ORIGINAL ARTICLE

Basic Study

Oral tolerance is inducible during active dextran sulfate sodium-induced colitis

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Abstract

AIM: To investigate whether oral tolerance is inducible during the active phase of dextran sulfate sodium (DSS)-induced colitis.

METHODS: Colitis was induced in 6- to 8-wk-old female BALB/c mice by the administration of 2% DSS. To induce oral tolerance, mice that received water with DSS [DSS (+)] and mice that received autoclaved water [DSS (-)] were intragastrically (i.g.) administered ovalbumin (OVA) as a tolerogen before systemic challenge with OVA. Following this, serum levels of OVA-specific IgE antibodies were measured. In mice with active

colitis, CD4^{+}CD25^{+}Foxp3^{+} cell and B10 cell frequencies were evaluated using flow cytometry. Cytokine mRNA expression profiles were evaluated by reverse transcription real-time polymerase chain reaction.

RESULTS: Regardless of the presence of DSS colitis, OVA-specific immunoglobulin E concentrations were significantly reduced in mice that were i.g. administered OVA compared to mice that were i.g. administered PBS [DSS (+): 4.4 (4.2-9.5) ng/mL vs 83.9 (66.1-123.2) ng/mL, $P < 0.01$; DSS (-): 27.7 (0.1-54.5) ng/mL vs 116.5 (80.6-213.6) ng/mL, $P < 0.01$]. These results demonstrated that oral tolerance was induced in both the presence and absence of colitis. In the spleen and mesenteric lymph nodes (MLN), the frequencies of CD4^{+}CD25^{+}Foxp3^{+} cells and B10 cells, both of which are associated with oral tolerance, did not significantly change. In the spleen, interferon-γ mRNA expression significantly decreased in mice with colitis [DSS (+): 0.42 (0.31-0.53) vs DSS (-): 1.00 (0.84-1.39), $P < 0.01$]. The expression levels of other cytokines did not significantly change.

CONCLUSION: Oral tolerance is inducible during active DSS colitis. The stability of regulatory cell populations in the spleen and MLN in colitis might correlate with these results.

Key words: Cytokine; Dextran sulfate sodium colitis; Oral tolerance; Regulatory T cell; Regulatory B cell

Core tip: Our study is the first to demonstrate that oral tolerance is inducible during the active phase of dextran sulfate sodium (DSS)-induced colitis. Lymphocytic infiltration into the large intestine mucosa associated with epithelial defects did not influence oral tolerance. In DSS colitis, the frequencies of CD4^{+}CD25^{+}Foxp3^{+} T cells and B10 cells in the spleen and mesenteric lymph nodes remained stable. This stability might have led to the induction of oral tolerance in DSS colitis. Accordingly, if an appropriate antigen is chosen, then oral immunotherapy may be applicable for the treatment of ulcerative colitis.

INTRODUCTION

Inflammatory bowel disease (IBD) includes Crohn's disease (CD) and ulcerative colitis (UC). The precise cause of IBD remains unknown. IBD is a multifactorial disease resulting from excessive immune responses to various environmental factors and is associated with genetic background. As a result of these excessive immune responses, T helper cell type (Th)0 cells differentiate into Th1, Th17 or Th2 cells in response to interleukin (IL)-12/IL-18, IL-6/tissue growth factor (TGF)-β or IL-4, respectively[1].

Regulatory T cells (Tregs) have been shown to suppress conventional T cells through multiple mechanisms, including the generation of immunosuppressive cytokines, such as TGF-β and IL-10, and via direct contact with effector T cells or antigen-presenting cells[2]. Decreases in the anti-inflammatory activity of Tregs may therefore be equal in importance to the enhancement of effector mechanisms in contributing to IBD pathogenesis[1].

Regulatory B cells (Bregs) are functionally characterized by their capacity to produce IL-10, a potent inhibitory cytokine. These cells have been designated as B10 cells because their ability to downregulate immune responses and inflammatory disease is attributable to IL-10. The absence of B10 cells exacerbates disease symptoms in mouse models[3]. Breg dysfunction has been reported to influence the pathogeneses of various autoimmune and allergic diseases. In addition to autoimmune and allergic diseases, intestinal inflammation is also regulated by Breg functions, a relationship that has been confirmed by several studies using mouse models of colitis[4]. Additionally, in humans, the depletion of B cells using anti-CD20 (rituximab) for various disorders has been reported to either exacerbate colitis or result in spontaneous colitis[5,6].

Oral tolerance is a phenomenon in which systemic immunity is suppressed relative to orally administered antigens. Treg involvement has been demonstrated as a mechanism for the induction of oral tolerance. Tregs are naturally produced in the thymus (nTreg) and are also induced in peripheral tissues (pTreg). Induced, antigen-specific Tregs can then circulate and establish systemic tolerance to their corresponding antigens. This phenomenon largely contributes to the induction of oral tolerance[7]. In recent years, Bregs have also been indicated to be involved in oral tolerance[8]. Consistent with the regulatory role of B cells, B cell-deficient mice are defective in developing oral tolerance[9].

The administration of dextran sulfate sodium (DSS) can induce colitis in animal models. This induced colitis is similar in appearance to human UC both clinically and histologically[10,11]. Several reports have evaluated Treg dynamics during active DSS colitis tolerance[12,13]; however, few reports have assessed Breg dynamics during active DSS colitis.

Although oral immunotherapy has been applied for various immune disorders, this treatment modality is considered ineffective for IBD because IBD patients have dysfunctional oral tolerance[14,15]. Although the effectiveness of oral immunotherapy for CD patients was recently reported[16,17], there are currently few reports regarding oral immunotherapy for UC patients.

In this study, we utilized a DSS colitis model to explore the potential use of oral immunotherapy during the active phase of UC. The oral administration of colon extracted protein (CEP) prior to the onset of DSS colitis has been shown to induce immune tolerance, downregulate the inflammatory immune response and alleviate DSS-induced colitis[18,19]. However, to the best of our knowledge, no report thus far has evaluated the effectiveness of the oral administration of CEP after the onset of DSS colitis, during the active phase of the disease.

The purpose of this study was to investigate whether oral tolerance is inducible during the active phase of DSS colitis. Additionally, we determined how cytokine levels and regulatory cell populations change in colitis in the mesenteric lymph nodes (MLN) and the spleen and how these changes influence the induction of oral tolerance. Furthermore, we explored the potential use of oral immunotherapy during the active phase of UC.

MATERIALS AND METHODS

Mice
Specific pathogen-free (SPF) BALB/c mice were purchased from Charles River Laboratories Japan (Yokohama, Kanagawa, Japan). All experiments were performed using 6- to 8-wk-old female mice. The protocol used in the current study was designed to minimize pain and discomfort to the animals and was approved by the Institutional Animal Care and Use Committee of Showa University. The mice had access to water and food *ad libitum* and were housed in SPF conditions with alternating light-dark cycles for one week prior to experimentation. Intragastric gavage was performed using straight gavage needles appropriate for each animal's size. All animals were euthanized using CO_2 prior to tissue collection.

