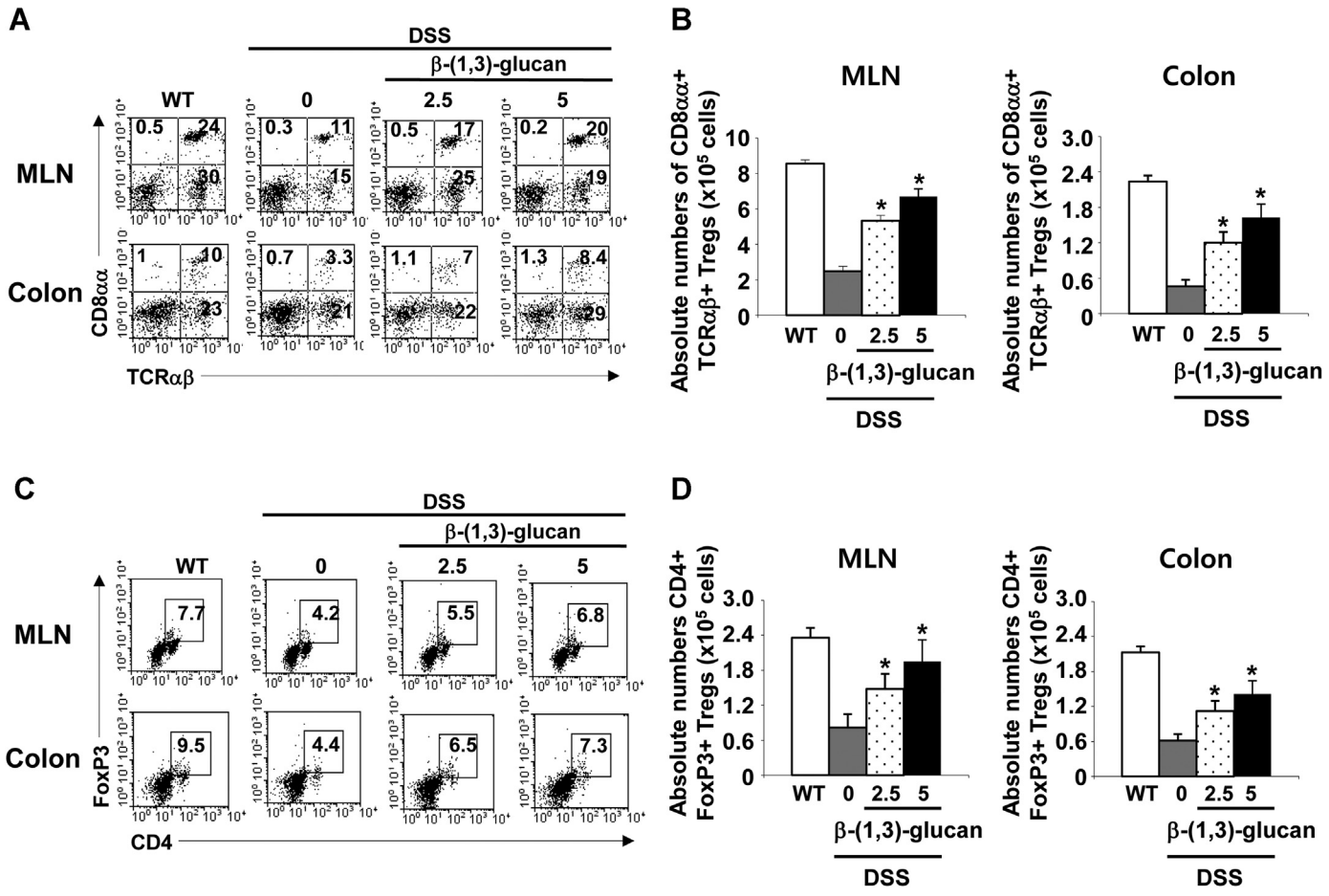

Fig. 4. Bacterial β-(1,3)-glucan reversed the DSS-induced reduction of Treg numbers. (A) Cells from the MLN and colon of mice were stained by antibodies for CD8α and TCRαβ, and the Tregs population was analyzed by flow cytometry. (B) The absolute number of $CD8\alpha\alpha^+TCR\alpha\beta^+$ Tregs in the MLN and colon was calculated from flow cytometry profiles and the total number of cells. (C) Cells from the MLN and colon of mice were stained by antibodies for CD4 and FoxP3. (D) The absolute numbers of $CD4^+FoxP3^+$ Tregs in the MLN and colon. Data are shown as the mean ± SEM and representative of five independent experiments ($n = 10$). *, $P < 0.05$.

