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A glycolipid adjuvant, 7DW8-5, provides a protective effect against colonic inflammation in mice by the recruitment of CD1d-restricted natural killer T cells

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Background/Aims: The modulation of CD1d-restricted natural killer T (NKT) cells by glycolipids has been considered as a potential therapy against immunologic diseases, including inflammatory bowel disease. A recently identified a glycolipid analog, 7DW8-5, which is derived from α-galactosylceramide (α-GalCer), is as much as 100-fold more active at stimulating both human and mice NKT cells when compared to α-GalCer. We explored the effects of 7DW8-5 in mouse models of acute and chronic colitis. **Methods:** We investigated the effects of 7DW8-5 on intestinal inflammation by assessing the effects of 7dW8-5 on a murine dextran sulfate sodium (DSS)-induced acute colitis model and a chronic colitis-associated tumor model. **Results:** The acute DSS-induced colitis model showed a dose-dependent response to 7DW8-5, as mice administered 7DW8-5 showed a significant improvement in DSS-induced colitis based on their disease activity index, histologic analysis, and serum C-reactive protein levels, when compared to mice administered vehicle alone. However, DSS-induced colitis in CD1d-KO mice showed no response to 7DW8-5. A fluorescence-activating cell sorting analysis revealed an increase in NKT cells in colonic tissues of 7DW8-5-treated mice. RNA-seq and real-time quantitative polymerase chain reaction showed a significant increase in the expression of interleukin (IL)-4, IL-13, and interferon-gamma in 7DW8-5-treated mice. In addition, 7DW8-5 treatment reduced colitis-associated tumor development in an azoxymethane/DSS mouse model. **Conclusions:** 7DW8-5 activates NKT cells through CD1d and provides a protective effect against intestinal inflammation in mice. Therefore, 7DW8-5 may be a promising therapeutic agent for treatment of inflammatory bowel disease. **(Intest Res 2020;18:402-411)**

Key Words: 7DW8-5; Alpha-galactosylceramide; Natural killer T-cells; Dextran sulfate sodium; Inflammatory bowel disease

INTRODUCTION

Certain types of glycolipids have remarkable immunomodulatory properties that arise from their ability to activate specific T lymphocyte populations that have a wide range of immune effector properties.[1] The most extensively studied glycolipid reactive T cells are the CD1d-restricted natural killer T (NKT) cells, which are characterized by the expression of semi-invariant T cell receptor (TCR) and surface antigens that are typically associated with NK cells (NK1.1).[2,3] The TCR on NKT cells is unique in that it recognizes glycolipid antigens presented by the major histocompatibility complex I-like molecule, CD1d.[4,5] Once activated, NKT cells can secrete a very diverse array of pro- and anti-inflammatory cytokines to modulate innate and adaptive immune responses.[1] NKT cells account for only a small percentage of lymphocytes; however, they are extremely potent and play central roles in immunity to infection, in some cancers, and in autoimmunity.[6-8] Thus,

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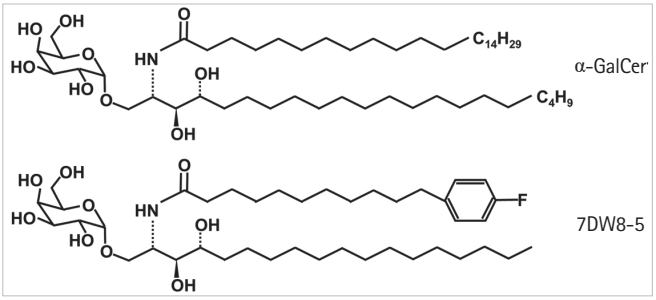

Fig. 1. Structure of α-galactosylceramide (α-GalCer) and 7DW8-5.

glycolipid-mediated activation of NKT cells has been explored for immunotherapy in a variety of infectious disease and cancers.[1]

Alpha-galactosylceramide (KRN7000, α-GalCer) was originally recognized for its antimetastatic properties in a marine sponge, and it became the first known CD1d-presented lipid antigen for NKT cells.[9] The synthetic form of α-GalCer is widely studied and is the best-known glycolipid antigen for activating NKT cells, both *in vivo* and *in vitro*.[10-12] However, α-GalCer displays only marginal biological activity.[12] A novel analog of α-GalCer (Fig. 1), 7DW8-5, has recently been identified. Stepwise screening assays confirm 7DW8-5 as a glycolipid that is as much as 100-fold more active at stimulating both human and mouse NKT cells when compared to α-GalCer.[11] 7DW8-5 has shown superior effects to α-GalCer as a malaria vaccine adjuvant in mouse models.[12,13] 7DW8-5 is also viewed as a promising immunotherapeutic agent; however, its effects are not yet well defined with respect to autoimmunity and inflammation. One commonly used experimental inflammation model is the mouse dextran sulfate sodium (DSS)-induced colitis model of inflammatory bowel disease (IBD). This model affords a high degree of uniformity and reproducibility for most lesions in the colon. The DSS model has confirmed an involvement of CD1d-mediated activation of NKT cells in improvement of the colitis symptoms induced by DSS administration.[14] In the present study, we used this model to investigate the effects of 7DW8-5 on colitis progression in DSS-induced mice and to define the changes in immune responses induced by activated NKT cells and mediated by 7DW8-5 treatment.

METHODS

1. Design of the Animal Experiment

C57BL/6 (wild type, WT) and C57BL/6-Cd1d1/J (CD1d knockout, $CD1d^{-/-}$) mice, 6 to 8 weeks old, were purchased from the Jackson laboratory (Sacramento, CA, USA). All mice were maintained in a temperature- and light-controlled facility and allowed ad libitum access to water and pellet chow. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Samsung Medical Center (IACUC No. 20150105005).

2. Acute Colitis Model

Acute colitis in mice was induced in mice by supplying DSS (MP Biomedicals, Santa Ana, CA, USA) in the drinking water at a 4% concentration. 7DW8-5 was purchased from the Funakoshi Company (Tokyo, Japan) and dissolved in dimethyl sulfoxide following the manufacturer's recommendation. The optimal dosage of 7DW8-5 was determined by administering 10, 50, 100, and 200 μg of 7DW8-5 per 1 kg of mouse body weight intraperitoneally to a minimum of 3 acute colitis model mice. The body weight change and colitis activity of the mice administered different dosages of 7DW8-5 were compared to those of 5 WT mice administered α-GalCer.

The effects of 7DW8-5 in the acute colitis model were evaluated by administering 7DW8-5 (DSS/7DW8-5 group, n = 10) or vehicle (DSS/vehicle group, n = 10) intraperitoneally at day 0 and repeating this administration every 48 hours. Day 0 was determined as the day of first administration of DSS and 7DW8-5 or vehicle. All groups of mice were sacrificed on day 7. The $CD1d^{-/-}$ mice were given a dose of only 100 μg/kg body weight of 7DW8-5 ($CD1d^{-/-}$ DSS/7DW8-5 group, n = 6) or vehicle ($CD1d^{-/-}$ DSS/vehicle group, n = 6) intraperitoneally. In addition, 200 mg of anti-NK1.1 was injected to WT mice intraperitoneally on day –1 to abolish NKT cell pharmacologically. Subsequently, DSS-induced colitis was induced with administration of 7DW8-5 (anti-NK1.1/DSS/7DW8-5 group, n = 3) or vehicle (anti-NK1.1/DSS/vehicle group, n = 3) intraperitoneally.

Colitis activity was monitored by scoring the disease activity index (DAI) on day 2, 4, and 6, as follows:[14] Weight loss: 0 (no loss), 1 (1%–5%), 2 (5%–10%), 3 (10%–20%), and 4 (>20%); Stool consistency: 0 (normal), 2 (loose stool), and 4 (diarrhea); Bleeding: 0 (no blood), 1 (hemoccult positive), 2 (hemoccult positive and visual pellet bleeding), and 4 (gross bleeding, blood around anus). A blood sample was obtained from each mouse at day 7. After sacrificing the mice, the colonic tissues were harvested and used for the measurement of colon length, RNA precipitation, single cell suspension for flow cytometry, and histologic analyses.