Induction of colitis
An overview of the experimental setup is provided in Figure 1A and B. DSS administration was performed as previously described with slight modification[11,20]. DSS colitis was induced by the administration of 2% DSS with a molecular weight ranging between 36 and 50 kDa (MP Biomedicals, Solon, OH, United States) *ad libitum* from day 1 through day 11. As a control, a subset of mice was provided with autoclaved water for the entire study period. All mice were clinically evaluated based on body weight and a scoring system comprising evaluations of stool consistency and fecal blood, as described previously[20].

Histological analysis of DSS-induced colitis
On day 8, the colons of the mice were removed and fixed in 10% buffered formalin and then embedded in paraffin, sliced into sections, and stained with hematoxylin and eosin. The stained sections were examined by two pathologists for evidence of colitis using a previously described histological scoring method[21].

Oral tolerance induction and immunization
To induce oral tolerance, the mice that received 2% DSS from day 1 through day 11 and the mice that received autoclaved water were intragastrically (i.g.) administered either 5 mg/d ovalbumin (OVA) or PBS as a control for 4 consecutive days from day 8 through day 11. To induce systemic antibody (Ab) production in response to OVA antigen, the mice were intraperitoneally (i.p.) injected with 1 μg of OVA antigen plus 0.1 mg of aluminum hydroxide (alum) (Thermo Scientific, Rockford, IL, United States) on days 14, 28, 42, and 56. Following this, blood samples were collected on day 63 to measure serum anti-OVA-specific IgE Ab concentrations, as described previously[22].

ELISA analysis to measure serum anti-OVA-specific IgE Ab concentrations
Serum OVA-specific IgE concentrations were measured by ELISA. Briefly, 50 μg/mL of OVA was dissolved in 0.1 mol/L sodium carbonate buffer (pH 9.5) and incubated with serum samples in a 96-well immunoplate at 4 ℃ overnight. The samples were treated with protein-free blocking buffer T20 (PBS) (Thermo Scientific) to inhibit nonspecific binding. After washing, the serum samples or OVA-specific IgE antibody (Ab) standards (Acris Antibodies, San Diego, CA, United States) and biotin-conjugated anti-mouse IgE Abs (Southern Biotech, Birmingham, AL, United States) were then plated in the wells and incubated for 1 h. All wells were sequentially incubated with HRP-conjugated streptavidin (eBioscience, San Diego, CA, United States). OVA-specific IgE Ab was detected using a TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD, United States) and measured at an absorbance of 450 nm after the addition of H_2SO_4.

Flow cytometric analysis to detect changes in $CD4^+Foxp3^+$ cell and B10 cell frequencies
Single-cell suspensions were prepared from the MLN and spleen on day 14. The following antibodies were used in this study: Anti-CD16/CD32 Ab as an Fc-blocker; FITC-conjugated anti-CD4 Ab; BV421-conjugated anti-CD25 Ab; Alexa Fluor-conjugated anti-Foxp3 Ab (BD Biosciences, San Diego, CA, United States); BV650-conjugated anti-CD3 Ab; FITC-conjugated anti-CD19 Ab; BV510-conjugated anti-CD5 Ab; PE-conjugated anti-CD1d Ab; and PE/Cy7-conjugated anti-IL-10 Ab (BioLegend, San Diego, CA, United States). Dead cells were detected using a Zombie Red Fixable Viability Kit (BioLegend) according to the manufacturer's recommended protocol. Following the staining of surface antigens, intracellular Foxp3 or IL-10 staining was performed using a Transcription Factor Buffer set (BD Biosciences) according to the manufacturer's recommended protocol. All cells were

analyzed using a LSRFortessa flow cytometer (BD Biosciences).

B cell stimulation for the analysis of B10 cells

Analysis of intracellular IL-10 was performed using flow cytometry as previously described[23]. Briefly, isolated spleen cells were resuspended (2×10^6/mL) in complete medium (RPMI 1640) (Wako Pure Chemical Industries, Osaka, Japan) containing 10% FBS with 5×10^{-5} mol/L 2-mercaptoethanol, 10 μg/mL lipopolysaccharide (LPS), 50 ng/mL phorbol 12-myristate 13-acetate (PMA), 500 ng/mL ionomycin (Sigma-Aldrich, St. Louis, MO, United States) and 1 μg/mL Brefeldin A (BioLegend) for 5 h in 24-well flat-bottom plates.

Reverse transcription real-time polymerase chain reaction analysis to detect cytokine mRNA expression

Total RNA was extracted from whole MLN and spleen cells on day 14 using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). Reverse transcription was performed using a QuantiTect Reverse Transcription Kit (Qiagen). Real-time polymerase chain reaction (PCR) was performed using a LightCycler 480 Ⅱ system (Roche Diagnostics, Mannheim, Germany) with LightCycler 480 Probes Master (Roche Diagnostics). The following PCR primers were used: TaqMan® Gene Expression Assays for mouse IL-4 (Assay ID: Mm00445259_m1), *IL-6* (Assay ID: Mm00446190_m1), *IL-10* (Assay ID: Mm 00439614_m1), interferon-γ (IFN-γ) (Assay ID: Mm01168134_m1), tumor necrosis factor (TNF)-α (Assay ID: Mm00443258_m1), and *GAPDH* (Assay ID: Mm99999915_g1) (Applied Biosystems, Foster City, CA, United States). All values were normalized against the expression of the housekeeping gene *GAPDH*.

Statistical analysis

Statistical analyses were performed using the Mann-Whitney *U* test, and a *P* value < 0.05 was considered to indicate a statistically significant difference.

RESULTS

Establishment of experimental colitis

DSS is widely used to induce intestinal inflammation. From day 8 to day 16, the mice that received water containing 2% DSS [DSS (+) mice] had significantly lower body weights than the mice that received autoclaved water [DSS (-) mice] (Figure 1B, *P* < 0.05 to *P* < 0.001). With the progression of colitis, the DSS (+) mice exhibited diarrhea and visible fecal blood. On day 12, the disease activity index (DAI) of the DSS (+) mice was significantly higher than that of the DSS (-) mice (Figure 1C, *P* < 0.01). On day 8, the average colon length of the DSS (+) mice was shorter than that of the DSS (-) mice (Figure 1D and E, *P* < 0.01). Histologically, on day 8, DSS colitis was characterized by epithelial defects, submucosal edema (F1) and inflammatory cell infiltration (F2) (Figure 1F). The histological scores for the DSS (+) mice were significantly higher than those

of the DSS (-) mice (Figure 1G, *P* < 0.05).