considerably reduced in the MLN and colon of Treg-depleted mice compared with WT controls (Supplementary Fig. 1). The reduced percentages and absolute numbers of $CD4^+FoxP3^+$ and $CD8\alpha\alpha^+TCR\alpha\beta^+$ Tregs were restored to almost WT control levels by preadministration of β-(1,3)-glucan in DSS-treated WT mice, but not in Treg-depleted mice. In good accordance, preadministration of β-(1,3)-glucan induced the restoration of body weight loss in DSS-treated WT mice, while it had no influence in Treg-depleted mice (20 ± 0.23 g in WT *vs.* 17 ± 0.16 g in Treg-depleted) (Fig. 5A). In addition, there was no alleviation in the DAI score from DSS-treated Treg-depleted mice preadministered with β-(1,3)-glucan compared to WT controls (7.5 ± 0.72 in WT *vs.*10.5 ± 0.71 in Treg-depleted) (Fig. 5B). In Treg-depleted mice, DSS-induced colon shortening was not recovered by preadministration of β-(1,3)-glucan compared to WT controls (Fig. 5C and D). More severe histological disease score was found in the colon of Treg-depleted mice compared to WT controls (Fig. 5E and F). In addition, the colon of DSS-treated Treg-depleted mice showed more increased gene expression of proinflammatory cytokines, such as IL-1β, IL-6, IL-17A and F than WT controls, regardless of preadministration with β-(1,3)-glucan (Supplementary Fig. 2A). In DSS-treated Treg-depleted mice, IL-10 gene expression was decreased by preadministration of β-(1,3)-glucan compare with WT controls, but no significant difference in TGF-β expression. The population of Mac-1 + Gr-1- macrophages was increased in the colon of Treg-depleted mice compared with WT controls, regardless of treatment with DSS and/or β-(1,3)-glucan (Supplementary Fig. 2B). These data suggested that bacterial β-(1,3)-glucan may play a preventive role in DSS-induced IBD by restoring the reduced population of Tregs.

Reversal of DSS-induced functional defects of NK cells and excessive IgA production by pretreatment with bacterial β-(1,3)-glucan

Several studies have reported that patients with IBD demonstrate an impairment of NK cell function (Yadav et al., 2011; Manzano et al., 1992) and amplification of IgA production in mucosal tissues (Sandborn, 2004; Wang et al., 2011). To determine whether β-(1,3)-glucan is capable of improving the DSS-induced impairment of mucosal immune responses, NK activity and IgA level were evaluated using NK cytotoxicity assay and ELISA. While NK cytotoxicity against target cells was significantly lower in DSS-only treated mice than in WT controls, preadministration of β-(1,3)-glucan before DSS treatment restored NK cytotoxicity to almost normal levels (Fig. 6A). Furthermore, DSS-only treatment resulted in excessive IgA production, which was significantly inhibited by preadministration of β-(1,3)-glucan before DSS treatment (Fig. 6B). These data implied that β-(1,3)-glucan is capable of reversing the DSS-induced functional defects of NK cells and excessive IgA production in mucosal immunity.

Discussion

This study aimed to elucidate the anti-inflammatory mechanism of bacterial β-(1,3)-glucan in DSS-induced IBD. Here, we

