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Daucosterol suppresses dextran sulfate sodium (DSS)-induced colitis in mice

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ABSTRACT

The effects of daucosterol have been identified in cancer therapy and neuronal diseases. However, the regulatory function of daucosterol in DSS-induced colitis has not yet been investigated. In this study, we evaluated the immunological and therapeutic effects of daucosterol in a mouse model of dextran sulfate sodium (DSS)-induced colitis. Unlike vehicle mice, mice pre- or post-treated with daucosterol showed inhibition of body weight loss and the decrease in the disease activity index (DAI). In addition, daucosterol treatment rescued the DSS-induced decrease in colon length and disruption of the epithelial lining. Furthermore, it reduced DSS-induced production of reactive oxygen species (ROS), infiltration of macrophages, and expression of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. Mice with colitis showed a decreased population of $Foxp3^+$ cells, which was upregulated by daucosterol treatment. Furthermore, daucosterol increased natural killer (NK) cell activity and inhibited excessive IgA levels in mice with DSS-induced colitis. Collectively, our findings demonstrated that daucosterol significantly alleviated DSS-induced colitis, indicating the possibility of daucosterol as a therapeutic option for colitis.

1. Introduction

Inflammatory bowel disease (IBD) comprises two main subtypes, ulcerative colitis (UC) and Crohn's disease (CD) base on the pathologic location in the gut [1]. Although UC develops in the colon and rectum, CD affects the whole gastrointestinal tract, from the mouth to the anus. Colitis is diagnosed based on clinical symptoms such as abdominal pain, bloody stool caused by rectal bleeding, diarrhea, and weight loss [2]. It is caused by a bacterial infection, an abnormal immune response, and the environment in the intestines; however, the precise underlying mechanism has not yet been identified [3]. Dextran sulfate sodium (DSS) is widely used to induce colitis so as to establish a mouse model of the disease. DSS induces disruption of the epithelial lining, infiltration of immune cells, and inflammatory response in the colon [4].

It has been well known that regulatory T (Treg) cells play an important role in maintaining immune homeostasis. Specifically, previous studies reported that Treg cells plays a critical role in the suppression of colitis-induced inflammatory responses by inhibiting the production of inflammatory cytokines and regulating of inflammation-related cells [5]. In addition, the alteration of balance between $Foxp3^+CD4^+$ Treg and effector T cells in the intestinal environment is considered a factor in inflammatory bowel disease (IBD) pathogenesis [6]. Foxp3 is a key transcription factor for regulation of Treg cell function and differentiation [7]. A significant reduction in the number of Treg cells is also observed in IBD animal model, suggesting that regulation of Treg cells might be an effective therapy for IBD [8].

Natural killer (NK) cells are a major component of the innate immune response and play an important role in mucosal immunity. Similar to T cells, mucosal NK cells promote an antipathogen response and maintenance of the intestinal epithelium [9]. Furthermore, recent studies

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show lower NK cell activity in colitis animals than is normal, indicating that NK cells have a potential role in the pathogenesis of IBD [10]. However, the precise mechanisms of NK cell-mediated alleviation of IBD pathogenesis remain unclear. With the reduction of NK cell activity, excessive IgA secretion in the colon is also observed in IBD patients, and IgA levels can be used as an indicator of IBD pathogenesis [11].

Daucosterol, a natural sterol, is a glucoside of β-sitosterol; it is mainly synthesized from plants. The effects of daucosterol have been reported in the suppression of cancer, promotion of neural stem cell proliferation, and induction of Th1 immune response [12–14]. However, the effects of daucosterol against colitis remain to be investigated. In the present study, we aimed to elucidate the beneficial effects of daucosterol against colitis. To this end, we evaluated the effects of the compound against the clinical symptoms of colitis and colitis-induced factors, such as Treg cells, NK cells, and IgA, in a mouse model of DSS-induced colitis after pre- or post-treatment with daucosterol.

2. Materials and methods

2.1. Animals

Eight-week-old female C57BL/6J mice were purchased from Damul Science (Daejeon, Republic of Korea). All mice were maintained in specific pathogen-free conditions, and all of the animal experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee of Chonnam National University.

2.2. DSS-induced colitis mouse model and daucosterol treatment

For induction of colitis, DSS (36,000–50,000 kDa; MP Biomedical, Solon, OH, USA) was dissolved in drinking water to 2% and administered for 9 days. Daucosterol (Sigma, St. Louis, MO, USA) was orally administered 3 times every week at 10 mg/kg in 0.5% carboxymethylcellulose (Sigma) solution. For pre-treatment, daucosterol was administrated for 2 weeks prior to DSS treatment, and lasted during the whole period of experiment. For post-treatment, daucosterol administration was started 2 days after DSS treatment.