3. Chronic Colitis-Induced Tumor Model (AOM/DSS Model)

The effects of 7DW8-5 on colitis-induced tumors were investigated by injecting mice intraperitoneally with azoxymethane (AOM; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 7.4 mg/kg body weight at day 0. At the same time, 3% DSS was supplied in the drinking water and maintained until day 4. The drinking water was then changed to regular water and maintained until day 14. This induction cycle was repeated twice. 7DW8-5 or vehicle were then administered to the mice every 48 hours during the DSS supply period and every 72 hours in the water supply period. All mice in all groups were sacrificed at day 45. After sacrificing the mice, colon tissues were harvested and the numbers of tumor ≥ 1 mm in diameter were counted after 4% indigo carmine staining.

4. Histological Analysis and Histological Scoring

Formalin-fixed, paraffin-embedded tissue was sectioned and stained with H&E via standard methods. Histologic scores were determined using a combined score of severity of inflammation, crypt damage, and ulceration. The histological scoring was defined as follows:[15] Severity of inflammation: 0, rare inflammatory cells in the lamina propria; 1, increased numbers of granulocytes in the lamina propria; 2, confluence of inflammatory cells extending into the submucosa; and 3, transmural extension of the inflammatory infiltrate. Crypt damage: 0, intact crypts; 1, loss of the basal one-third; 2, loss of the basal two-thirds; 3, entire crypt loss; 4, change of epithelial surface with erosion; and 5, confluent erosion. Ulceration: 0, absence of ulcer; 1, one or two foci of ulcerations; 2, three or four foci of ulcerations; and 3, confluent or extensive ulceration.

5. RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from appropriate cells using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and the concentration measured with an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The real-time quantitative polymerase chain reaction (qPCR) assays were run using 1–5 μg RNA per sample using a qScript™ One-STEP qRT-PCR Kit (Quanta Biosciences, Beverly, MA, USA). Relative mRNA expression levels were measured by quantitative PCR using a TaqMan probe (BD Bioscience, San Jose, CA, USA). The plate documentation/experimental parameters for qPCR followed the manufacturer's instructions and analysis was done using a 7300 real-time PCR detection system (BD Bioscience). Three independent qPCR reactions were performed and target mRNA levels were normalized relative to β-actin expression levels.

6. Measurement of Serum CRP Level

The serum level of C-reactive protein (CRP) was measured in mouse blood obtained at day 7 using a mouse CRP ELISA kit (R&D Systems, Minneapolis, MN, USA). ELISA assays were performed in accordance with the manufacturer's instructions. The optical densities of the samples were determined using a microplate reader set at 450 nm. The experiments were run in triplicate.

7. Fluorescence-Activating Cell Sorting

Single cell suspensions were prepared from the colon tissues and incubated in DMEM (Dulbecco's modified Eagle's medium) with collagenase 1 (Sigma-Aldrich) and dispase (Sigma-Aldrich) for 1 hour. After incubation, the cells were collected by filtering the cell solution through a 40 μm strainer. The flow cytometry analysis was conducted on 5×10^4 cells to detect each marker. Cells were incubated for 20 minutes in the presence of an appropriate dose of antibodies in fluorescence-activating cell sorting (FACS) buffer. In this study, we used anti-mouse CD3e (BD bioscience), NK1.1 (BD bioscience), and Cd1d tetramer (Proimmune Ltd., Oxford, UK) to measure the population of NKT cells. Colonic NKT cells were sorted by first enriching the stained cells for CD3+ leukocytes and the CD3-enriched samples were then sorted with anti-NK1.1 and CD1d tetramer. Cell sorting was conducted using a FACS Aria 1 instrument (BD Bioscience). The percentages of cell populations were calculated by acquiring a total of 10,000 events.

8. RNA Sequencing

Total RNA was extracted from colonic tissues obtained from DSS/vehicle mice (n = 3), DSS/7DW8-5 mice (n = 3), and DSS/α-GalCer mice (n = 2). An mRNA library was constructed and sequencing was carried out using the 100 bp paired-end mode of the TruSeq Rapid PE Cluster kit and TruSeq Rapid SBS kit. The RNA sequencing data were subjected to quality control, and then the trimmed reads were mapped to a reference genome with the TopHat splice-aware aligner, and the transcript was assembled by Cufflinks with aligned reads that contained paired-end information. The fragments per kilobase of transcript per million mapped reads of assembled transcripts were calculated for each sample using Cufflinks. All RNA-seq experiments and analyses were performed by Macrogen (Seoul, Korea).

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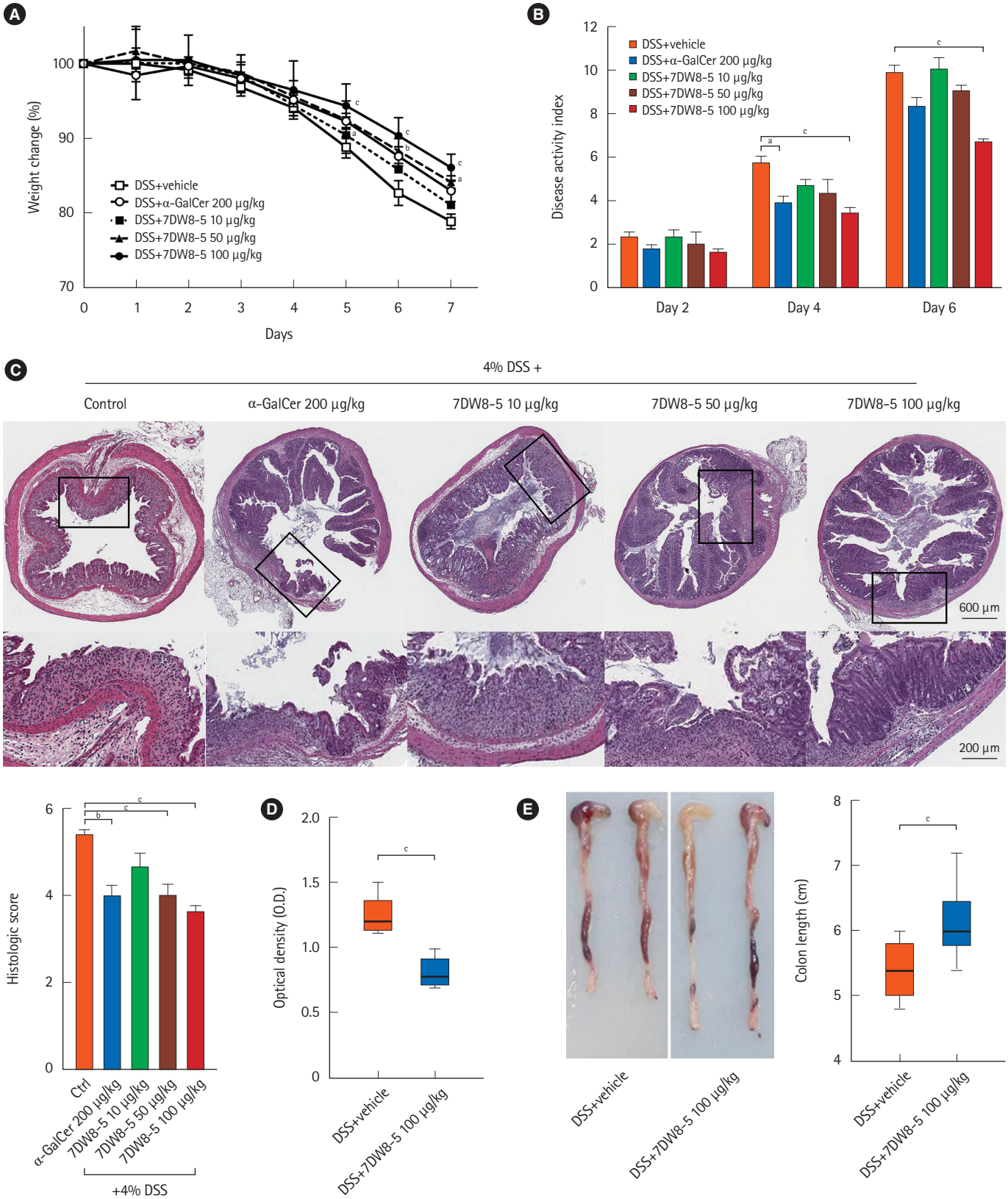

Fig. 2. The effect of 7DW8-5 on dextran sulfate sodium (DSS)-induced acute colitis. (A) The changes in body weight. (B) Disease activity index. (C) Histological score. Magnified image of the top black square is the bottom picture (H&E). (D) Serum C-reactive protein level. (E) Colon length. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$. α-GalCer, α-galactosylceramide.