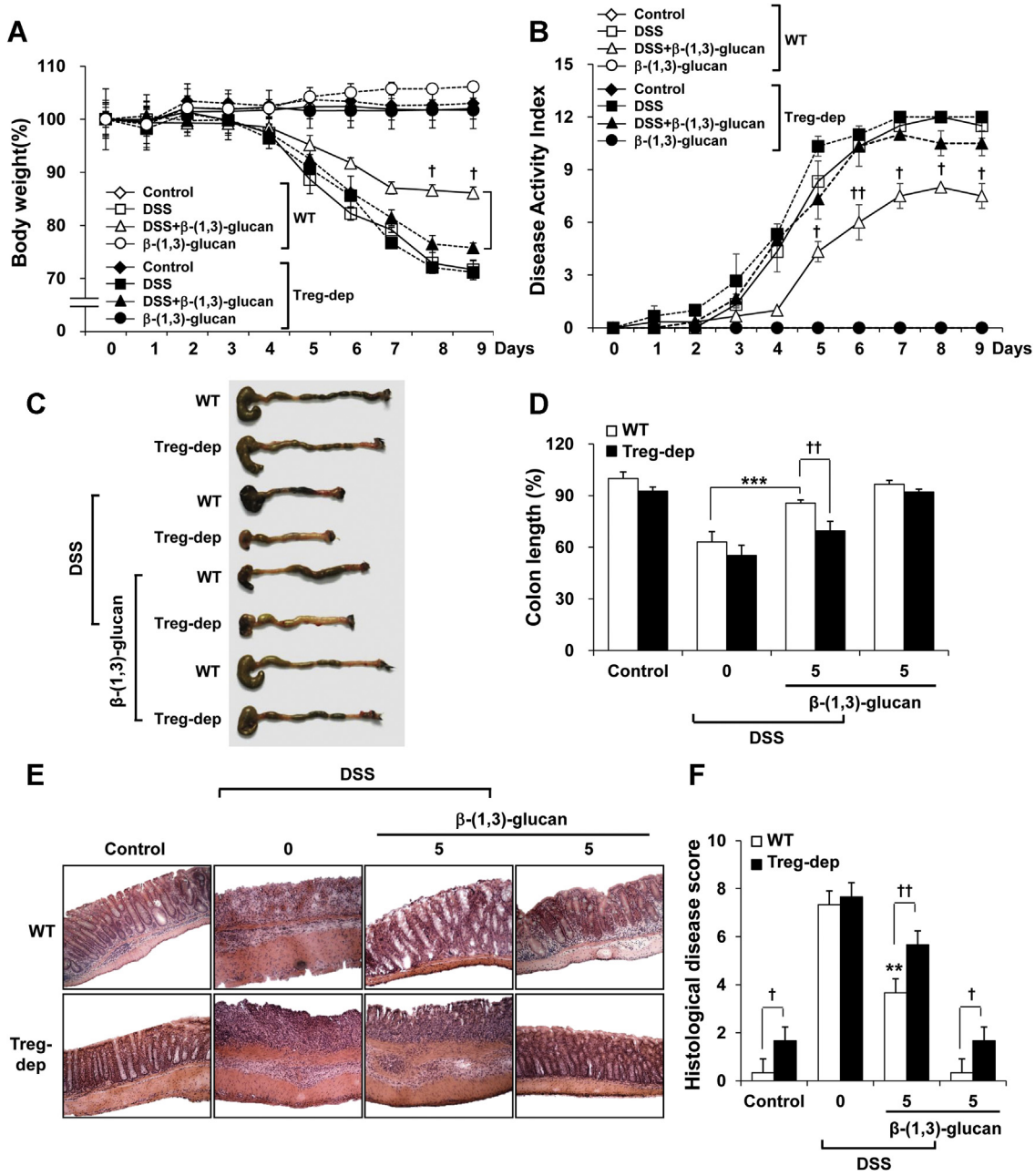

Fig. 5. Bacterial β-(1,3)-glucan failed to prevent clinical signs of DSS-induced IBD in Treg-depleted mice. (A) Changes in body weights were measured from Control, DSS-only treated mice (DSS), mice pretreated with 5 mg/kg bacterial β-(1,3)-glucan before DSS treatment (DSS + β-(1,3)-glucan) and β-(1,3)-glucan-only treated mice (β-(1,3)-glucan) in both WT and Treg-depleted mice. Data are represented as a percentage of body weight relative to initial body weight. (B) The clinical DAI was calculated as described in the Materials and Methods. (C) Macroscopic appearance of the colon tissues. (D) Colon length was measured on day 9 after DSS treatment. (E) Representative microscopic views (200×) of H&E-stained colon tissues from WT and Treg-depleted mice. (F) The histological scores were calculated as described in the Materials and Methods. Data are shown as the mean ± SEM and representative of three independent experiments ($n = 9$). Significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) difference between value for DSS and DSS + β-(1,3)-glucan in WT mice; Significant (†, $P < 0.05$; ††, $P < 0.01$) difference between value for WT and Treg-depleted mice.

provided the first demonstration that β-(1,3)-glucan prevented the progression of DSS-induced IBD through Treg-mediated inhibition of inflammatory responses as well as maintenance of intestinal immune cell homeostasis. Our findings that β-(1,3)-glucan inhibited the expression of inflammatory cytokines and ROS due to the colonic infiltration of macrophages and fibroblasts were consistent with several previous studies suggesting the anti-inflammatory effects of non-bacterial β-(1,3)-glucan. Remarkably, we elucidated a novel mechanism by which β-(1,3)-glucan pretreatment reversed the DSS-induced reduction of Tregs and thereby induced an anti-inflammatory effect. Furthermore, our study is the first to show that β-(1,3)-glucan regulated intestinal immune cell homeostasis by recovering the functional defect of NK cells and excessive IgA production in DSS-induced IBD mice.