Induction of oral tolerance in an experimental colitis model

DSS (+) mice and DSS (-) mice were i.g. administered 5 mg/d OVA or PBS, respectively, for 4 consecutive days before undergoing an i.p. administered challenge with 1 μg OVA plus 0.1 mg of alum every two weeks for a total of four times. Serum samples were collected from the mice 1 wk after each challenge, and OVA-specific IgE concentrations were measured. Regardless of the presence of DSS colitis, the mice that were i.g. administered OVA had significantly lower OVA-specific IgE concentrations than the mice i.g. administered PBS [DSS (+): 4.4 (4.2-9.5) ng/mL *vs* 83.9 (66.1-123.2) ng/mL, *P* < 0.01; DSS (-): 27.7 (0.1-54.5) ng/mL *vs* 116.5 (80.6-213.6) ng/mL, *P* < 0.01] (Figure 2). These results demonstrated that oral tolerance was inducible with or without colitis.

Determination of Treg frequency in the spleen and MLN using flow cytometric analysis

As indicated above, oral tolerance was inducible with or without colitis. We hypothesized that MLN and spleen populations of CD4+CD25+Foxp3+ cells, which are involved in oral tolerance, remained stable regardless of the presence of colitis, although the colitis mice did exhibit epithelial defects and inflammatory cell infiltration into the colonic mucosa. In the spleen, the frequency of CD4+CD25+ cells among CD4+ T cells and the frequency of CD4+Foxp3+ cells among CD4+ T cells were 5.6% (5.35%-5.75%) for the control mice and 6.6% (5.4%-6.6%) for the DSS (+) mice (Figure 3B) and 8.0% (7.0%-8.6%) for the control mice and 7.9% (7.2%-8.5%) for the DSS (+) mice (Figure 3C), respectively. The CD4+Foxp3+ cells included both CD4+CD25+Foxp3+ T cell and CD4+CD25-Foxp3+ T cell populations. In the spleen, the frequencies of CD4+CD25+Foxp3+ cells and CD4+CD25-Foxp3+ cells among CD4+ T cells were 5.0% (4.4%-5.2%) for the control mice and 4.5% (4.5%-5.1%) for the DSS (+) mice (Figure 3D) and 3.0% (2.6%-3.4%) for the control mice and 3.4% (2.7%-3.4%) for the DSS (+) mice (Figure 3E), respectively. In the MLN, the frequencies of CD4+ CD25+ T cells among CD4+ T cells and CD4+Foxp3+ T cells among CD4+ T cells were 7.4% (6.8%-7.9%) for the control mice and 7.1% (4.9%-16.6%) for the DSS (+) mice (Figure 3G) and 9.4% (9.1%-9.8%) for the control mice and 12.2% (8.3%-20.4%) for the DSS (+) mice (Figure 3H), respectively. In the MLN, the frequencies of CD4+CD25+Foxp3+ cells and CD4+CD25-Foxp3+ cells among CD4+ T cells were 7.0% (6.6%-7.2%) for the control mice and 6.5% (4.5%-13.6%) for the DSS (+) mice (Figure 3I) and 2.6% (2.2%-2.8%) for the control mice and 5.7% (3.5%-7.1%) for the DSS (+) mice (Figure 3J), respectively. These findings demonstrate that CD4+CD25+ cell frequency among CD4+ T cells in the spleen tended to increase in colitis, while the
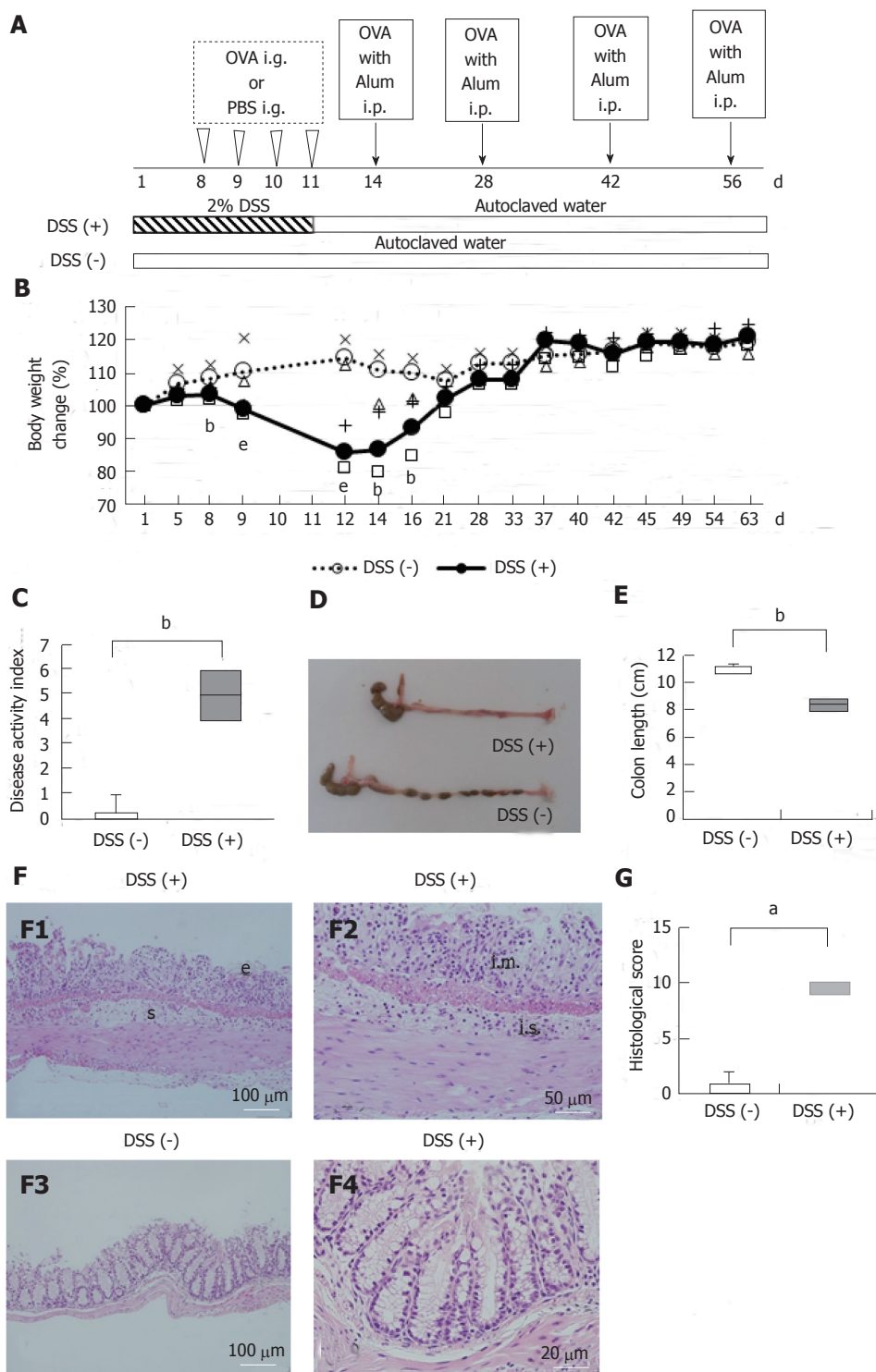
Figure 1 Administration of dextran sulfate sodium to induce colitis. A: Experimental design for the induction of oral tolerance and dextran sulfate sodium (DSS) colitis; B: Percent changes in the body weights of mice that received 2% DSS [DSS (+) mice] and mice that received autoclaved water (control mice). The body weights of the DSS (+) mice (median: Closed circles, first quartile: Plus, third quartile: Open box) were significantly lower than those of the control mice (median: Open circles, first quartile: X-mark, third quartile: Open triangle) from day 8 to day 16; C: Disease activity index (DAI) for the DSS (+) mice and the control mice on day 12. The DAI of the DSS (+) mice (closed bar) was significantly increased compared to that of the control mice (open bar); D: A representative image of colons collected from the DSS (+) mice and the control mice on day 8. The median colon length in the DSS (+) mice was shorter than that in the control mice; E: Colon lengths for the DSS (+) mice and the control mice on day 8. On day 8, the median colon length for the DSS (+) mice (closed bar) was significantly shorter than that for the control mice (open bar); F: Representative hematoxylin and eosin staining of colon sections. On day 8, the colons were removed, fixed in 10% buffered formalin, embedded in paraffin, cut into sections, and stained with hematoxylin and eosin. The DSS (+) mice showed pathological changes characterized by epithelial defects, submucosal edema (F1) and inflammatory cell infiltration in the mucosal layer and submucosal layer (F2). The control mice showed minimal chronic inflammatory cells in the lamina propria with regularly spaced crypts and did not show epithelial defects or submucosal edema (F3, F4); G: Histological scores for the DSS (+) mice and the control mice on day 8. The DSS (+) mice (closed bar) had significantly higher histological scores than the control mice (open bar). The data are shown as the median and interquartile range of three to six mice per group. [a]$P < 0.05$, [b]$P < 0.01$, [e]$P < 0.001$. e: Epithelial; s: Submucosal edema; i.m.: Inflammatory cell infiltration in the mucosal layer; i.s.: Inflammatory cell infiltration in the submucosal layer.
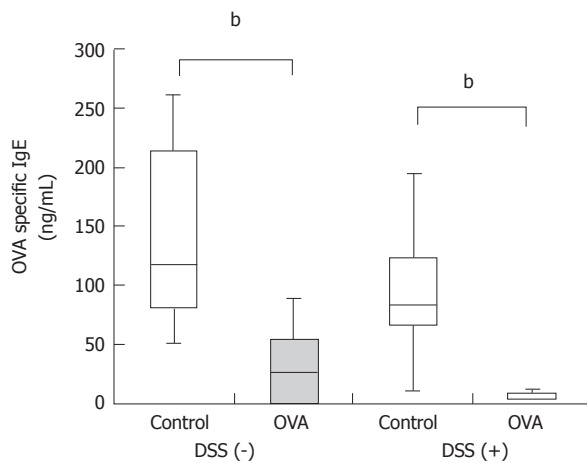
Figure 2 Ovalbumin-specific IgE concentrations in the sera of mice with and without dextran sulfate sodium colitis. On day 63, serum OVA-specific immunoglobulin E (IgE) concentrations were measured by ELISA. The mice that were i.g. administered OVA exhibited significantly lower OVA-specific IgE concentrations (closed bar) than the control mice (open bar) both in the presence and absence of DSS colitis. All results represent at least two independent experiments with four to six mice in each group. The data are shown as the median and interquartile range. [b]$P < 0.01$. OVA: Ovalbumin; DSS: Dextran sulfate sodium; ELISA: Enzyme-linked immuno sorbent assay; i.g.: Intragastrically.

