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Specific substance of Maruyama (SSM) suppresses immune responses in atopic dermatitis-like skin lesions in DS-Nh mice by modulating dendritic cell functions

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ABSTRACT

Background: Specific substance of Maruyama (SSM) is a carcinostatic immunotherapeutic agent extracted from *Mycobacterium tuberculosis*. The efficacy of SSM induced interleukin(IL)-12 and IFN- γ production, and inhibition of IL-4, resulting in a shift from Th2 to Th1 in vivo.

Objective: The DS-Nh mice are a model of human atopic dermatitis (AD), which spontaneously develop dermatitis under conventional conditions. In this study, to determine whether SSM can prevent the development of skin lesions in a murine model of AD.

Methods: DS-Nh mice were injected with SSM 5 days per week for 11 weeks. Pharmacological, histological and serological studies were performed to investigate the therapeutic effect of SSM for DS-Nh mice. Analysis of cytokines responses to SSM using quantitative RT-PCR and flow cytometry were also performed to evaluate their therapeutic mechanisms in these AD model mice.

Results: Clinically, erythema, erosions, excoriation, and edema were observed in DS-Nh mice at 16 weeks of age, which advanced with age. Histologically, the relative number of mast cells increased in DS-Nh mice. SSM treatment alleviated the clinical and histological findings in accord with reduced serum IgE level, and increased IgG2a level. TSLP expression was not induced, but IL-1 β , IL-12, IL-17A, and IFN- γ were induced in SSM-treated DS-Nh mice. Overall, SSM treatments increased the number of activated DCs in lesions. SSM induced CD80, CD86, and MHC class II expression on bone marrow-derived DCs. **Conclusions:** SSM enhanced IL-12 production, but suppressed TSLP expression, resulting in a shift from Th2 to Th1 responses. This shift suppressed AD-like skin lesions in a similar fashion as the BCG vaccine. Therefore, SSM may be a useful adjuvant for suppressing skin lesions in AD models.

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1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory disease mediated by Th2 cells and characterized by pruritic and eczematous skin lesions [1,2]. It is known as a Th2 cell-mediated disease and *staphylococcal enterotoxin*-producing *staphylococcus aureus* is frequently isolated from skin lesions [3]. The important role that Th1 and Th2 cytokines play in the skin inflammatory response has been demonstrated in experimental models of allergen-induced

allergic skin inflammation in mice with targeted deletions or overexpression of these cytokines. In this regard, transgenic mice genetically engineered to overexpress IL-4 in their skin develop inflammatory pruritic skin lesions similar to AD, suggesting that local skin expression of Th2 cytokines plays a critical role in AD [4]. Intriguingly, IL-17 producing cells are detected in the acute lesion of AD [5], however, the role of Th17 in the development of AD remains unknown. Moreover, it was reported that TSLP is highly expressed by keratinocytes of AD and TSLP acts on dendritic cells to strongly prime naïve CD4⁺T cells to produce Th2 cytokines [6].

DS non-hair (DS-Nh) mice are spontaneous hairless mutants developed from a colony of inbred DS strain mice, which were developed from an outbred dd stock at the Central Institute for Experimental Animals, Tokyo, Japan [3,7–10]. The DS-Nh mice are a model of human AD [3,7–10], which spontaneously develops dermatitis under conventional conditions, that is strongly associated with *staphylococcal enterotoxin* C-producing bacterial infections

Abbreviations: SSM, specific substance of Murayama; DS-Nh, DS non-hair mice; IL, interleukin; TSLP, thymic stromal lymphoprotein; DC, dendritic cell; RT, reverse transcription.

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[9]. On the contrary, no dermatitis is observed in these mice under specific pathogen-free (SPF) conditions [9].