9. Statistical Analysis

The statistical significance of differences observed between experimental groups was analyzed using the Mann-Whitney *U*-test, Kruskal-Wallis test, one-way analysis of variance (ANOVA) and a two-way ANOVA with Bonferroni multiple comparisons with the statistical software GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Statistical significance was set at the level of $P<0.05$.

RESULTS

The concentration of 7DW8-5 was variable, in agreement with previous work,[12,13,16-20] so we administered 200 μg/kg of α-GalCer or 10, 50, and 100 μg/kg of 7DW8-5 to the acute colitis model mice. The changes in DSS-induced acute colitis due to α-GalCer or 7DW8-5 administration were evaluated by comparing the values for body weight change, DAI, colon length, serum CRP, and histologic score for α-GalCer or 7DW8-5 administered mice to those for the mice administered vehicle only (Fig. 2). Increasing the injected dose of 7DW8-5 to 10, 50, and 100 μg/kg resulted in improvements in body weight, DAI score, and histologic scores.

A two-way ANOVA with Bonferroni multiple comparisons revealed that weight loss was significantly increased in DSS/vehicle group compared with the α-GalCer 200 μg/kg (day 6, $P<0.01$ and day 7, $P<0.01$) and the 7DW8-5 100 μg/kg group (day 5, day 6, and day 7; $P<0.001$). The DAI scores were lower in the DSS/vehicle group than the α-GalCer 200 μg/kg (day 4,

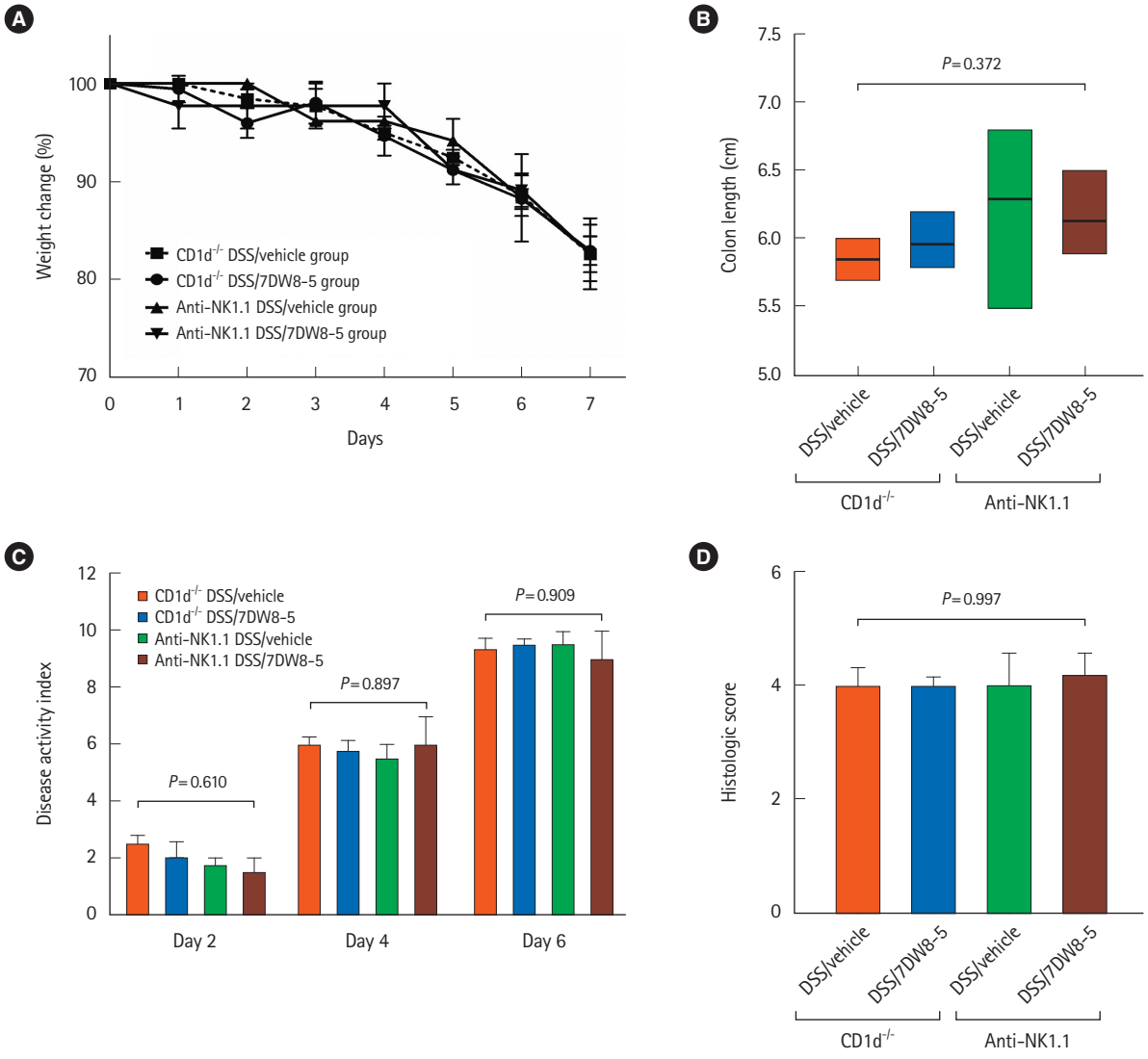

Fig. 3. The effect of 7DW8-5 on dextran sulfate sodium (DSS)-induced acute colitis in CD1d$^{-/-}$ mice and anti-NK1.1 administrated C57BL/6 mice. (A) The changes in body weight. (B) Colon length. (C) Disease activity index. (D) Histological score.

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$P<0.05$) and the 7DW8-5 100 μg/kg group (day 4 and day 6, $P<0.001$). The histologic scores were significantly lower in the α-GalCer 200 μg/kg ($P=0.01$), the 7DW8-5 50 μg/kg ($P=0.001$), and the 7DW8-5 100 μg/kg ($P=0.001$) than in the 10 μg/kg group. However, injection of a 200 μg/kg dose of 7DW8-5 into mice did not provide any additional improvement in colitis progression when compared to the 100 μg/kg dose (data not shown). The weight change, DAI score, and histologic analysis in mice administered α-GalCer at 200 μg/kg were similar to those in mice administered 7DW8-5 at 50 μg/kg and were ameliorated compared with those in mice administered 100 μg/kg of 7DW8-5. Therefore, administration of 7DW8-5 affected colitis in a dose-dependent manner up to a concentration of 100 μg/kg. The total colon length was significantly shorter in the DSS/vehicle group than in the 7DW8-5 group 100 μg/kg group ($P<0.001$). The level of CRP was significantly lower at day 7 in the serum of 7DW8-5 group 100 μg/kg group than in the DSS/vehicle group ($P=0.013$).

The CD1d dependency of the reduction of the effects of 7DW8-5 was confirmed by administering 7DW8-5 or vehicle intraperitoneally to CD1d$^{-/-}$ mice and WT mice with anti-NK1.1 administration (Fig. 3). Subsequently, DSS-induced colitis was induced. No significant differences in weight loss ($P=0.993$), DAI (day 2, $P=0.610$; day 4, $P=0.897$; day 6, $P=0.909$), colon length ($P=0.372$), or histologic score ($P=0.997$) were observed. These findings indicated that 7DW8-5 could not protect against colitis in the CD1d$^{-/-}$ mice or in mice injected with anti-NK1.1 when compared with WT mice. Thus, 7DW8-5 showed its effects on intestinal inflammation in a CD1d-restricted manner.