2.3. Clinical assessment of DSS-induced colitis

The disease activity index (DAI) of colitis was determined as described previously [15]. Briefly, stool consistency, occult/gross blood in stools, and body weight loss were recorded daily, and DAI was calculated based on these results.

2.4. Histological analysis and scoring

Colon tissues were fixed with 10% formalin, embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E), and histological scores were determined as previously described [15].

2.5. Isolation of primary colon cells

Whole colon tissue was isolated from mice; after the feces was removed, tissue samples were cut and transferred to a 50-ml conical tube. Ten milliliters of RPMI-1640 (GIBCO, Big Cabin, OK, USA) containing 2% fetal bovine serum (FBS) was added with 1 mg/ml type IV collagenase (Sigma, St. Louis, MO, USA). The mixture was shaken and incubated at 250 rpm for 30 min. After filtering using a 100-mesh (150 μm) screen, it was centrifugation at 500 ×*g* for 10 min at 4 °C. The supernatant was removed and the isolated colon cells were used for flow cytometry analysis.

2.6. Isolation of primary mesenteric lymph node and spleen cells

Mesenteric lymph node (MLN) and spleen were isolated from the animals, crushed, and filtered using with a 70-μm cell strainer. After centrifugation, primary spleen cells were added to ACK lysis buffer (Red Blood Cell lysis, Thermo Scientific, Waltham, MA USA) to remove red blood cells by incubation for 5 min following by washing twice with RPMI-1640 medium containing 10% FBS. The isolated primary MLN and spleen cells were resuspended in RPMI-1640 medium containing 10% FBS and used for the experiment.

2.7. Flow cytometry analysis

Primary cells were stained with individual fluorescent conjugated antibodies for 20 min at 4 °C in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline [PBS] containing 3% FBS and 0.1% NaN_3). After washing twice with FACS buffer, the cells were analyzed by FACS Calibur using Modifit LT 3.0 software (Verity Software House, San Diego, CA, USA).

2.8. Quantitative real-time PCR

Total RNA was extracted from the colon cells using TRIreagent (MRC, Cincinnati, OH, USA), according to the manufacturer's guideline. cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). cDNA samples synthesized by reverse transcription were added to AccuPower® 2X GreenStar™ qPCR Master Mix (Bionner, Daejon, Korea) containing 2× mixture, ROX dye, and 10 pmol of primers for each gene. The sequences of the primers used in quantitative real-time PCR are as follows: GAPDH: Forward, 5′-CCC TTA TTG ACC TCA ACT ACA TGG T-3′ and Reverse, 5′-GAG GGG CCA TCC ACA GTC TTC TG-3′; TNF-α: Forward, 5′-CAT CTT CTC AAA ATT CGA GTG ACA A-3′ and Reverse, 5′-TGG GAG TAG ACA AGG TAC AAC CC-3′; IL-6: Forward, 5′-CCG GAG AGG AGA CTT CAC AG-3′ and Reverse, 5′-CAG AAT TGC CAT TGC ACA AC-3′; and IL-1β: Forward, 5′-CAA CCA ACA AGT GAT ATT CTC CAT G-3′ and Reverse, 5′-GAT CCA CAC TCT CCA GCT GCA-3′. Quantitative real-time PCR was performed under the following conditions: 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 15 s.

2.9. Immunohistofluorescence

Tissue sections were fixed with acetone for 5 min and then dried at room temperature for 10 min. Tissue sections were incubated in TBS-T (50 mM Tris, 150 mM NaCl, pH adjustment with HCl to 7.6 along with 0.05% Tween-20) for 15 min. Thereafter, they were washed twice with TBS-T and permeabilized using 1% Triton X-100. Blocking was performed using 3% bovine calf serum (BSA) dissolved in TBS-T. Mouse Foxp3-Alexa Fluor 488 antibody (MF-14, Biolegend, San Diego, CA, USA) was added to the tissue sections and incubated was performed at room temperature for 1 h. After washing with TBS-T, the cells were stained with 4′6-diamidino-2-phenylindole (DAPI), and the expression of Foxp3 was confirmed using fluorescence microscopy.

2.10. NK cytotoxicity assay

NK cytotoxicity was measured using the cytoTox 96 lactate dehydrogenase (LDH)-release assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. NK cells isolated from mouse spleen were pretreated with recombinant murine IL-2 (20 ng/ml; Peprotech Inc., Rocky Hill, NJ, USA). After 24 h, the NK cells were co-cultured with Yac-1 cells as target cells for 4 h at 37 °C in a 5% CO_2 incubator. LDH activity in the culture supernatant was measured using a chromogenic substrate. Absorbance was measured at 490 nm by using a plate reader. Specific lysis percentage was calculated using the following formula: (experimental effector spontaneous release – experimental target spontaneous release) / (target maximum release – target spontaneous release) × 100.