The specific substance of Maruyama (SSM) is a carcinostatic immunotherapeutic agent that was originally extracted from *Mycobacterium tuberculosis* isolated from human patients by Maruyama [11]. The clinical usefulness of non-specific immunotherapies, predominantly as biological response modifiers (BRMs), has been investigated for the treatment of patients with various carcinomas [12–15]. SSM solution A (SSMA) and B (SSMB) have been obtained and contain 2 µg/mL and 0.2 µg/mL of tubercle bacillus extracts, respectively. Subsequently, the use of SSMA and SSMB was approved by the Japanese Ministry for Health and Welfare (MHW) in 1981. In 1991, the Japanese MHW approved the use of 20 µg/mL Z-100 solution (ZERIA Pharmaceutical, Co., Ltd.) for injection [16]. Hayashi et al. reported that IFN-γ is induced by SSM in mice [17]. Similarly, Satomura et al. proved that IFN-γ production is generated by SSM stimulation for patients with chronic hepatitis B [18]. Subsequently, Oka et al. demonstrated that interleukin (IL)-12 production by CD11c⁺ cells were also significantly increased in mice treated with Z-100 compared with control mice [19]. The anti-cancer mechanism of action for SSMA, SSMB, and Z-100 was determined to be the induction of IL-12 and IFN-γ, which results in suppression of carcinoma proliferation [14–17]. The efficacy of such a BRMs is at least partly mediated via the production of IFN-γ and IL-12 and the inhibition of IL-4, resulting in a shift from Th2 towards Th1 responses in vivo [18,19].

In this study, we tested the hypothesis that SSM can inhibit Th2 cytokine production and prevent the development of skin lesions in Ds-Nh mice. Five-week-old Ds-Nh mice were used in this study because skin lesions have not yet developed at this age. We subcutaneously injected SSMB or saline into the abdominal region of conventionally housed DS-Nh mice 5 days per week for 11 weeks. We examined the levels of Th1 and Th2 cytokines, and IL-1β and TSLP mRNA in lesional skin samples by quantitative real-time RT-PCR. The levels of serum IgE, and IgG2a were measured by enzyme-linked immunosorbent assay (ELISA). Through immunohistochemistry, we examined the distribution of DCs within the dermis of facial skin lesions using a CD209 antibody. Finally, we

demonstrated SSM activation of DCs by measuring CD40, CD80, CD86, and MHC class II expression on SSM-treated and untreated bone marrow-derived DCs.

2. Materials and methods

2.1. Mice and treatments

Male Ds-Nh mice used in this study were obtained as F1 heterozygous progeny (Nh/+) from male Ds-Nh (Nh/Nh) × female DS (+/+) mice housed under SPF conditions. All mice used in this study were maintained in cages, exposed to a 12-h light/12-h dark cycle, and provided with standard food and water *ad libitum*. The animals were housed in rooms under SPF conditions for 5 weeks and then moved to conventional conditions. All animal experiments complied with the standards set out in the guidelines. We injected SSMB (1 mL) or saline (1 mL) subcutaneously in the abdominal region of conventionally housed DS-Nh mice 5 days per week for 11 weeks.

2.2. Histopathology of the skin

The tissues obtained by biopsies of half of the facial lesions from the mice at 10, 12, 14, and 16 weeks of age were fixed in 10% formalin in neutral buffer for at least 16 h and embedded in paraffin. Deparaffinized sections were prepared for hematoxylin and eosin (HE) staining and immunohistochemistry. Paraffin-embedded sections were used for histopathological and immunohistochemical analyses. For histological examination, tissues were fixed with 10% formalin in PBS and then embedded in paraffin. Sections with a thickness of 5 mm were prepared and subjected to staining with H&E. The histological findings were evaluated as reported previously [20]. In brief, samples were scored for the severity and character of the inflammatory response using a subjective grading scale. Responses were graded as follows: 0, no response; 1, minimal response; 2, mild response; 3, moderate response; and 4, marked response. The slides were blinded, randomized, and reread to determine the histology score. All studies were read by the same pathologist using the same

Table 1
Primer and probe sequences for real-time RT-PCR of mouse cytokines and chemokines.