Therefore, we determined whether 7DW8-5 in the presence of CD1d molecules activates NKT cells by measuring the percentage of NKT cells in colon tissues by FACS analysis. The

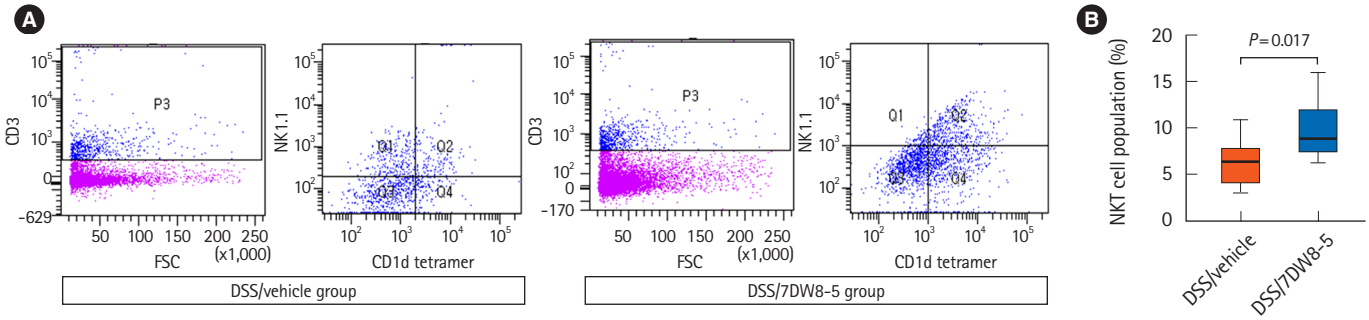

Fig. 4. Fluorescence-activating cell sorting for invariant natural killer T (iNKT) cell. (A) Gating strategy for flow cytometry analysis. (B) Percentage of NKT cell population in the colon tissue of the DSS/7DW8-5 group. FSC, forward scatter; DSS, dextran sulfate sodium.

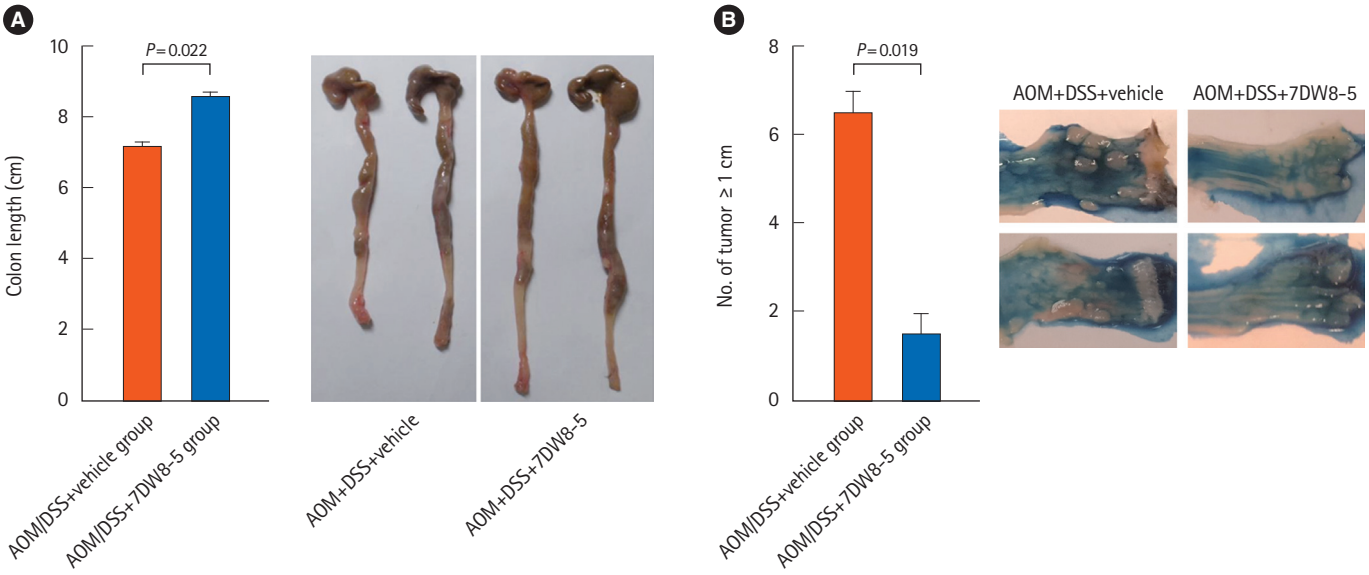

Fig. 5. The effect of 7DW8-5 in the chronic colitis-induced tumor model. (A) Colon length. (B) The number of tumors ≥ 1 mm. AOM, azoxymethane; DSS, dextran sulfate sodium.

A

Gene symbol	Log_2FC
IL-1α	-0.35
IL-1β	-0.42
IL-4	8.41
IL-5	1.00
IL-6	0.44
IL-10	-1.26
IL-13	17.42
IL-12α	0.68
IL-12β	-1.10
IL-17α	0.86
IL-18	-1.35
IL-21	0.91
IL-22	3.53
IFN-γ	2.28

B

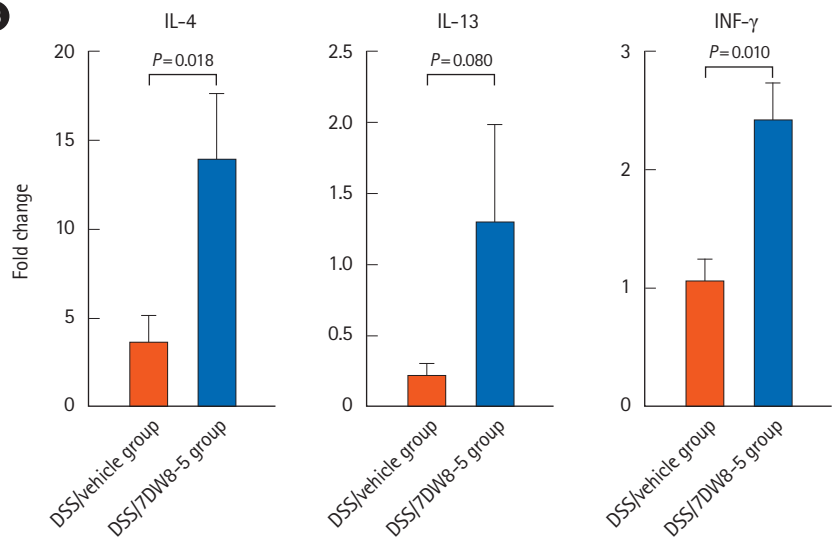

Fig. 6. Expression pattern of T helper type 1 (Th1) and Th2 cytokines in 7DW8-5 or vehicle only administrated dextran sulfate sodium (DSS)-induced acute colitis model. (A) Heatmap of differentially expressed cytokine genes. (B) Real-time quantitative polymerase chain reaction analysis for interleukin (IL)-4, IL-13, and interferon γ (IFN-γ).

population of NKT cells, which is characterized by CD3, NK1.1, and CD1d tetramer positive cells, was significantly higher (P=0.017) in the colon tissue of the DSS/7DW8-5 group than in colon tissues of the DSS/vehicle group (Fig. 4).