frequencies of CD4^{+}CD25^{+}Foxp3^{+} cells and CD4^{+}CD25^{−}Foxp3^{+} cells among CD4^{+} T cells did not change. Our findings additionally revealed that CD4^{+}CD25^{+}Foxp3^{+} cell frequency among CD4^{+} T cells in the MLN did not change during colitis, while CD4^{+}CD25^{−}Foxp3^{+} Treg frequency increased significantly (Figure 3J, $P < 0.05$). Inflammatory cell infiltration into the colonic mucosa did not influence the stability of CD4^{+}CD25^{+}Foxp3^{+} T cell populations in the spleen, although CD4^{+} T cells in the spleen were activated during colitis. Moreover, inflammatory cell infiltration did not influence the stability of CD4^{+}CD25^{+}Foxp3^{+} T cell populations in the MLN; however, inflammatory cell infiltration increased the population of CD4^{+}CD25^{−}Foxp3^{+} cells in the MLN. These results suggest that the stability of CD4^{+}CD25^{+}Foxp3^{+} T cell populations in the spleen and MLN may play a role in oral tolerance induction in DSS colitis, and elevated numbers of CD4^{+}CD25^{−}Foxp3^{+} Tregs in the MLN may help sustain homeostasis during colitis.

Measurement of Breg frequency in the spleen using flow cytometric analysis
B10 cells are associated with the induction of oral tolerance[8]. Consistent with the regulatory role of B cells, B cell-deficient mice are defective in the ability to develop oral tolerance[9]. We hypothesized that B10 cell populations, similarly to CD4^{+}CD25^{+}Foxp3^{+} cell populations, remain stable during colitis. Therefore, we examined whether any differences existed in CD19^{+}IL-10^{+} cell populations in the spleens of mice with and without DSS colitis. The frequencies of CD19^{+}IL-10^{+} cells among CD19^{+} cells and CD19^{+}CD5^{+}CD1d[Hi] cells among CD19^{+} cells, as well as CD19^{+}CD5^{+}CD1d[Hi]