		Sequence (5' → 3')	Length (bp) ^a	Accession ^b
IL-1β	F	GAAGAAGAGCCCATCTCTG	98	NM_008361
	R	TCATCTCGGAGCCTGTAGTG		
	P	TGGGAACGTCACACACACAGCA		
IL-4	F	ATCGATAAGCTGCACCATGA	79	NM_021283
	R	TTGCATGATGCTCTTTAGGC		
	P	TCCACATCACTGAAAGACTTCTGGAA		
IL-12	F	TTCTAGACAAGGGCATGCTG	72	NM_008351
	R	GCAGAGTCTCGCCATTATGA		
	P	TGCATCAGCTCATCGATGGCC		
IL-17A	F	GCCCTCAGACTACCTCAACC	150	NM_010552
	R	GAATTCATGTGGTGGTCCAG		
	P	TCCGCAITGACACAGCGCTG		
CCL24	F	TCTCTGTGTGGCTCAAAGG	147	NM_019577
	R	TTACCCAGAGATGGACAGA		
	P	CAGCTGCAAGCAAGGTCCGC		
IFN-γ	F	CATTGAGAGCTGCAGTGACC	89	NM_008337
	R	CTGTCTGGCTGTCTGTTAAA		
	P	TCCCATCAGCAGCACCTCCC		
TSLP	F	CAGCTGTCAAAGGGTTCAGA	72	NM_021367
	R	TCGTAGATGTGCAAGGCTCT		
	P	CCCAGGACAGTACAATTCGTAGCA		
GAPDH	F	CATCACTGCCACCCAGAAGA	78	NM_008084
	R	ATGTTCTGGGAGCC		
	P	TGGATGGCCCTCTGAAAGCTG		

F, forward primer; R, reverse primer; P, TaqMan probe, dual labeled with 5'FAM and 3'TAMRA.

^a Amplicon length in base pairs.

^b Genebank accession number of cDNA, available at <http://www.ncbi.nlm.gov/>.

subjective grading scale. The each histology score was calculated, including inflammation, neutrophils, mononuclear cells, edema, epithelial hyperplasia, and mast cells.

2.3. Immunohistochemistry

Paraffin sections of facial lesions were autoclaved in citrate buffer and stained using an ABC kit according to the supplier's instructions (Vector Laboratories, Burlingame, CA). Cryostat sections (3–4 μm) were fixed in acetone and stained as previously described [21]. Sections were incubated with the following

primary antibodies: Toluidine blue (TB) (Muto Pure Chemicals, Tokyo), and rabbit anti-mouse CD209 (Santa Cruz Biotechnology, CA) in blocking buffer overnight at 4 °C. Stained sections were counterstained with hematoxylin.

2.4. Immunofluorescence analysis

For CD209 immunofluorescent staining of facial lesions, tissues were cryosectioned, air-dried, and rinsed with PBS. Sections were blocked with 10% normal serum in PBS for 30 min and preincubated overnight with primary antibody at room tempera-