2.11. ELISA

PBS containing a protease inhibitor was added to mouse feces. After centrifugation, fecal extracts were harvested from the supernatant. Ninety-six–well plates (Nunc Maxisorp, Roskilde, Denmark) were coated with the fecal extracts and then blocked with PBS containing 5% BSA for 1 h at room temperature. After washing thrice, HRP-conjugated IgA antibody was added and the reaction was allowed for 2 h at room temperature. Thereafter, tetramethylbenzidine (TMB) solution was added and stopping buffer (distilled water containing 10% sulfuric acid) added within 30 min. Absorbance was measured at 450 nm using a plate reader.

Protein amounts of IL-10 and IFN-g in the colon were measured using ELISA kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

2.12. Measurement of reactive oxygen species (ROS)

The isolated colon cells were rinsed with PBS twice and re-suspended in 500 µl PBS including 2′7′-dichlorofluorescin diacetate (DCF-DA, 10 mM). After incubation at room temperature 30 min, the cells were rinsed with PBS twice and analyzed by FACS Calibur using Modifit LT 3.0 software (Verity Software House). The distribution of DCF-DA positive cells was presented by histogram and the shift level was measured by mean fluorescence intensity (MFI).

2.13. Statistical analysis

For statistical analyses, *p*-values were calculated using a two-way analysis of variance or Student's *t*-test. Data were considered statistically significant when p-values were < 0.05.