IL-10^{+} cells among CD19^{+}CD5^{+}CD1d[Hi] cells were 0.8% (0.7%-1.2%) for the control mice and 1.4% (0.9%-1.9%) for the DSS (+) mice (Figure 4B), 1.5% (1.2%-2.5%) for the control mice and 2.4% (1.3%-2.8%) for the DSS (+) mice (Figure 4C), and 7.6% (6.4%-8.4%) for the control mice and 11.3% (6.9%-14.5%) for the DSS (+) mice (Figure 4D), respectively. The frequencies of CD19^{+}IL-10^{+} cells in the spleens of the DSS colitis mice were comparable to those in the spleens of the control mice. CD19^{+}CD5^{+}CD1d[Hi] cell frequency among CD19^{+} cells did not change during DSS colitis. However, IL-10^{+} cell frequency among CD19^{+}CD5^{+}CD1d[Hi] cells tended to increase in the spleens of the DSS colitis mice relative to the control mice. These results suggest that DSS colitis may act either directly or indirectly to promote IL-10 production within CD19^{+}CD5^{+}CD1d[Hi] cells from the spleen.
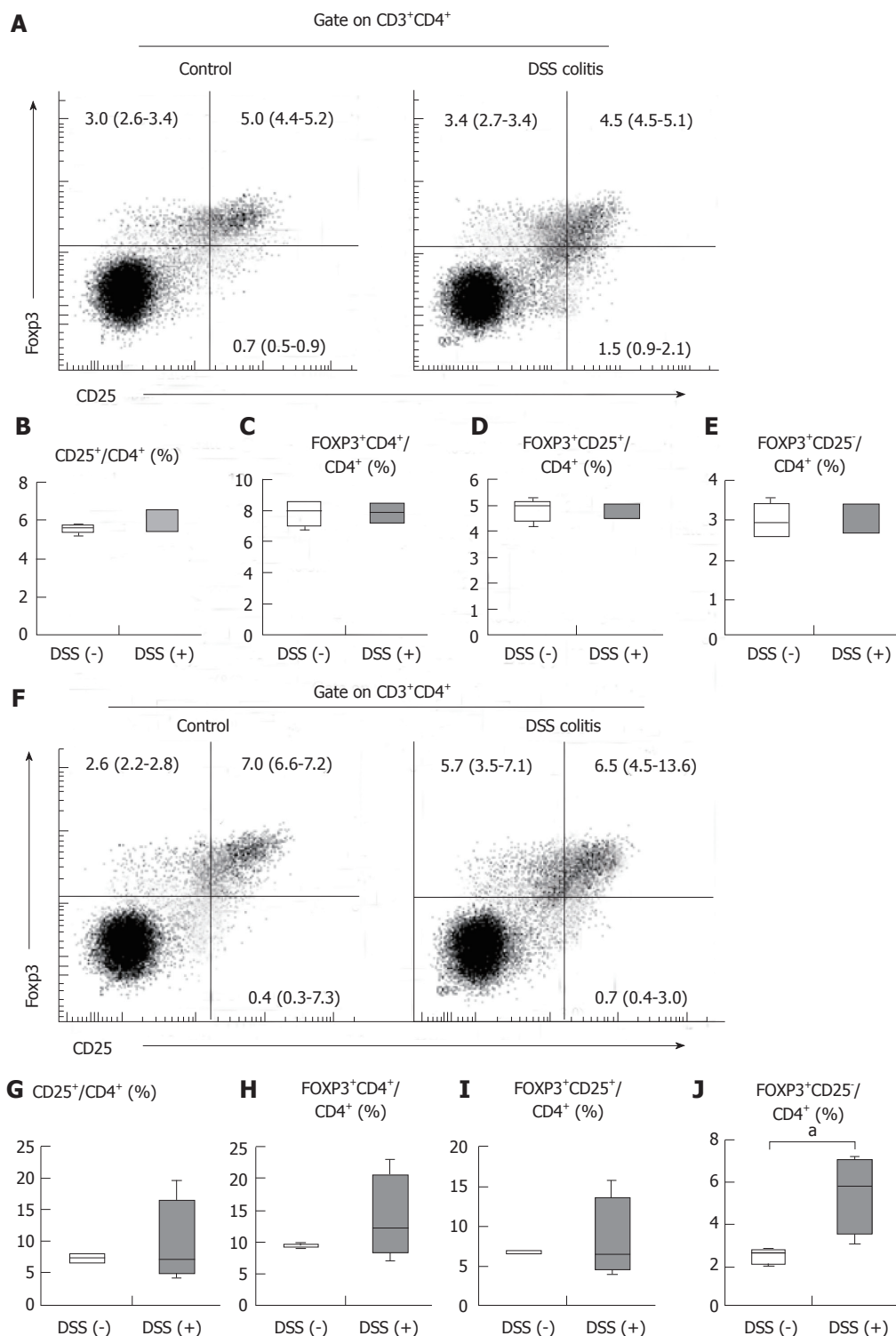
Reverse transcription real-time PCR evaluation of cytokine levels in the spleen and MLN during colitis
As indicated above, CD4^{+}CD25^{+}Foxp3^{+} Treg and B10 cell populations did not decrease during DSS colitis. We next investigated how the levels of cytokines, which influence the function and differentiation of Tregs and B10 cells, change during colitis. In the spleens of the mice with colitis, IFN-γ mRNA expression was significantly lower than that in the mice without colitis ($P < 0.01$) (Figure 5A). However, IFN-γ expression in the MLN were comparable between mice with and without colitis (Figure 5B). Additionally, there were no significant differences in IL-4, IL-6, IL-10 or TNF-α mRNA expression in the spleens or MLNs of mice with and without colitis (Figure 5).

DISCUSSION
In the current study, we revealed that oral tolerance is inducible during the active phase of DSS colitis. We hypothesized that the MLN and spleen, both of which are involved in oral tolerance induction, maintained stability during colitis. We also investigated the manners in which cytokine levels and regulatory cell populations, such as those for Foxp3^{+} T cells and B10 cells, change during colitis.

Histologically, DSS colitis was characterized by epithelial defects and inflammatory cell infiltration. In previous studies, DSS colitis has been shown to exhibit a Th1-predominant profile[24] or a Th1-Th17-predominant profile within the colonic mucosa[25]. During the acute phase of DSS colitis, no differences were found in Foxp3 mRNA expression in colonic tissues from DSS colitis mice and normal mice; however, during the chronic phase, *Foxp3* mRNA expression increased[12]. In the current study, we revealed that CD4^{+}CD25^{+} cell frequency among CD4^{+} T cells in the spleen tended to increase during colitis, while CD4^{+}Foxp3^{+} cell frequency among CD4^{+} T cells did not change. CD25 is known as an activation marker. These results suggest that DSS colitis

A

Gate on CD3$^+$CD4$^+$

Control

3.0 (2.6-3.4)   5.0 (4.4-5.2)

0.7 (0.5-0.9)

Foxp3

CD25

DSS colitis

3.4 (2.7-3.4)   4.5 (4.5-5.1)

1.5 (0.9-2.1)

B CD25$^+$/CD4$^+$ (%)

C FOXP3$^+$CD4$^+$/ CD4$^+$ (%)

D FOXP3$^+$CD25$^+$/ CD4$^+$ (%)

E FOXP3$^+$CD25$^-$/ CD4$^+$ (%)

F

Gate on CD3$^+$CD4$^+$

Control

2.6 (2.2-2.8)   7.0 (6.6-7.2)

0.4 (0.3-7.3)

Foxp3

CD25

DSS colitis

5.7 (3.5-7.1)   6.5 (4.5-13.6)

0.7 (0.4-3.0)

G CD25$^+$/CD4$^+$ (%)

H FOXP3$^+$CD4$^+$/ CD4$^+$ (%)

I FOXP3$^+$CD25$^+$/ CD4$^+$ (%)

J FOXP3$^+$CD25$^-$/ CD4$^+$ (%)