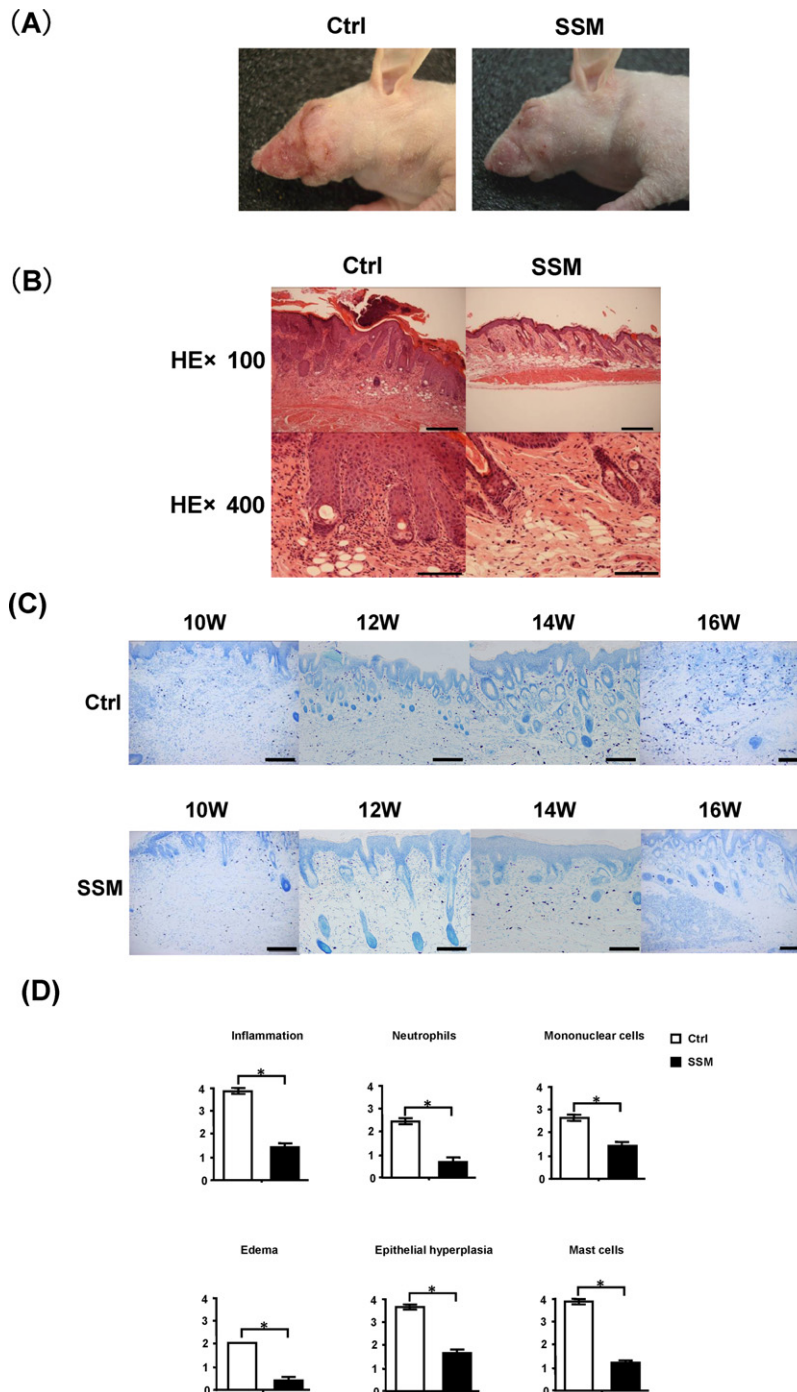


Fig. 1. Clinicohistopathological and immunohistochemical changes in facial skin lesions of DS-Nh mice. (A) Clinical features of conventionally housed DS-Nh mice treated with or without SSM at 16 weeks of age. (B) Histopathological changes in skin lesions from DS-Nh mice with or without SSM treatments at 16 weeks of age. Scale bars = 300 μm and 100 μm . (C) Toluidine blue staining of mast cell in skin lesions from DS-Nh mice with or without SSM treatments at 10, 12, 14, and 16 weeks of age. Scale bar = 300 μm . The histological findings were scored by inflammation, neutrophil infiltration, mononuclear cell infiltration, edema, epithelial hyperplasia, and mast cells infiltration. (D) The each histology score is showed. Data represent means $\pm$ standard error of the mean (SEM). (N = 5) * $P \leq 0.05$ (Mann-Whitney two-sided U test).

ture. Samples were washed and incubated with a fluorescein isothiocyanate (FITC)-labeled secondary antibody at room temperature for 60 min. After washing, each sample was mounted with a glass coverslip using Dako glycerol mounting medium (Dako, Carpinteria, CA). To evaluate localization and distribution of DCs in the epidermis and dermis, after staining with these antibodies, images were captured using a microscopic digital camera (DP70, Olympus, Tokyo). The number of immunoreactive DCs per mm² was counted in the epidermis and dermis of each immunostained section.

2.5. Measurement of serum IgE and IgG2a

Sera from Ds-Nh mice were collected throughout the course of the experiment (10, 12, 14, and 16 weeks of age) and stored at –80 °C. IgE and IgG2a serum levels were measured using murine ELISA kits (BD Pharmingen, and Bethyl Laboratory, Inc., Tokyo, respectively). Data are shown as the means ± standard deviations (SDs) of triplicate experiments.

2.6. Quantitative real-time RT-PCR

The tissues obtained by biopsies of half of the facial lesions of Ds-Nh mice were frozen and stored at –80 °C. Total RNA was extracted using RNeasy Mini Kits as per the manufacturer's instructions (QIAGEN, Valencia, CA). After digestion with RNase-free DNase I, poly(A)⁺ RNA was purified using a mRNA Purification Kit (GE Healthcare, Piscataway, NJ), and then reverse transcribed using SuperScript II reverse transcriptase (Invitrogen Life Technology, Tokyo). For quantitative RT-PCR, 5 ng of cDNA was amplified using a Real Time System (Takara, Otsu) in triplicate assays for 40 cycles in Premix Ex Taq (Takara, Otsu). The oligonucleotide primers and probes specific for IL-1 β , IL-4, IL-12, IL-17A, CCL24, IFN- γ , and TSLP used in this study are listed in Table 1. For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for expression of the genes, results were normalized to those of the 'housekeeping' glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA. Plasmids were assayed with the tissue RNAs and used for construction of standard curves. To construct standard curves, PCR products were inserted into pGEM-T Easy Vector (Promega, Madison, WI) by using TA cloning method.