Although it has recently become apparent that the demand for β-(1,3)-glucans has been gradually increasing due to their nutritional value as well as pharmacological effects (Akramiene et al., 2007; Chen and Seviour, 2007; Zeković et al., 2005), the use of fungi, yeasts or natural products as a source of β-(1,3)-glucans required a more expensive and complicated process for their purification. To solve these problems, we previously developed a novel mutant strain, *Agrobacterium* sp. R259, which exhibited a higher yield of β-(1,3)-glucan with an optimized fermentation system than the parent strain, *Agrobacterium* sp. ATCC 31750 (Farooq et al., 2009).

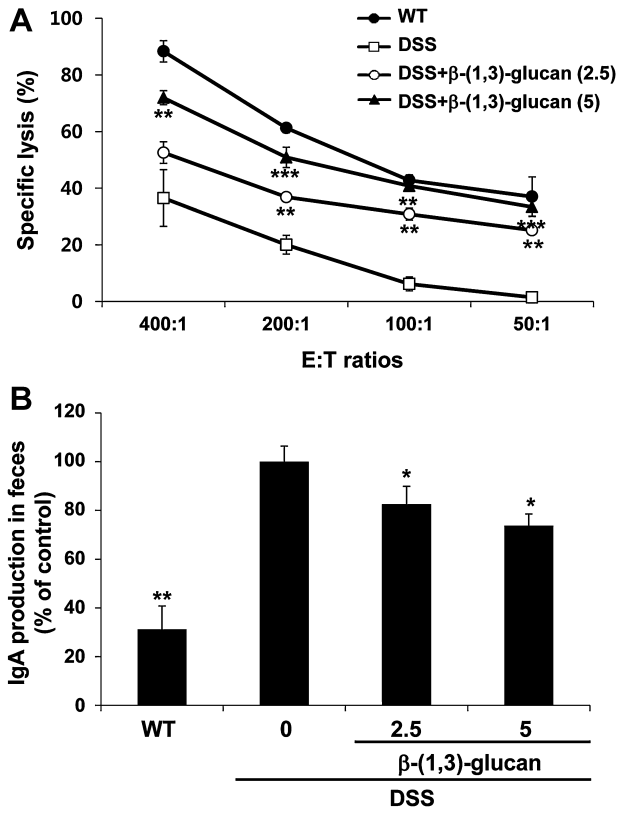

Fig. 6. Bacterial β-(1,3)-glucan restored DSS-induced functional defects of NK cells and excessive IgA production. (A) NK cytotoxicity was analyzed by LDH-release assay using NK cells isolated from the spleen as effectors cells and MC38 colon cancer cells as targets at different E:T ratios. (B) Total IgA concentrations were measured by ELISA in the fecal extracts. Data are shown as the mean ± SEM and representative of five independent experiments ($n = 10$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

It has been shown that low molecular weight β-(1,3)-glucans (<5–10 kDa) are biologically inactive *in vivo* (Akramiene et al., 2007; Chen and Seviour, 2007; Zeković et al., 2005). The molecular weight of β-(1,3)-glucan that we developed and used in this study was approximately 300 kDa, and its administration led to an anti-inflammatory response in DSS-induced IBD. These findings are consistent with the previous observations that fungus- or mushroom-derived β-(1,3)-glucans that have molecular weights ranging from 100 to 670 kDa had anti-inflammatory activities (Chen and Seviour, 2007; Zeković et al., 2005). A comparison of the therapeutic effects between β-(1,3)-glucans derived from our mutant strain and other sources needs to be investigated further in IBD. Despite previous studies that explored the medicinal properties of fungus- or mushroom-derived β-(1,3)-glucans in various diseases, including microbial infection, cancer, hypercholesterolemia and diabetes mellitus (Akramiene et al., 2007; Chen and Seviour, 2007; Zeković et al., 2005), only limited information exists regarding the therapeutic role of β-(1,3)-glucan in IBD. In the present study, we demonstrated the functional mechanism and therapeutic efficacy of β-(1,3)-glucan in DSS-induced IBD.