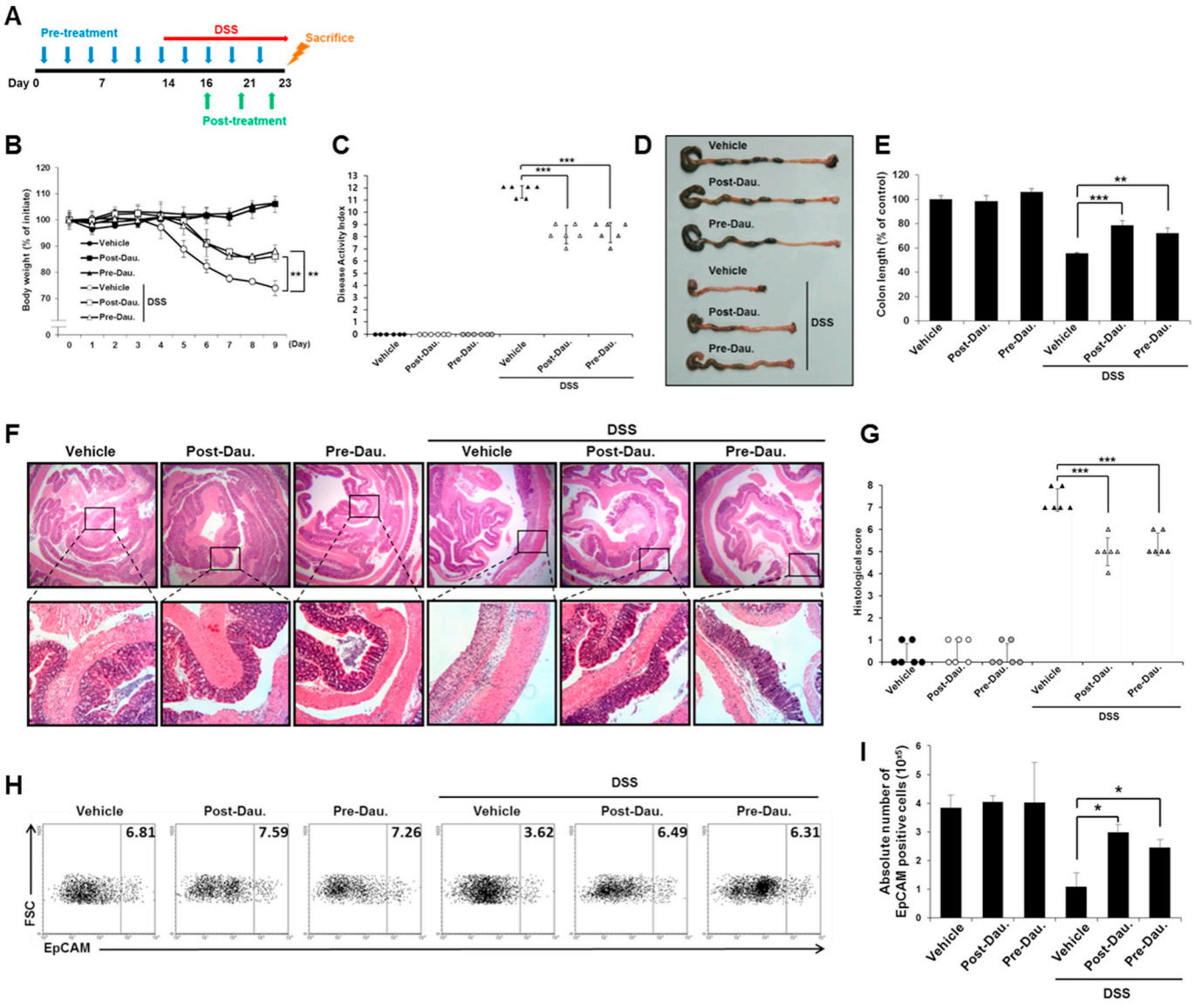

Fig. 1. Daucosterol ameliorated the clinical symptoms of DSS-induced colitis.
(A) Administration schedule of daucosterol in DSS-induced colitis mouse model. (B) Body weight was measured during drinking 2% DSS for 9 days. (C) DAI value was calculated as described in the Materials and Methods. (D) Macroscopic observation of the colon. (E) Colon length was measured and presented as a line graph compared to the vehicle length. (F) Colon tissues were embedded in paraffin, sectioned, and observed under a microscope (100×) after H&E staining. (G) The histological score was determined as described in the Materials and Methods. (H) The isolated colon cell Flow cytometry analysis showed that the population and absolute number s were stained with a fluorescent conjugated anti-EpCAM antibody, and were measured using flow cytometry. (I) The absolute number of $EpCAM^+$ cells analyzed using flow cytometry is shown in a bar graph. Data are shown as the mean ± SEM values and are representative of three independent experiments ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

3. Results

3.1. Daucosterol prevented clinical symptoms of DSS-induced colitis

To investigate the effects of daucosterol on colitis, the clinical symptoms of colitis, such as body weight loss, DAI, a decrease in colon length, and disruption of the epithelial cell lining, were observed in DSS-induced colitis mice treated with post and pre-daucosterol (Fig. 1A). DSS-induced colitis led to a decrease in mouse body weight, but the ratio of the reduced body weight was lower in the daucosterol-treated mice than in the vehicle-treated mice (Fig. 1B). Furthermore, DAI was lower in the daucosterol-treated mice than in the controls (Fig. 1C). Daucosterol treatment led to the prevention of the DSS-induced shortening of colon length (Fig. 1D and E). Furthermore, DSS-induced disruption of epithelial cell lining and muscle layer thickness was lesser in the daucosterol-treated mice than in the controls (Fig. 1F). Expectedly, the histological disease scores of the daucosterol-treated mice were lower than those of the vehicle-treated mice (Fig. 1G). To more accurately determine the disruption of epithelial cells, the distribution of epithelial cells in the colon was analyzed by flow cytometry using the anti-EpCAM antibody as a marker of epithelial cells (Fig. 1H). Distribution of epithelial cells, represented as EpCAM$^+$ absolute cell number, was decreased by DSS, which was increased in daucosterol-treated mice (Fig. 1I). These results suggest that pre- and post-daucosterol treatment may have a preventive and therapeutic role against DSS-induced colitis.

3.2. Daucosterol ameliorated inflammation in DSS-induced colitis

Previous studies reported that DSS-induced colitis increases the total number of immune cells, including macrophages [4] and that the increased immune cells lead to an inflammatory response characterized by the production of reactive oxygen species (ROS) and inflammatory cytokines including TNF-α, IL-6, and IL-1β [4]. Accordingly, to determine whether daucosterol regulates the secondary immune responses induced by colitis, the distribution of macrophages, production of ROS and inflammatory cytokines were estimated in DSS-induced colitis mice with or without daucosterol treatment. DSS-induced ROS production and MFI values were decreased in daucosterol-treated mice compared to those in controls (Fig. 2A and B). Distribution of F4/80$^+$ macrophages was upregulated in mice treated with only DSS, which was reduced by both pre- and post-treatment of daucosterol (Fig. 2C and D). In addition, daucosterol specifically reduced mRNA levels of inflammatory cytokines, TNF-α, IL-6, and IL-1β, increased by DSS (Fig. 2E). Furthermore, protein levels of IL-10 were reduced by DSS treatment in the colon, but restored by daucosterol treatment. In contrast, DSS-induced overproduction of IFN-γ protein was suppressed by daucosterol treatment (Fig. 2F). These results suggested that daucosterol can alleviate inflammation in colitis through inhibition of ROS and inflammatory cytokine production associated with macrophage