2.7. Cell culture and flow cytometry

Bone marrow-derived DCs (BMDCs) were obtained from C57BL/6J Slc (B6) mice (Japan SLC Inc., Shizuoka). BM cells were cultured at 5×10^5 cells/ml in cRPMI [RPMI-1640 (Sigma–Aldrich, St. Louis, MO) with 10% heat-inactivated fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM HEPES, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μ g/ml streptomycin] supplemented with 5 ng/ml recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ) for 7 days. SSM (Zeria Pharmaceutical Co. Ltd., Tokyo) or saline was added to the cultures during the last 2 days of culture. Flow cytometry was used to measure the expression levels of MHC class II, CD40, CD80, and CD86. HL3 (anti-CD11c-FITC), HM40-3 (anti-CD40-PE), 16-10A1 (anti-CD80-APC), GL1 (anti-CD86-APC-Cy7), and M5/114.15.2 (anti-MHC class II-Pacific Blue) monoclonal antibodies were purchased from BD Bioscience (San Jose, CA). Flow cytometry was performed on a BD FACSCanto II (BD Biosciences).

2.8. Statistical methods

Statistical analysis of the experimental data was performed to determine differences between cases using χ^2 analyses to compare categorical variables and the Mann–Whitney *U* test to compare

continuous measures. Significance testing was analyzed using the SPSS software package (SPSS Inc., Version 17.0, Chicago, IL). A two-sided 0.05 level test was used to determine statistical significance for all analyses. *P*-values were considered to indicated statistical significance if <0.05 . The values are expressed as mean ± SD.

3. Results

3.1. Clinicohistopathological and immunohistochemical features of facial skin lesions

Five-week-old Ds-Nh mice were used in this study because skin lesions had not yet developed. We subcutaneously injected SSMB (1 mL) or saline (1 mL) into the abdominal region of conventionally housed Ds-Nh mice 5 days per week for 11 weeks. Clinically, erythema, excoriation, and edema were observed in the DS-Nh mice at 16 weeks of age, which advanced with age (Fig. 1A). Histopathologically, hyperkeratosis, acanthosis, and infiltration of lymphocytes were apparent in skin lesions of the control Ds-Nh mice, and these characteristics were greatly attenuated by