The immune balance between inflammatory Th17 cells and anti-inflammatory Tregs is important for maintenance of immune homeostasis in the intestinal mucosa (Waite and Skokos, 2012). The uncontrolled activation of Th17 cells stimulates inflammatory responses in IBD by inducing the suppression of Treg differentiation followed by reduced anti-inflammatory activity (Waite and Skokos, 2012). In this study, we did not evaluate changes in the Th17 cell populations, but DSS-only treated mice showed elevated gene expression of the Th17 cytokines IL-17A and IL-17F (Fig. 2A). Thus, it is assumed that the population of activated Th17 cells would be increased in DSS-induced IBD mice. In addition, given the reduced number of Tregs in DSS-only treated mice (Fig. 4), the suppression of Treg differentiation may be induced by the uncontrolled activation of Th17 cells. Whereas Th17 cell differentiation from naive CD4 T cells is mainly stimulated by IL-6 and TGF-β (Xavier and Podolsky, 2007), the differentiation and activation of Tregs are promoted by IL-10 and TGF-β or TGF-β alone (Xavier and Podolsky, 2007; Asseman et al., 1999). Here, we showed that increased IL-6 and decreased IL-10 gene expression was found in DSS-induced IBD mice, which was reversed in β-(1,3)-glucan-pretreated mice (Fig. 2A). However, there was no significant difference in TGF-β gene expression among all mice examined, implying constitutive expression of TGF-β. Therefore, the increased expression of IL-6 may induce the differentiation of Th17 cells, which leads to the inhibition of Tregs and consequent suppression of IL-10 in DSS-induced IBD. These findings suggest that β-(1,3)-glucan may play a role in improving the homeostatic imbalance between Th17 cells and Tregs in DSS-induced IBD; potentially, β-(1,3)-glucan may induce Treg-mediated suppression of uncontrolled Th17 activation through the modulation of cytokines associated with their differentiation and activation.

Recently, it has been reported that NK cytotoxicity is impaired in patients with active UC (Yadav et al., 2011; Manzano et al., 1992) and that there is an increased risk of developing colorectal cancer in both UC and CD patients (Itzkowitz and Yio, 2004). However, the mechanisms responsible for the defective NK activity and the resultant effect on the development or progress of IBD have not been investigated. Moreover, little is known about the functional defects of NK cells in IBD mouse models. Importantly, NK cytotoxicity in this study was defective in DSS-induced IBD mice, which was restored by preadministration of β-(1,3)-glucan (Fig. 5A). The restoration of NK cell function implied that β-(1,3)-glucan may have therapeutic potential for the suppression of IBD-induced colorectal cancer. The mechanism by which β-(1,3)-glucan rescues NK cell function in DSS-induced IBD remains to be further investigated.

Previous clinical and *in vitro* experiments have demonstrated that administration of β-(1,3)-glucan enhances humoral and cellular immunity (Mantovani et al., 2008; Kim et al., 2006; Brown and Gordon, 2005). There are two contradictory reports with respect to the association between disease course and IgA levels in IBD, while a remarkable decrease in IgA levels is observed in biopsy specimens from UC patients (Liu et al., 2012), the intestinal IgA levels are increased in DSS-induced colitis rats (Wang et al., 2011). IgA in humans acts as an immune regulator by maintaining immune homeostasis through protection against microbial infections, but the involvement of IgA in the pathogenesis of IBD has not been previously reported. In this study, preadministration of β-(1,3)-glucan resulted in the recovery of Treg numbers in DSS-induced IBD and lowered the excessive production of IgA to almost normal values. These findings are strongly supported by the previous report that Tregs suppress B cell-dependent IgA production in secondary lymphoid organs (Lim et al., 2005). Given the insignificant difference in B cell numbers between DSS-only treated mice and β-(1,3)-glucan-pretreated mice (data not shown), these findings raised the possibility that β-(1,3)-glucan may suppress DSS-induced overproduction of IgA by increasing the Tregs population that inhibits B cell activation but not by changing B cell numbers.

In summary, the primary novel finding of this study was that *Agrobacterium* sp. R259-derived bacterial β-(1,3)-glucan prevented DSS-induced IBD by inducing the recovery of Treg numbers. This finding was achieved mainly by an anti-inflammatory mechanism involving the modulation of altered NK cytotoxicity and IgA production by β-(1,3)-glucan, which consequently led to the maintenance of intestinal immune homeostasis. Therefore, β-(1,3)-glucan may have a therapeutic potential for the treatment of gastroenterologic diseases and provide information that will help to further elucidate

the regulatory mechanism of β-(1,3)-glucan in various inflammatory diseases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2014.07.003.

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