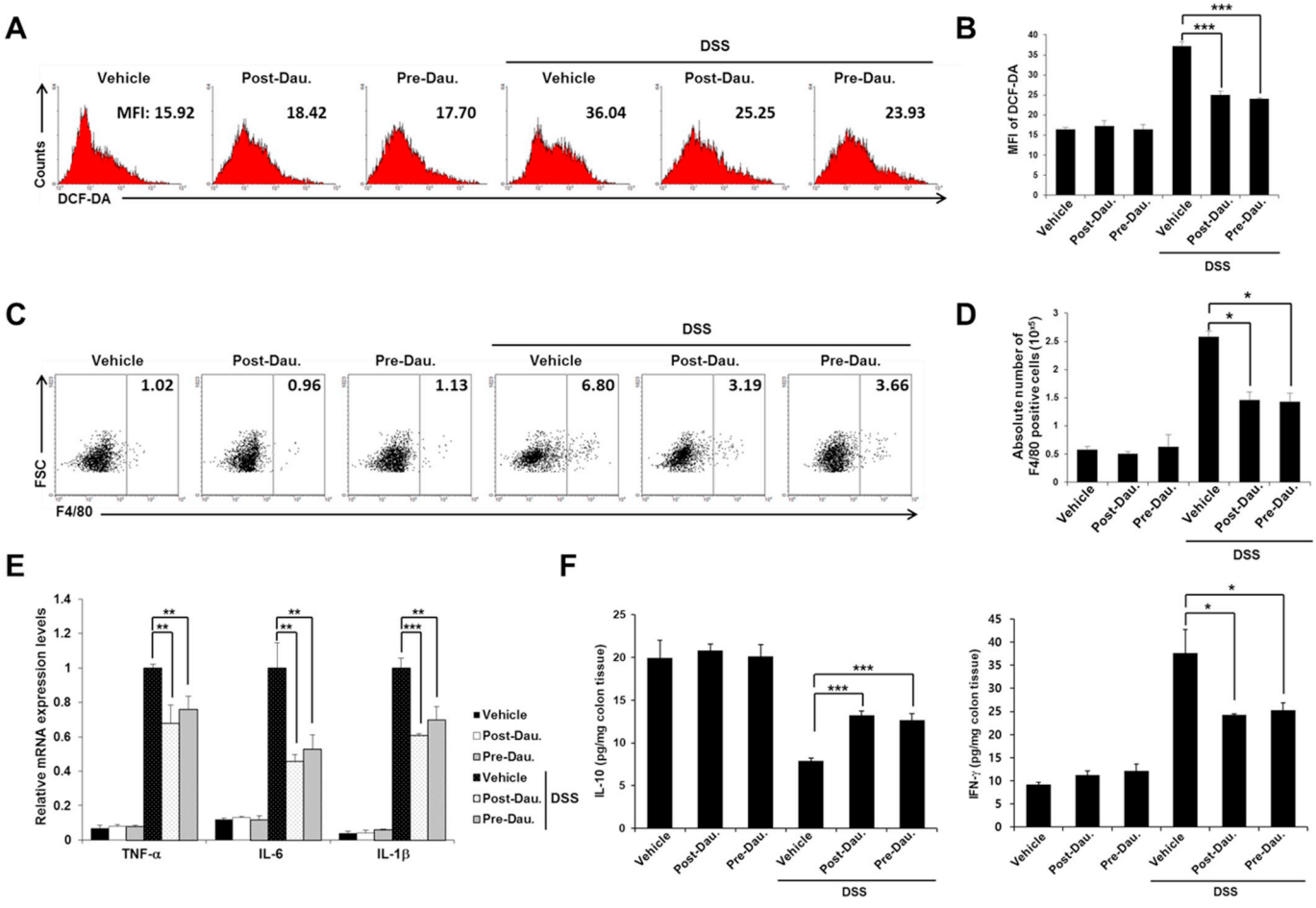

Fig. 2. Daucosterol decreased DSS-induced inflammation in the colon.
(A) For analysis of intracellular ROS production, cells from the colon were stained with DCFH-DA and MFI values were measured using flow cytometry. (B) The MFI values of DCF-DA were represented as bar graphs. (C) Analysis of macrophage population was performed by flow cytometry analysis using fluorescent conjugated anti-F4/80. (D) The absolute number of F4/80$^+$ cells were shown as bar graphs. (E) Gene expression levels of cytokines were analyzed in primary total colon cells using quantitative real-time PCR. (F) The amount of protein produced in the colon was measured using ELISA. Data are shown as the mean ± SEM values and are representative of three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

infiltration.