Figure 3 Regulatory T cell frequencies in the spleen and mesenteric lymph nodes as determined by flow cytometry. A: Analysis of CD25 and Foxp3 expression in the spleen. Representative data are shown for control mice (left panel) and DSS colitis mice (right panel); B: CD4$^+$CD25$^+$ cell frequencies in the spleens of DSS colitis mice and control mice. CD4$^+$CD25$^+$ cell frequency tended to increase in the spleens of DSS colitis mice (closed bar) compared to those of control mice (open bar); C: CD4$^+$Foxp3$^+$ cell frequencies in the spleens of DSS colitis mice and control mice. CD4$^+$Foxp3$^+$ cell frequency was comparable in the spleens of DSS colitis mice (closed bar) and control mice (open bar); D: CD4$^+$CD25$^+$Foxp3$^+$ cell frequencies in the spleens of DSS colitis mice and control mice. CD4$^+$CD25$^+$Foxp3$^+$ cell frequency was comparable in the spleens of DSS colitis mice (closed bar) and control mice (open bar); E: CD4$^+$CD25$^-$Foxp3$^+$ cell frequencies in the spleens of DSS colitis mice and control mice. CD4$^+$CD25$^-$Foxp3$^+$ cell frequency was comparable in the spleens of DSS colitis mice (closed bar) and control mice (open bar); F: Analysis of CD25 and Foxp3 expression in the MLN. Representative data are shown for control mice (left panel) and DSS colitis mice (right panel); G: CD4$^+$CD25$^+$ cell frequencies in the MLNs of DSS colitis and control mice. CD4$^+$CD25$^+$ cell frequency was comparable in the MLNs of DSS colitis mice (closed bar) and control mice (open bar); H: CD4$^+$Foxp3$^+$ cell frequencies in the MLNs of DSS colitis mice and control mice. CD4$^+$Foxp3$^+$ cell frequency tended to increase in the MLNs of DSS colitis mice (closed bar) compared with control mice (open bar); I: CD4$^+$CD25$^+$Foxp3$^+$ cell frequencies in the MLNs of DSS colitis mice and control mice. CD4$^+$CD25$^+$Foxp3$^+$ cell frequency was comparable in the MLNs of DSS colitis mice (closed bar) and control mice (open bar); J: CD4$^+$CD25$^-$Foxp3$^+$ cell frequencies in the MLNs of DSS colitis mice and control mice. CD4$^+$CD25$^-$Foxp3$^+$ cell frequency was significantly higher in the MLNs of DSS colitis mice (closed bar) compared with control mice (open bar). All results represent at least two independent experiments with four to six mice in each group. The data are shown as the median and interquartile range. [a]$P < 0.05$. MLN: Mesenteric lymph nodes; DSS: Dextran sulfate sodium.
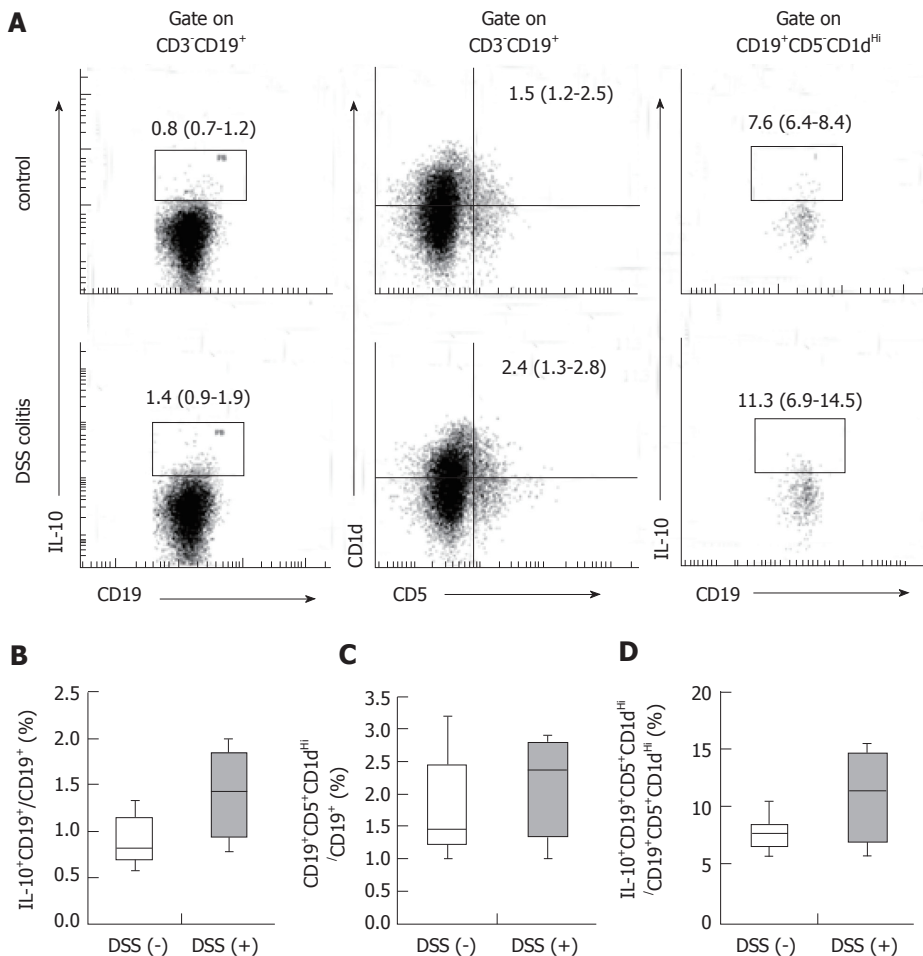
Figure 4 Regulatory B cell frequency in the spleen as determined by flow cytometry. A: Analysis of CD19+IL-10+ cell populations (left panel), CD19+CD5+CD1dHi cell populations (center panel) and CD19+CD5+CD1dHiIL-10+ cell populations (right panel) in the spleen based on flow cytometry. Representative data are shown for control mice (top panel) and DSS colitis mice (bottom panel); B: CD19+IL-10+ cell frequencies among CD19+ cells in the spleens of DSS colitis mice and control mice. CD19+IL-10+ cell frequency among CD19+ cells was comparable in the spleens of DSS mice (closed bar) and control mice (open bar); C: CD19+CD5+CD1dHi cell frequencies among CD19+ cells in the spleens of DSS colitis mice and control mice. CD19+CD5+CD1dHi cell frequency among CD19+ cells was comparable in the spleens of DSS mice (closed bar) and control mice (open bar); D: IL-10+ cell frequencies among CD19+CD5+CD1dHi cell populations in the spleens of DSS colitis mice and control mice. IL-10+ cell frequency among the CD19+CD5+CD1dHi cell population tended to increase in the spleens of the DSS mice (closed bar) compared with those of the control mice (open bar). All results represent at least two independent experiments with four to six mice in each group. The data are shown as the median and interquartile range. DSS: Dextran sulfate sodium; IL: Interleukin.

activated CD4+ cells in the spleen while sustaining the stability of CD4+Foxp3+ cell populations in the spleen. We further revealed that CD4+CD25+ cell frequency among CD4+ T cells in the MLN did not change during colitis, while CD4+Foxp3+ cell frequency among CD4+ T cells tended to increase. In previous studies, CD4+Foxp3+ Treg frequencies in the MLNs and spleens of mice with colitis were lower than those in mice without colitis[13]. The target organs and mouse species assessed, as well as the concentrations of DSS, evaluation timing and evaluation methodology used may explain the differences in these results.