We evaluated the expression pattern of the DSS-induced acute colitis model after administration of 7DW8-5 by performing RNA-seq using total RNA obtained from colon tissues of the DSS/7DW8-5 and DSS/vehicle groups. The differential gene expression analysis revealed 325 differentially expressed genes filtered by absolute values of a fold change >2 or <0.5 between the 2 groups. A heatmap and hierarchical tree to compare the differences in gene expression between the group are illustrated in Supplementary Fig. 1A, and the functional category in gene ontology was selected for functional annotation (Supplementary Fig. 1B). We then focused on the cytokines of the DSS/7DW8-5 group and compared them to those of DSS/vehicle group. Among the T helper type 1 (Th1) cytokines, interferon γ (IFN-γ), interleukin (IL)-12A, IL-17A, IL-21, and IL-22 showed increases and IL-1α, IL-1β, IL-12β, and IL-18 showed decreases (Fig. 5A). Conversely, the expression of Th2 cytokines, such as IL-4, IL-5, IL-6, and IL-13, showed an increasing pattern, whereas the levels of anti-inflammatory cytokine IL-10 decreased (Fig. 6A). The qPCR for IL-4, IL-13, and IFN-γ confirmed the expression of these cytokines (Fig. 6B).

We also tested 7DW8-5 for its effects on the chronic colitis-induced tumorigenesis in the AOM/DSS mouse model. Administration of 7DW8-5 to the AOM/DSS model resulted in a greater colon length (P=0.022) (Fig. 5A) and a lower number of tumors ≥1 mm (P=0.019) (Fig. 5B) when compared to the AOM/DSS model administered vehicle only.

DISCUSSION

NKT cells are rare, but they play a pivotal role in the immune system. Exposure of these cells to glycolipid antigens causes them to secrete a multitude of specific cytokines that modulate both innate and adaptive immunity. Recent evidence has clearly established the importance of NKT cells in human health and disease.[1] Several clinical trials using α-GalCer, the first synthetic and now a well-known glycolipid, demonstrated the safety of parenteral administration; however, its efficacy was only marginal.[13,21-24] 7DW8-5 is a biologically more potent analog of α-GalCer[12] and is viewed as a promising immunotherapeutic agent, especially as a potential vaccine adjuvant for malaria, HIV, and tuberculosis.[12,13,18] Previous reports have indicated that α-GalCer ameliorates the intestinal inflammation induced by DSS;[10] however, 7DW8-5 has not yet been evaluated for its effects on intestinal inflammation. In the present study, we demonstrated that 7DW8-5 activates CD1d-restricted NKT cells through CD1d and provides a protective effect against

intestinal inflammation in mice. Not surprisingly, this effect of 7DW8-5 on intestinal inflammation was totally abolished in mice lacking CD1d expression, confirming that the immunomodulatory effect of 7DW8-5 is driven by a CD1d-based mechanism.

RNA-seq was performed to identify the underlying immune response and the differential gene expression between the DSS/7DW8-5 group and the control, focusing on the cytokines, were evaluated. We selected and analyzed all detectable expression data for the Th1 and Th2 cytokines from the entire data set of RNA-seq. The RNA-seq data included the expression level of 27 genes associated with Th1 pathway, 23 genes associated with the Th2 pathway, and 45 genes associated with the Th17 pathway (Supplementary Table 1). However, our data indicated an insufficient expression change for most of the Th1 and Th2 cytokines and the results did not meet statistical significance. As a well-known Th1-biasing synthetic glycolipid,[1] 7DW8-5 elicited a strong IFN-γ response, a Th1 response. In addition, we found that Th2 cytokine like IL-4 and IL-13, most of which may serve in protecting the intestine from inflammation, was increased in DSS/7DW-8 group.[5] Our opinion regarding this finding is that even though an accumulation of CD1d-restricted NKT cells occurred in the DSS/7DW8-5 group, these cells still represented a small population in the whole colon tissue. Thus, the absolute level of cytokines derived from NKT cells represents only a limited amount of the total RNA samples and could be below the cutoff threshold value. Furthermore, DSS administration causes colitis in NKT cell-deficient mice, suggesting that NKT cells may not be critical in the induction of colitis.[14] This could be one of the reasons that the present study could not show any significant change of Th1 and Th2 cytokines

NKT cells have been proposed to have either protective or pathogenic roles, depending on the nature of the inflammatory stimuli and the possible lipid antigens that are recognized.[2,25] In animal models, the protective role of NKT cells against intestinal inflammation has been reported in murine colitis models.[15,25] 7DW8-5 elicited mixed effects on the Th1, Th2, and Th17 pathways, even though there were no significantly different expression patterns of the Th1, Th2, and Th17 pathway associated genes between the DSS/vehicle group, DSS/α-GalCer group, and DSS/7DW8-5 group. Nevertheless, when compared to the DSS/vehicle group, the expression was increased for 7 genes in the Th1 pathway, 5 genes in the Th2 pathway, and 11 genes in Th17 pathway in the DSS/α-GalCer group, whereas the expression was increased for 13 genes in Th1 pathway, 10 genes in Th2 pathway, and 23 genes in Th17 pathway in DSS/α-7DW8-5 group. A comparison with the DSS/α-GalCer group revealed increased expression of 22 genes in the Th1 pathway, 15 genes in the Th2 pathway, and 36 genes in the Th17 pathway in DSS/7DW8-5 group. Our data indicated that 7DW8-5 treatment could induce the expression of Th1/Th17 associated genes, as well as the expression of Th2 associated genes. These results suggested that an alteration in the balance between the Th1/Th17 and Th2 was induced by 7DW8-5 administration as part of the response against DSS-induced colitis, as if the well-known glycolipid, α-GalCer, induced an alteration of the Th1/Th2 balance and improved colitis in the DSS colitis model.[25-27] NKT cells have been proposed to have either protective or pathogenic roles, depending on the nature of the inflammatory stimuli and the possible lipid antigens that are recognized.[2,25]

In conclusion, the activation of NKT cells by 7DW8-5 in the presence of CD1d provides protection against colitis in mice. 7DW8-5 has been studied as a vaccine adjuvant and potential cancer treatment to date, but investigations into its use as a potential immune regulatory agent in immunologic disease, and especially IBD, have been limited. In the present study, we demonstrated that the 7DW8-5 glycolipid adjuvant provides a protective effect against colonic inflammation in mice, catalyzed by the recruitment of CD1d-restricted NKT cells, and that 7DW8-5 is more potent than the well-established glycolipid, α-GalCer. This concept can translate to human trials, because 7DW8-5 offers a promising potential mechanism for the regulation of the immune response at a relatively low dosage and cost.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTION

Conceptualization: Lee C, Hong SN, Kim YH. Methodology: Lee C. Formal analysis: Lee C, Hong SN. Funding acquisition: Hong SN, Kim YH. Visualization: Lee C, Hong SN. Writing -

original draft: Lee C. Writing - review and editing: Lee C, Hong SN, Kim YH. Approval of final manuscript: all authors.

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SUPPLEMENTARY MATERIAL

Supplementary materials are available at the Intestinal Research website (https://www.irjournal.org).