3.3. Daucosterol regulates the number of Treg cells reduced by colitis

Treg cells play key a role as suppressors of the inflammatory response [16]. Specifically, $CD4^+$ Treg cells have a critical role in the maintenance of the microenvironment in colitis [17]. To determine whether daucosterol can regulate the distribution of Treg cells in DSS-induced colitis, the population of Treg cells was analyzed by flow cytometry. The distribution of $CD4^+Foxp3^+$ cells was dramatically reduced in the MLN and colon treated with DSS alone, but restored to about 70% of WT level by both pre- and post-treatment of daucosterol (Fig. 3A and B). The immunohistofluorescence analysis showed that Treg cells were decreased by DSS. The number of Treg cells decreased by DSS was increased by daucosterol treatment (Fig. 3C). The colitis-related reduction in the $Foxp3^+$ cell number was also upregulated by daucosterol treatment (Fig. 3D). These results imply that daucosterol-mediated regulation of Treg cells may have a crucial role in colitis for the suppression of inflammatory responses.

3.4. Daucosterol involved to the regulation of NK cell activity and IgA production in colitis

Impairment of NK cell activity has been found in colitis [10]. Additionally, colitis patients show high levels of IgA, which is produced by mainly B_1 cells [11]. To determine whether daucosterol improves colitis-induced mucosal immune responses, impairment of NK cell activity and elevation of IgA production were evaluated with NK and B_1 cell populations. Flow cytometry analysis showed that the population and absolute number of $CD69^+NK1.1^+$ cells (activated NK cells) were reduced by DSS, but recovered by administration of daucosterol (Fig. 4A and B). Expectedly, colitis-induced downregulation of NK cytotoxicity was also restored by daucosterol treatment (Fig. 4B). Furthermore, the DSS-induced excessive production of IgA levels (Fig. 4C) and the enhanced IgA-producing B1 cell activity were suppressed in the daucosterol-treated mice (Fig. 4D). These results indicate that daucosterol can improve indirectly the colitis-induced impairments in NK cells and excessive IgA production.

4. Discussion

Colitis is caused by a variety of reasons, in particular, several compounds such as formononetin [18], *N*-methylcytisine [19] and magnolon [20] have been reported to be effective in DSS-induced colitis. In this study, we demonstrated that pre- or post-treatment with daucosterol alleviated the clinical symptoms of colitis. Furthermore, our findings provide evidence that the suppressive effects of daucosterol against colitis-induced clinical symptoms are mediated by inhibition of macrophage infiltration and secondary inflammatory products, such as ROS and inflammatory cytokines. The suppressive effects