CD4+Foxp3+ T cells include CD4+CD25+Foxp3+ cell and CD4+CD25-Foxp3+ cell populations. The function of CD4+CD25-Foxp3+ Tregs remains unclear. One previous study indicated that CD4+CD25-FoxP3+ Tregs act similarly to conventional Tregs to a certain extent[26]; however, another study demonstrated that CD4+CD25-Foxp3+ Tregs differ from CD4+CD25+ Tregs

both phenotypically and functionally[27,28]. We revealed that mice with and without colitis had comparable frequencies of CD4+CD25+Foxp3+ Tregs within the MLN, while CD4+CD25-Foxp3+ Treg frequency significantly increased during colitis. CD4+CD25-FoxP3+ Tregs may retain a suppressive function in an inflammatory environment[28]. Taken together, the above data indicate that CD4+CD25-Foxp3+ Tregs may play roles in maintaining homeostasis in the MLN and in inducing oral tolerance during DSS colitis.

Recently, Bregs have been shown to play an important role in oral tolerance in addition to Tregs. Allergen-specific, IL-10-producing B cells are involved in the development of tolerance to food allergens[8]. The proportion of IL-10-producing B cells following antigen stimulation was shown to decrease in an allergy group, whereas it increased in a tolerant group[29]. We hypothesized that, similarly to CD4+CD25+Foxp3+ cell populations, B10 cell populations remain stable

A

B
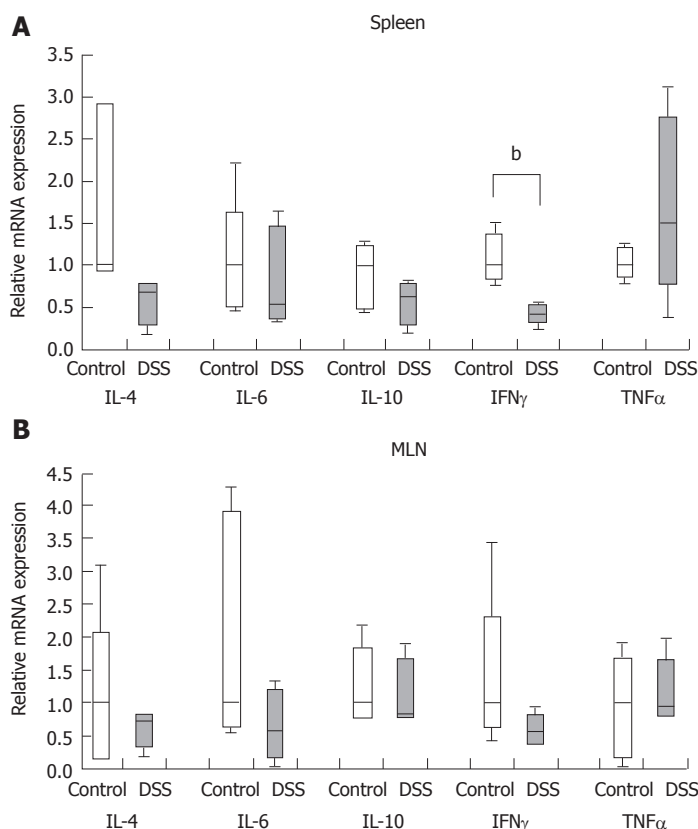
Figure 5 Evaluation of cytokine mRNA expression in the spleen and mesenteric lymph nodes during colitis. A: Evaluation of cytokine mRNA expression in the spleen during colitis. IFN-γ mRNA expression was significantly lower in the spleens of mice with colitis compared to those of mice without colitis. There were no significant differences in IL-4, IL-6, IL-10 or TNF-α mRNA expression; B: Evaluation of cytokine mRNA expression in the MLN in mice with colitis. In the MLN, there was no significant difference in IL-4, IL-6, IL-10, IFN-γ, or TNF-α expression between mice with and without colitis. Expression values were normalized to the expression of the housekeeping gene *GAPDH*. All results represent at least two independent experiments with four to six mice in each group. The data are shown as the median and interquartile range. [b]$P < 0.01$. MLN: Mesenteric lymph nodes; DSS: Dextran sulfate sodium; TNF: Tumor necrosis factor; IFN: Interferon; IL: Interleukin.

during colitis. The frequencies of CD19+IL-10+ cells in the spleens of DSS colitis mice were comparable to those in the spleens of control mice. CD19+CD5+CD1d^Hi is the predominant source of IL-10 production[30]. CD19+CD5+CD1d^Hi cell frequency among CD19+ cells did not change during DSS colitis; however, CD19+CD5+CD1d^HiIL-10+ cell frequency within the CD19+CD5+CD1d^Hi cell population tended to increase. These results suggest that DSS colitis may act either directly or indirectly to promote IL-10 production from CD19+CD5+CD1d^Hi cells in the spleen. B10 cells have also been shown to inhibit intestinal injury in DSS colitis mice[31]. A previous study showed that B10 cell populations did not decrease in the spleen during DSS colitis, similar to the present results[32]. Both the current study and the referenced study suggest that DSS colitis does not decrease B10 cell frequency, inhibit B cell IL-10 production, or inhibit B10 cell functions associated with oral tolerance. However, there were limitations associated with our analysis of B10 cells. Whole cells from the spleen were stimulated with LPS, PMA and ionomycin. Thus, it is not possible to exclude the effects of cells other than B cells on Bregs.

Cytokines can influence Treg function[33] and Breg differentiation[34,35]. As indicated above, the frequencies of CD4+CD25+Foxp3+ Tregs and B10 cells did not decrease during DSS colitis. We therefore investigated how cytokines, which influence the functions of Tregs and B10 cells, change during colitis.

IFN-γ appears to play an important role in food allergen tolerance induction. Specific oral immunotherapy using IFN-γ may induce tolerance induction in both IgE-mediated[36,37] and non-IgE-mediated food allergies[37]. IFN-γ can both promote and subvert Treg suppressive activity in various settings, and the balance between these opposing functions likely depends on contextual factors, such as the timing and extent of the expression[33]. IFN-γ induces murine CD5+ B1 cells to adopt a macrophage-like morphology. Macrophage-like B1 cells express high levels of CD5[38]. Moreover, IFN-γ induces allergen-specific B10 responses and promotes tolerogenic function[29,34]. In previous studies, during the acute phase of DSS colitis, colonic Th cells have been shown to exhibit a Th1 profile, rather than a Th2 or Th17 profile[24]. Moreover, DSS colitis leads to a Th1-Th17 response during its active phase[25]. In the current study, we revealed that IFN-γ mRNA expression was reduced in the spleens of mice with DSS, while its expression did not change in the MLN. This change in IFN-γ mRNA expression in the spleen did not influence oral tolerance, whereas the stability of IFN-γ expression in the MLN may have influenced the induction of oral tolerance.