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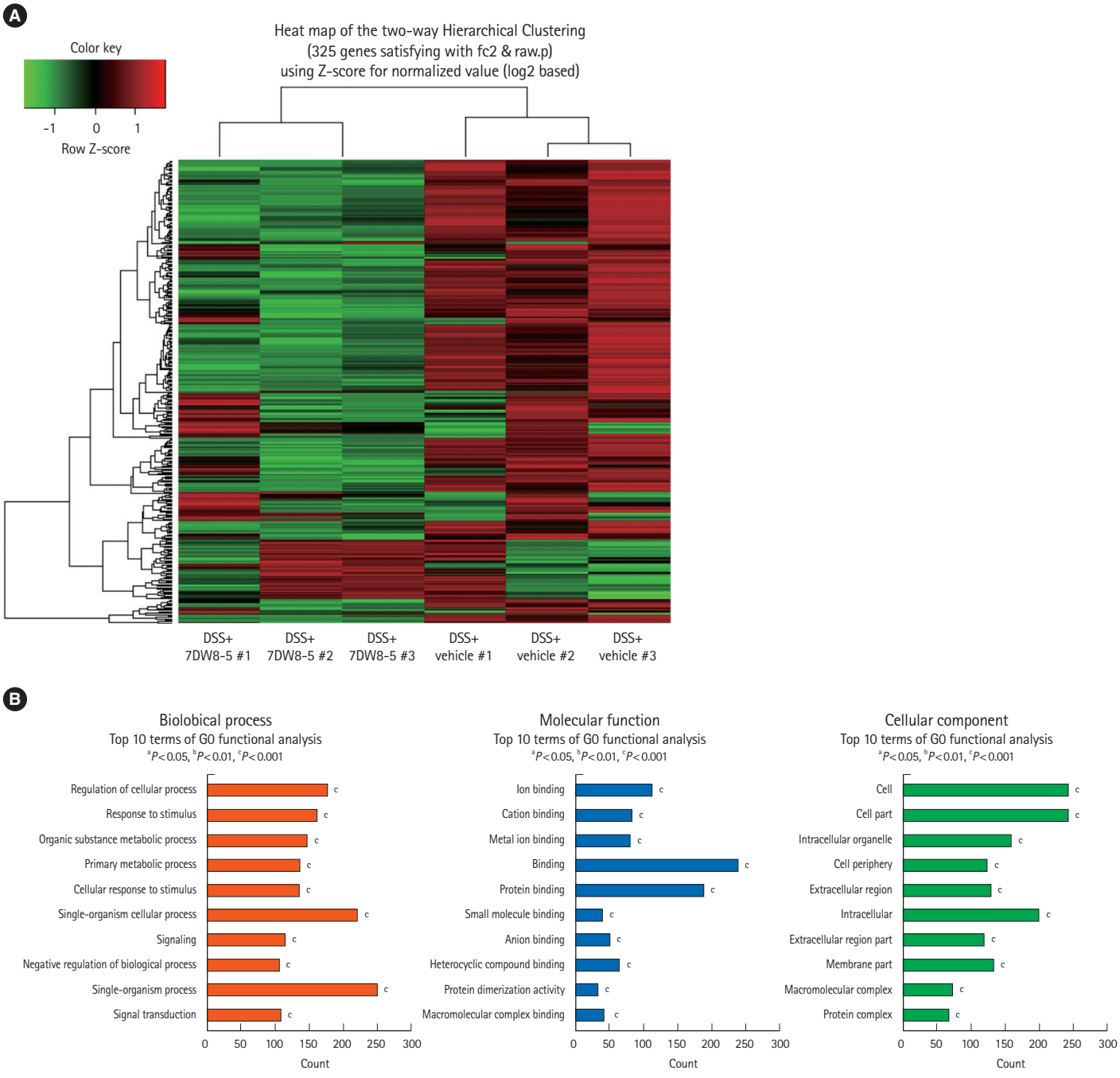
See "A glycolipid adjuvant, 7DW8-5, provides a protective effect against colonic inflammation in mice by the recruitment of CD1d-restricted natural killer T cells" on page 402-411.



Supplementary Fig. 1. RNA-seq. (A) Heatmap and hierarchical tree comparing gene expression differences in colon tissue of, dextran sulfate sodium (DSS)/7DW8-5 mice compared to DSS/vehicle mice, (B) top 10 terms of gene ontology functional analysis.

Supplementary Table 1. Expression of Th1, Th2, and Th17 Pathway Associated Genes

	Gene_Symbol	Gene_ID	Transcript_ID	Gene_Description	7DW8-5 treated DSS-induced colitis mice vs. only DSS administrated mice			α-GalCer treated DSS-induced colitis mice vs. only DSS administrated mice			7DW8-5 treated DSS-induced colitis mice vs. α-GalCer treated DSS-induced colitis mice		
					Fold change	*P*-value	q-value	Fold change	*P*-value	q-value	Fold change	*P*-value	q-value
Th1 pathway													
Th1 Markers:	Ccr5	12774	NM_009917	chemokine (C-C motif) receptor 5	1.14	0.736	0.913	-1.12	0.647	0.886	1.28	0.549	0.699
	Cxcr3	12766	NM_009910	chemokine (C-X-C motif) receptor 3	-1.34	0.476	0.900	-1.37	0.449	0.867	1.02	0.895	0.929
	Ifng	15978	NM_008337	interferon gamma	1.17	0.588	0.900	1.41	0.063	0.867	-1.21	0.524	0.682
	Stat4	20849	NM_011487	signal transducer and activator of transcription 4	-1.35	0.636	0.900	-1.67	0.435	0.867	1.24	0.640	0.757
	Tbx21	57765	NM_019507	T-box 21	-1.02	0.763	0.920	-1.01	0.918	0.975	-1.02	0.675	0.780
Th1 Immune Response:	Ccr2	12772	NM_009915	chemokine (C-C motif) receptor 2	-1.11	0.810	0.932	-1.33	0.247	0.867	1.20	0.673	0.779
	Cd80	12519	NM_009855	CD80 antigen	-1.18	0.635	0.900	-1.22	0.315	0.867	1.03	0.917	0.944
	Il12b	16160	NM_008352, NM_001303244	interleukin-12 subunit beta precursor	1.09	0.768	0.921	-1.05	0.549	0.867	1.15	0.645	0.760
	Il18	16173	NM_008360	interleukin 18	-2.21	0.031	0.900	-1.65	0.242	0.867	-1.34	0.386	0.606
	Il18bp	16068	NM_010531	interleukin 18 binding protein	1.90	0.632	0.900	1.24	0.451	0.867	1.53	0.739	0.825
	Il1rl1	17082	NM_001294171, NM_010743, NM_001025602	interleukin-1 receptor-like 1 isoform b precursor	-2.10	0.214	0.900	-6.50	0.054	0.867	3.10	0.049	0.590
	Il27ra	50931	NM_016671	interleukin 27 receptor, alpha	-1.90	0.434	0.900	-2.08	0.401	0.867	1.10	0.626	0.747
	Nfkb1	18033	NM_008689	nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105	1.05	0.755	0.918	-1.15	0.233	0.867	1.21	0.357	0.600
	Socs5	56468	NM_019654	suppressor of cytokine signaling 5	1.28	0.238	0.900	-1.16	0.415	0.867	1.48	0.068	0.590
	Tlr4	21898	NM_021297	Toll-like receptor 4	1.13	0.744	0.915	-1.10	0.507	0.867	1.24	0.584	0.721
	Tlr6	21899	NM_011604	Toll-like receptor 6	-1.30	0.293	0.900	1.04	0.827	0.941	-1.35	0.056	0.590

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Supplementary Table 1. Continued

	Gene_Symbol	Gene_ID	Transcript_ID	Gene_Description	7DW8-5 treated DSS-induced colitis mice vs. only DSS administrated mice			α-GalCer treated DSS-induced colitis mice vs. only DSS administrated mice			7DW8-5 treated DSS-induced colitis mice vs. α-GalCer treated DSS-induced colitis mice		
					Fold change	*P*-value	q-value	Fold change	*P*-value	q-value	Fold change	*P*-value	q-value
	Vegfa	22339	NM_001025257, NM_001287057, NM_001025250, NM_001287058, NM_001110268, NM_001110267, NM_001110266, NM_009505, NM_001287056	vascular endothelial growth factor A	1.52	0.553	0.900	-1.17	0.807	0.935	1.78	0.356	0.600
Other Th1-Related Genes:	Cd28	12487	NM_007642	CD28 antigen	-1.51	0.470	0.900	-1.54	0.459	0.867	1.02	0.815	0.875
	Cd40	21939	NM_170704, NM_011611, NR_027852, NM_170702, NM_170703	CD40 antigen	-1.17	0.781	0.924	-1.61	0.172	0.867	1.38	0.593	0.726
	Csf2	12981	NM_009969	colony stimulating factor 2 (granulocyte-macrophage)	1.29	0.057	0.900	-1.11	0.103	0.867	1.42	0.019	0.590
	Il12rb2	16162	NM_008354	interleukin 12 receptor, beta 2	-1.02	0.775	0.922	1.02	0.463	0.867	-1.04	0.545	0.696
	Il18r1	16182	NM_008365, NM_001161843, NM_001161842	interleukin 18 receptor 1	-1.11	0.597	0.900	-1.38	0.013	0.867	1.25	0.348	0.598
	Il2ra	16184	NM_008367	interleukin 2 receptor, alpha chain	-1.45	0.524	0.900	-1.82	0.297	0.867	1.25	0.651	0.764
	Irf1	16362	NM_001159396, NM_001159393, NM_008390	interferon regulatory factor 1	1.63	0.400	0.900	1.16	0.551	0.867	1.41	0.529	0.685
	Socs1	12703	NM_001271603, NM_009896	suppressor of cytokine signaling 1	1.72	0.485	0.900	1.44	0.303	0.867	1.19	0.797	0.863
	Stat1	20846	NM_001205314, NM_009283, NM_001205313	signal transducer and activator of transcription 1	1.48	0.515	0.900	1.14	0.335	0.867	1.30	0.642	0.758
	Tnf	21926	NM_001278601, NM_013693	tumor necrosis factor	1.21	0.858	0.950	-1.01	0.982	0.996	1.23	0.834	0.887