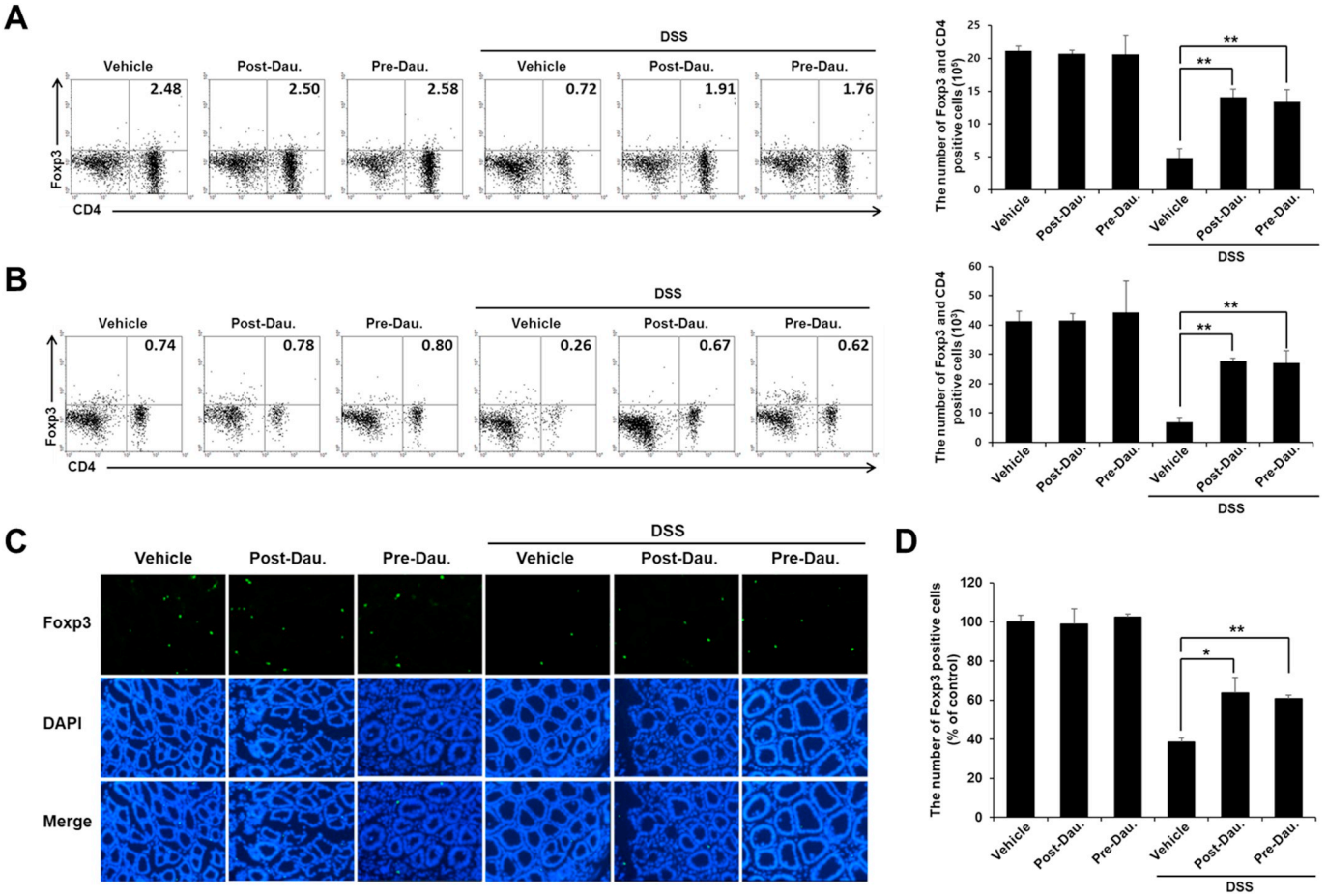

Fig. 3. Daucosterol restored the reduced number of Treg cells by DSS.
Cells were isolated from the MLN (A) and colon (B), and flow cytometry analysis was performed after staining with fluorescent-conjugated Foxp3 and CD4 antibodies. The numbers in each quadrant represent the percentages of $CD4^+Foxp3^+$ cells (left panel). The absolute number of each population was determined by calculating from flow cytometry profiles (right panel). (C) The expression levels of Foxp3 in colon tissue were examined by immunofluorescence staining. (D) The number of $Foxp3^+$ cells were counted in the equal areas of the colon. Data are shown as the mean ± SEM values and are representative of three independent experiments ($*p < 0.05$, $**p < 0.01$).