IL-4 inhibits Treg function[33]. IL-4 receptor signaling has been shown to impair the capacity of Tregs to suppress mast cell activation and expansion, which in turn drives Th2-cell reprogramming of Tregs[39]. Moreover, IL-4 inhibits mouse CD5+ B1 cells from adopting a macrophage-like morphology[38]. In previous studies, DSS colitis mice have not exhibited increased IL-4 production from colonic T cells[24]. Similarly, in the current study, we revealed that DSS colitis mice did not exhibit increased IL-4 mRNA expression in either the spleen or MLN. This stability of IL-4 mRNA expression

in the spleen and MLN may influence oral tolerance induction.

IL-6 subverts Treg cell function[33] and is essential for the differentiation of IL-10-producing B cells. Bregs are induced by the gut microbiota; this induction is driven by IL-6 production[35]. During both the acute and chronic phases of DSS colitis, serum IL-6 concentrations increase[25]. In the current study, we revealed that IL-6 mRNA expression was stable in the spleens and MLNs of mice with DSS colitis. This stability may influence oral tolerance induction.

IL-10 signaling is required to maintain Treg and Breg functions. IL-10 exhibits anti-inflammatory effects in part through its regulation of Treg stability and function both under steady-state conditions and during inflammation[33]. Autocrine stimulation of IL-10 is critical toward enriching IL-10 production in CD40^{Hi}CD5^{+} Bregs both *in vitro* and *in vivo*[40]. In DSS colitis, serum IL-10 levels have been shown to remain stable during the acute phase, whereas these levels increase during the chronic phase[25]. In the current study, we revealed that IL-10 mRNA expression was stable in the spleens and MLNs of mice with DSS colitis. This stability may influence oral tolerance induction.

TNF-α can both promote and subvert Treg cell function[34]. In DSS colitis, serum TNF-α concentrations increase during the acute phase but remain stable during the chronic phase[25]. We revealed that TNF-α mRNA expression was stable in the spleens and MLNs of mice with DSS colitis. This stability may influence oral tolerance induction.

In our cytokine analysis, IFN-γ expression decreased in the spleens of mice with DSS colitis, whereas IFN-γ expression in the MLN and IL-4, IL-6, IL-10 and TNF-α expression in both the spleen and the MLN remained stable. The cytokine profiles associated with DSS colitis may help to maintain the function and differentiation of Tregs and Bregs, which in turn are associated with oral tolerance. However, it should be noted that we only evaluated cytokine mRNA expression and not cytokine production.

Oral immunotherapy has been utilized for various immune disorders. However, oral immunotherapy is considered only poorly effective for IBD because IBD patients have dysfunctional oral tolerance[14,15]. One mechanism underlying this dysfunction is small intestinal permeability. In an IL-10 knock out model, increasing small intestinal permeability was shown to prevent the development of oral tolerance[41]. Several studies have also shown that a defect in intestinal epithelial permeability may be involved in the pathogenesis of IBD. Supporting this concept, other studies have shown that increased intestinal permeability precedes the onset of colitis in experimental animal models of IBD[41-43]. Conversely, IBD family members with no clinical symptoms exhibit dysfunctional oral tolerance, although small intestinal permeability is within the normal range. Thus, other genetic backgrounds are likely involved in dysfunctional oral tolerance[44]. In

previous reports, the absence of functional inducible nitric oxide synthase (iNOS) enhanced the efficacy of oral tolerance[45]. Conversely, nitric oxide (NO) and iNOS production were increased both in colonic tissues collected from IBD patients and in a DSS-induced colitis model[46,47]. These data suggest that dysfunctional oral tolerance in IBD patients might be due to NO induction in addition to inflammation.

Although the effectiveness of oral immunotherapy for CD patients has recently been reported[16,17], there are few reports regarding the use of oral immunotherapy for UC patients. DSS-induced colitis serves as an experimental animal model of UC[10,11]. In the present study, we used this DSS colitis model to explore the potential use of oral immunotherapy as a treatment during the active phase of UC. Oral administration of CEP prior to the onset DSS colitis has been shown to induce immune tolerance, downregulate the inflammatory immune response and alleviate DSS-induced colitis[18,19]. However, no reports have evaluated oral tolerance following the oral administration of CEP after DSS colitis has developed.

To the best of our knowledge, the current study is the first to demonstrate that oral tolerance is inducible during the active phase of DSS colitis. Lymphocytic infiltration into the large intestine mucosa associated with epithelial defects did not influence oral tolerance. In addition to that used here, there are many other mouse models of IBD available for use. Further research evaluating oral tolerance in these models is warranted prior to clinical translation. Our study suggests that the choice of an appropriate antigen will enhance the effectiveness of oral immunotherapy for the treatment of UC.

COMMENTS

Background
Oral immunotherapy is considered only poorly effective for inflammatory bowel disease (IBD) because IBD patients have dysfunctional oral tolerance. Although the effectiveness of oral immunotherapy for Crohn's disease patients has recently been reported, there are few reports regarding the use of oral immunotherapy for ulcerative colitis (UC) patients. Dextran sulfate sodium (DSS) colitis serves as an animal model of UC. Oral administration of colon extract protein (CEP) prior to the onset of DSS colitis has been shown to alleviate colitis; however, the effectiveness of oral administration of CEP after the onset of DSS colitis has not been evaluated.

Research frontiers
The purpose of this study was to investigate whether oral tolerance is inducible during the active phase of DSS colitis. Additionally, The authors determined how cytokine levels and regulatory cell populations, such as those of Foxp3^{+} T cells and B10 cells, which are associated with oral tolerance, change in colitis in the mesenteric lymph nodes and the spleen.

Innovations and breakthroughs
This study is the first to demonstrate that oral tolerance is inducible during the active phase of DSS colitis. Lymphocytic infiltration into the large intestine mucosa associated with epithelial defects did not influence oral tolerance. The frequency of CD4^{+}CD25^{+}Foxp3^{+} cells and B10 cells, which are associated with oral tolerance, did not change significantly. In the spleen, IFN-γ mRNA expression decreased in mice with colitis, but the expression levels of other

cytokines did not significantly change. This stability in regulatory cell populations and the observed cytokine profiles might influence oral tolerance induction during DSS colitis.

Applications

This study suggests that if an appropriate antigen is chosen, then oral immunotherapy may be applicable for the treatment of UC.

Terminology

Oral tolerance is a phenomenon in which systemic immunity is suppressed following the oral administration of antigens. Oral immunotherapy has been applied for various immune disorders.

Peer-review

The manuscript by Ino *et al* is an interesting study and first to demonstrate that oral tolerance is inducible in the active phase of DSS colitis. In addition the authors tried to make a link between oral tolerance and the numbers of Treg and B10 cells.

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