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Supplementary Table 1. Continued

	Gene_Symbol	Gene_ID	Transcript_ID	Gene_Description	7DW8-5 treated DSS-induced colitis mice vs. only DSS administrated mice			α-GalCer treated DSS-induced colitis mice vs. only DSS administrated mice			7DW8-5 treated DSS-induced colitis mice vs. α-GalCer treated DSS-induced colitis mice		
					Fold change	*P*-value	q-value	Fold change	*P*-value	q-value	Fold change	*P*-value	q-value
Th2 pathway													
Th2 Markers:	Ccr4	12773	NM_009916	chemokine (C-C motif) receptor 4	-1.38	0.430	0.900	-1.36	0.449	0.867	-1.01	0.857	0.903
	Gata3	14462	NM_008091	GATA binding protein 3	-1.03	0.818	0.935	1.21	0.514	0.867	-1.25	0.461	0.643
	Stat6	20852	NM_009284	signal transducer and activator of transcription 6	1.13	0.110	0.900	-1.02	0.581	0.870	1.16	0.087	0.590
Th2 Immune Response:	Bcl6	12053	NM_009744	B cell leukemia/ lymphoma 6	1.50	0.224	0.900	-1.29	0.376	0.867	1.93	0.043	0.590
	Cd27	21940	NM_001033126, NM_001286753, NM_001042564	CD27 antigen	-2.20	0.423	0.900	-2.19	0.427	0.867	-1.01	0.905	0.936
	Cd86	12524	NM_019388	CD86 antigen	-1.13	0.818	0.935	-1.37	0.014	0.867	1.21	0.721	0.812
	Il10	16153	NM_010548	interleukin 10	-1.78	0.325	0.900	-2.54	0.208	0.867	1.42	0.450	0.638
	Il6	16193	NM_031168	interleukin 6	-1.03	0.978	0.992	-1.72	0.398	0.867	1.67	0.674	0.779
Other Th2-Related Genes:	Ccl5	20304	NM_013653	chemokine (C-C motif) ligand 5	-1.24	0.812	0.933	1.08	0.903	0.972	-1.33	0.772	0.846
	Ccl7	20306	NM_013654	chemokine (C-C motif) ligand 7	1.17	0.812	0.933	-1.95	0.268	0.867	2.27	0.290	0.590
	Cebpb	12608	NM_001287738	CCAAT/enhancer-binding protein beta isoform b	1.80	0.347	0.900	-1.47	0.486	0.867	2.65	0.052	0.590
	Gfi1	14581	NM_010278, NM_001267621	growth factor independent 1	-1.46	0.033	0.900	1.41	0.382	0.867	-2.05	0.191	0.590
	Icos	54167	NM_017480	inducible T cell co-stimulator	-1.56	0.367	0.900	-1.95	0.270	0.867	1.25	0.507	0.670
	Il1r1	16177	NM_008362, NM_001123382	interleukin 1 receptor, type I	1.09	0.850	0.946	-1.82	0.287	0.867	1.98	0.211	0.590
	Il4ra	16190	NM_001008700	interleukin 4 receptor, alpha	1.35	0.485	0.900	-1.15	0.640	0.883	1.56	0.330	0.593
	Irf4	16364	NM_013674	interferon regulatory factor 4	-1.48	0.541	0.900	-1.72	0.445	0.867	1.16	0.546	0.697

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Supplementary Table 1. Continued

Gene_Symbol	Gene_ID	Transcript_ID	Gene_Description	7DW8-5 treated DSS-induced colitis mice vs. only DSS administrated mice			α-GalCer treated DSS-induced colitis mice vs. only DSS administrated mice			7DW8-5 treated DSS-induced colitis mice vs. α-GalCer treated DSS-induced colitis mice		
				Fold change	*P*-value	q-value	Fold change	*P*-value	q-value	Fold change	*P*-value	q-value
Jak1	16451	NM_146145	Janus kinase 1	1.09	0.742	0.915	-1.57	0.019	0.867	1.71	0.233	0.590
Junb	16477	NM_008416	jun B proto-oncogene	1.81	0.308	0.900	-1.23	0.696	0.898	2.22	0.163	0.590
Maf	17132	NM_001025577	avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog	1.17	0.443	0.900	-1.07	0.700	0.899	1.26	0.353	0.599
Nfatc1	18018	NM_001164110, NM_001164111, NM_001164112, NM_016791, NM_001164109, NM_198429	nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1	-1.17	0.322	0.900	-1.25	0.244	0.867	1.07	0.278	0.590
Nfatc2	18019	NM_001291173, NR_111897, NM_001291171, NM_001291170, NM_001291177, NM_001291176, NM_001291175, NM_001291174, NM_010899, NM_001291169, NM_001291179, NM_001291178, NM_001291172, NM_001037177, NM_001291168, NR_111898, NM_001136073, NM_001037178	nuclear factor of activated T-cells, cytoplasmic 2 isoform f	-1.11	0.551	0.900	-1.09	0.567	0.868	-1.02	0.896	0.930
Pcgf2	22658	NM_001163308, NM_009545, NM_001163307	polycomb group ring finger 2	1.26	0.296	0.900	1.35	0.240	0.867	-1.08	0.730	0.819
Il7	16196	NM_008371	interleukin 7	-1.50	0.337	0.900	1.04	0.877	0.961	-1.55	0.311	0.592

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Supplementary Table 1. Continued

	Gene_Symbol	Gene_ID	Transcript_ID	Gene_Description	7DW8-5 treated DSS-induced colitis mice vs. only DSS administrated mice			α-GalCer treated DSS-induced colitis mice vs. only DSS administrated mice			7DW8-5 treated DSS-induced colitis mice vs. α-GalCer treated DSS-induced colitis mice		
					Fold change	*P*-value	q-value	Fold change	*P*-value	q-value	Fold change	*P*-value	q-value
Th17 pathway													
Surface Molecules:	Cd34	12490	NM_133654, NM_001111059	CD34 antigen	-1.14	0.615	0.900	-2.57	0.101	0.867	2.26	0.008	0.590
	Cd4	12504	NM_013488	CD4 antigen	-1.87	0.366	0.900	-2.37	0.291	0.867	1.27	0.573	0.714
	Cd8a	12525	NM_009857, NM_001081110	CD8 antigen, alpha chain	-1.83	0.538	0.900	-1.37	0.725	0.908	-1.34	0.043	0.590
	Icam1	15894	NM_010493	intercellular adhesion molecule 1	1.09	0.889	0.961	-1.71	0.036	0.867	1.86	0.408	0.616
Chemokines:	Ccl2	20296	NM_011333	chemokine (C-C motif) ligand 2	1.09	0.852	0.947	-3.14	0.166	0.867	3.42	0.139	0.590
	Ccl20	20297	NM_001159738, NM_016960	chemokine (C-C motif) ligand 20	-1.67	0.675	0.902	-3.86	0.082	0.867	2.32	0.524	0.682
	Ccl22	20299	NM_009137	chemokine (C-C motif) ligand 22	-1.34	0.785	0.925	-2.62	0.226	0.867	1.95	0.558	0.705
	Cx3cl1	20312	NM_009142	chemokine (C-X3-C motif) ligand 1	1.22	0.502	0.900	1.08	0.792	0.931	1.13	0.622	0.744
	Cxcl1	14825	NM_008176	chemokine (C-X-C motif) ligand 1	-1.08	0.934	0.976	-5.21	0.107	0.867	4.81	0.255	0.590
	Cxcl12	20315	NM_021704, NM_001012477, NM_013655	chemokine (C-X-C motif) ligand 12	1.38	0.500	0.900	-2.24	0.216	0.867	3.10	0.041	0.590
	Cxcl2	20310	NM_009140	chemokine (C-X-C motif) ligand 2	-1.51	0.708	0.908	-2.39	0.359	0.867	1.58	0.676	0.781
	Cxcl5	20311	NM_009141	chemokine (C-X-C motif) ligand 5	-1.32	0.763	0.920	-3.24	0.062	0.867	2.46	0.412	0.618
	Mmp3	17392	NM_010809	matrix metallopeptidase 3	1.40	0.699	0.906	-3.68	0.219	0.867	5.16	0.183	0.590
	Mmp9	17395	NM_013599	matrix metallopeptidase 9	-1.60	0.447	0.900	-2.91	0.177	0.867	1.81	0.237	0.590
Cytokines:	Csf3	12985	NM_009971	colony stimulating factor 3 (granulocyte)	1.06	0.966	0.987	-2.63	0.178	0.867	2.78	0.501	0.666