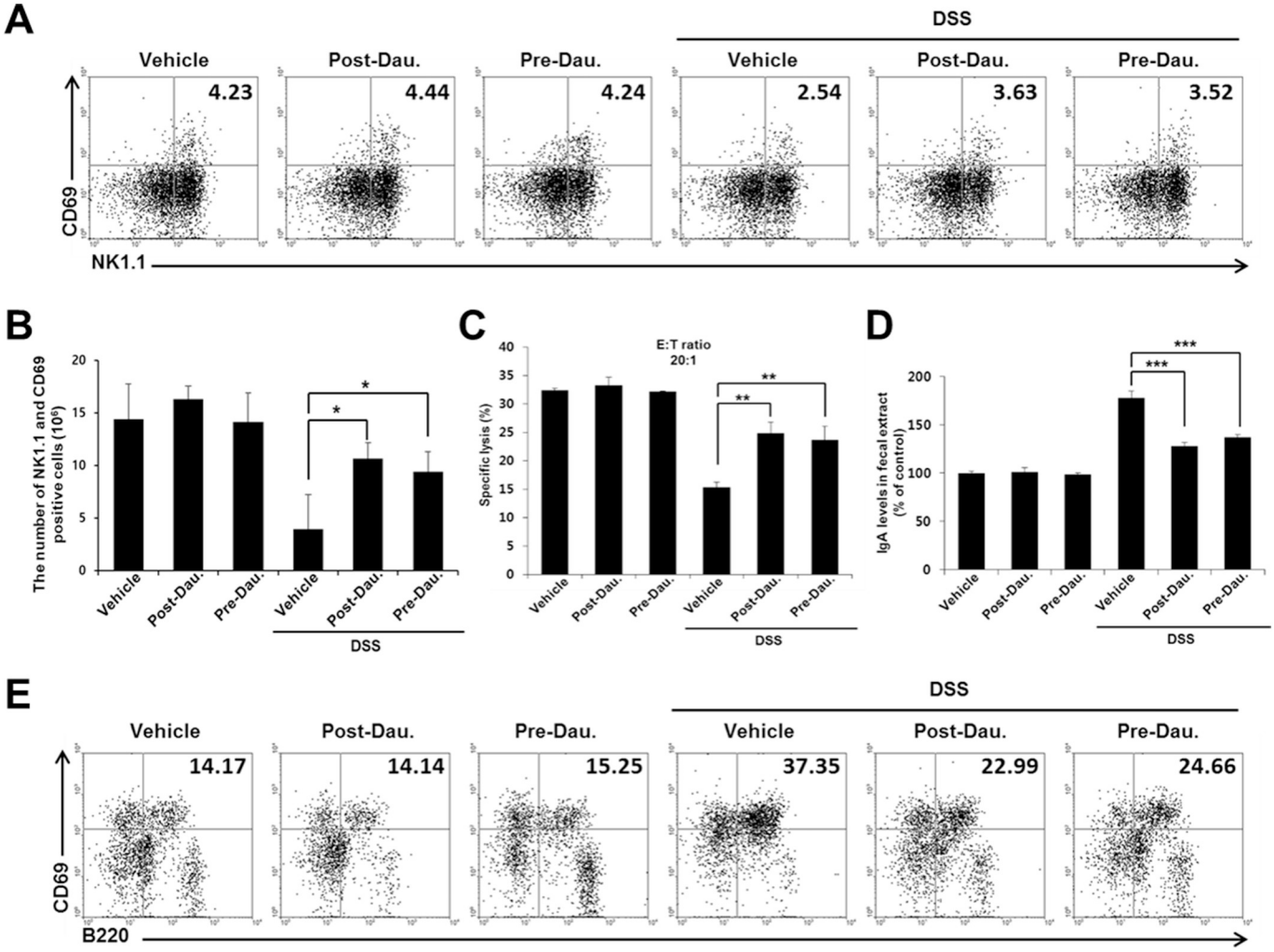

Fig. 4. Daucosterol prevented the reduction of NK cell activity and excessive production of IgA levels in DSS-induced colitis.
(A) The cells were isolated from the spleen, and analyzed by flow cytometry using the indicated fluorescent-conjugated antibodies. The numbers in each quadrant represent the percentages of $CD69^+NK1.1^+$ cells. (B) The absolute number of each population was determined by calculating from flow cytometry profiles. (C) NK cells, used as effector cells, were isolated from the spleen. NK cell cytotoxicity against Yac-1 cells used as target cells was examined by LDH-assay at a ratio of 20:1 (E:T). (D) IgA levels in feces were measured by ELISA using fecal extracts. (E) Cells isolated from the colon were stained and analyzed using flow cytometry. Data are shown as the mean ± SEM values and are representative of three independent experiments ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

of daucosterol are also corroborated by Treg cell number, NK cell activity and IgA production, which are considered as indicators of colitis severity. Collectively, the findings of the present study suggest that daucosterol significantly ameliorates DSS-induced colitis through the regulation of inflammation and immune cell infiltration in the colon.

Several studies have reported beneficial effects of daucosterol with regard to the promotion of neural stem cell proliferation and protection against fungal infection [13,14]. However, the present study identified daucosterol effects in DSS-induced colitis mouse model, which have not been reported previously. Specifically, this study showed that daucosterol regulates the population and activation of immune cells, such as macrophages, Treg cells, NK cells, and B1 cells in colitis. Given that macrophages, Treg cells, NK cells, and B_1 cells contribute to allergies, infections, tumors and mucosal immunity, our findings imply that daucosterol can be used to improve various immune responses. However, further detailed studies are required to define the precise mechanism in daucosterol-mediated regulation of macrophage and regulatory T cell infiltration.

It is well known that infiltration of immune cells induces inflammation with secondary inflammatory products during the progression of colitis [4], and Treg cells play an essential role in the downregulation of colitis -induced inflammation [5]. We found that daucosterol inhibits colitis-induced ROS and inflammatory cytokines, such as TNF-α, IL-6, IL-1β and IFN-γ, accompanied by a regulation of Treg cell number and reduction of macrophage infiltration. In addition, decreased by DSS, anti-inflammatory cytokines, such as IL-10, increased by daucosterol treatment. Based on previous reports and our results, it is likely that daucosterol regulates Treg cells in early stages of colitis prior to infiltration of macrophages.

In summary, we provide the first evidence that pre- or post-treatment with daucosterol prevents DSS-induced colitis by regulation regulatory T cell number. The preventive effects of daucosterol involve suppression of macrophage infiltration and inflammatory cytokines. Moreover, daucosterol enhances NK cell activity and reduces excessive production of IgA in colitis. Therefore, daucosterol may be good candidate therapeutic compound for colonic diseases including colitis.

Author contributions

J. Jang, Y.S. Lee, W.K. Yang, H.S. Kang, and S.H. Kim designed and performed experiments, analyzed data, and wrote the manuscript. S.M. Kim, S.M. Yee, E.M. Kim, E.H. Lee, and H.R. Choi performed experiments and analyzed data. H.Y. Kim and K.H. Kim provided materials, performed sample compound preparation, contributed to the

discussion, and edited the manuscript. H.S. Kang and S.H. Kim supervised the manuscript. All co-authors contributed to the manuscript.

Competing interests

The authors have no competing interest to declare in regard to this manuscript.

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