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Supplementary Table 1. Continued

	Gene_Symbol	Gene_ID	Transcript_ID	Gene_Description	7DW8-5 treated DSS-induced colitis mice vs. only DSS administrated mice			α-GalCer treated DSS-induced colitis mice vs. only DSS administrated mice			7DW8-5 treated DSS-induced colitis mice vs. α-GalCer treated DSS-induced colitis mice		
					Fold change	*P*-value	q-value	Fold change	*P*-value	q-value	Fold change	*P*-value	q-value
	Il15	16168	NM_001254747, NM_008357	interleukin 15	1.23	0.383	0.900	1.57	0.116	0.867	-1.27	0.361	0.600
	Il17a	16171	NM_010552	interleukin 17A	1.08	0.907	0.966	-1.06	0.729	0.909	1.14	0.838	0.890
	Il17c	234836	NM_145834	interleukin 17C	1.18	0.653	0.901	1.02	0.872	0.958	1.16	0.696	0.794
	Il17d	239114	NM_145837	interleukin 17D	1.95	0.043	0.900	1.31	0.230	0.867	1.49	0.127	0.590
	Il17f	257630	NM_145856	interleukin 17F	1.69	0.602	0.900	-1.01	0.836	0.945	1.71	0.595	0.726
	Il1b	16176	NM_008361	interleukin 1 beta	-1.47	0.766	0.921	-1.50	0.650	0.886	1.02	0.986	0.991
	Il22	50929	NM_016971	interleukin 22	2.83	0.599	0.900	-1.10	0.643	0.884	3.12	0.570	0.712
	Il23a	83430	NM_031252	interleukin 23, alpha subunit p19	1.06	0.762	0.920	1.10	0.660	0.890	-1.04	0.862	0.907
	Tgfb1	21803	NM_011577	transforming growth factor, beta 1	-1.31	0.524	0.900	-2.78	0.124	0.867	2.13	0.223	0.590
Cytokine Receptors:	Ccr6	12458	NM_009835, NM_001190338, NM_001190337, NM_001190336, NM_001190335, NM_001190334, NM_001190333	chemokine (C-C motif) receptor 6	-1.40	0.660	0.901	-2.02	0.374	0.867	1.45	0.566	0.710
	Il12rb1	16161	NM_008353	interleukin 12 receptor, beta 1	-1.11	0.715	0.910	-1.08	0.767	0.921	-1.03	0.895	0.929
	Il17ra	16172	NM_008359	interleukin 17 receptor A	-1.26	0.278	0.900	-1.27	0.134	0.867	1.01	0.956	0.970
	Il17rb	50905	NM_019583	interleukin 17 receptor B	1.04	0.793	0.927	1.16	0.028	0.867	-1.11	0.535	0.689
	Il17rc	171095	NM_178942, NM_134159	interleukin 17 receptor C	-1.10	0.459	0.900	1.15	0.359	0.867	-1.27	0.108	0.590
	Il17re	57890	NM_001034029, NM_145826, NM_001034031	interleukin 17 receptor E	-1.30	0.570	0.900	1.20	0.271	0.867	-1.56	0.397	0.611
	Il23r	209590	NM_144548	interleukin 23 receptor	1.01	0.953	0.984	-1.06	0.385	0.867	1.06	0.509	0.671
	Il6ra	16194	NM_010559	interleukin 6 receptor, alpha	1.03	0.916	0.969	-1.54	0.152	0.867	1.59	0.257	0.590

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Supplementary Table 1. Continued

	Gene_Symbol	Gene_ID	Transcript_ID	Gene_Description	7DW8-5 treated DSS-induced colitis mice vs. only DSS administrated mice			α-GalCer treated DSS-induced colitis mice vs. only DSS administrated mice			7DW8-5 treated DSS-induced colitis mice vs. α-GalCer treated DSS-induced colitis mice		
					Fold change	*P*-value	q-value	Fold change	*P*-value	q-value	Fold change	*P*-value	q-value
Signaling Pathway Molecules and Transcriptional Factors:	Clec7a	56644	NM_020008	C-type lectin domain family 7, member a	1.22	0.792	0.927	1.04	0.688	0.897	1.17	0.830	0.885
	Foxp3	20371	NM_001199348, NM_001199347, NM_054039	forkhead box P3	-1.42	0.419	0.900	-1.24	0.569	0.868	-1.14	0.160	0.590
	Isg20	57444	NM_001113527, NM_001291221, NM_001291220, NM_020583	interferon-stimulated gene 20 kDa protein isoform a	-2.11	0.457	0.900	-2.18	0.453	0.867	1.03	0.934	0.956
	Jak2	16452	NM_001048177, NM_008413	Janus kinase 2	1.12	0.766	0.921	-1.14	0.468	0.867	1.28	0.557	0.704
	Rora	19883	NM_001289916, NM_001289917, NM_013646	nuclear receptor ROR-alpha isoform 3	1.37	0.151	0.900	-1.20	0.277	0.867	1.64	0.102	0.590
	Rorc	19885	NM_011281, NM_001293734, NR_121656	nuclear receptor ROR-gamma isoform 2	1.17	0.538	0.900	1.05	0.690	0.897	1.11	0.663	0.772
	Runx1	12394	NM_001111021, NM_001111022, NM_001111023, NM_009821	runt related transcription factor 1	-1.01	0.927	0.973	-1.13	0.236	0.867	1.11	0.541	0.694
	S1pr1	13609	NM_007901	sphingosine-1-phosphate receptor 1	-1.44	0.348	0.900	-3.44	0.041	0.867	2.40	0.122	0.590
	Socs3	12702	NM_007707	suppressor of cytokine signaling 3	1.85	0.238	0.900	-1.17	0.789	0.930	2.17	0.288	0.590
	Stat3	20848	NM_213659, NM_011486, NM_213660	signal transducer and activator of transcription 3	1.51	0.261	0.900	-1.35	0.362	0.867	2.03	0.081	0.590
	Stat5a	20850	NM_001164062, NM_011488	signal transducer and activator of transcription 5A	-1.14	0.374	0.900	-1.08	0.551	0.867	-1.06	0.654	0.766
	Syk	20963	NM_001198977, NM_011518	spleen tyrosine kinase	-1.09	0.863	0.952	-1.33	0.037	0.867	1.22	0.708	0.803
	Traf6	22034	NM_009424, NM_001303273	TNF receptor-associated factor 6	1.19	0.401	0.900	1.18	0.367	0.867	1.01	0.957	0.971

Th1, T helper type 1; DSS, dextran sulfate sodium; α-GalCer, α-galactosylceramide.