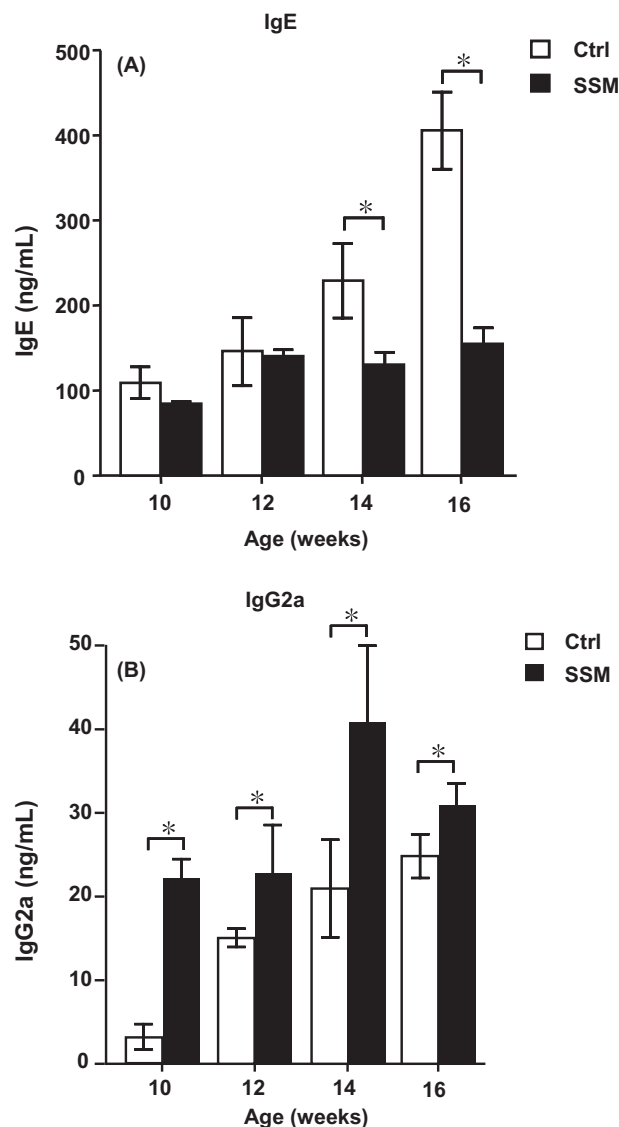


Fig. 2. Serum IgE (A) levels were differentially regulated in conventionally housed Ds-Nh mice treated with or without SSM. In contrast, serum IgG2a (B) levels increased with age in Ds-Nh mice treated with or without SSM. Data represent means ± SEM. **P* < 0.05 (Mann–Whitney two-sided *U* test).

treatment with SSM (Fig. 1B). In contrast, the infiltration of mast cells was observed in facial skin lesions from both groups of Ds-Nh mice under conventional conditions. The relative number of mast cells increased more in control Ds-Nh mice than in SSM-treated Ds-Nh mice. The total number of mast cells gradually increased in both Ds-Nh groups throughout the course of the experiment (Fig. 1C). The each histology score in SSM-treated Ds-Nh mice was lower than that in control Ds-Nh mice (Fig. 1D).

3.2. Suppression of serum IgE levels and enhancement of serum IgG2a levels by SSM administration

It has been reported that IgG2a antibody formation is typical for a Th1 response, and IgE antibody production results from a Th2 immune response [22]. The levels of serum IgE in control Ds-Nh mice increased with age and were higher than those of Ds-Nh mice treated with SSM throughout the experiment (Fig. 2A). On the contrary, serum IgG2a levels significantly increased in both groups of conventionally housed Ds-Nh mice during the course of the experiment. More specifically, the serum IgG2a levels increased more in Ds-Nh mice treated with SSM than in the control Ds-Nh mice at ages 14 and 16 weeks (Fig. 2B).

3.3. Induction of Th1 cytokines and inhibition of IL-4 and CCL24 by SSM

Total RNA was isolated from facial skin biopsies obtained from Ds-Nh mice treated with or without SSM at ages 10, 12, 14, and 16

weeks for use in quantitative RT-PCR analyses. In the lesional skin from mice administered SSM, IL-12 and IFN- γ mRNA levels increased slightly during the experiment (Fig. 3A and B). Additionally, IL-12 and IFN- γ mRNA levels were significantly higher in the Ds-Nh mice treated with SSM than in the control mice at the age of 16 weeks. SSM treatment significantly inhibited the production of IL-4 and CCL24 mRNA at age 12, 14, and 16 weeks. In contrast, IL-4 and CCL24 mRNA levels gradually increased with age from 10 to 16 weeks in the control Ds-Nh mice (Fig. 3C and D).

3.4. Inhibition of TSLP and increased IL-1 β /IL-17A by SSM

Quantitative real-time RT-PCR analysis of tissues isolated from facial lesions indicated that TSLP mRNA expression in Ds-Nh mice treated with SSM was lower than that in control Ds-Nh mice throughout the course of the experiment (Fig. 4A). In contrast, the levels of IL-1 β and IL-17A mRNA increased in Ds-Nh mice treated with SSM and were significantly higher than in control Ds-Nh mice from 10 to 16 weeks of age (Fig. 4B and C).

3.5. Activation of DCs in Ds-Nh mice by SSM

Immunohistochemical analysis of facial skin lesions indicated an increase in the number of CD209⁺ cells, which are mainly DCs, in the dermis of Ds-Nh mice treated with SSM at 10–16 weeks of age as compared to that of the control mice (Fig. 5A). In addition, we evaluated the effect of SSM on BMDCs. Flow cytometric analysis indicated that expression levels of MHC class II, CD80, and CD86,

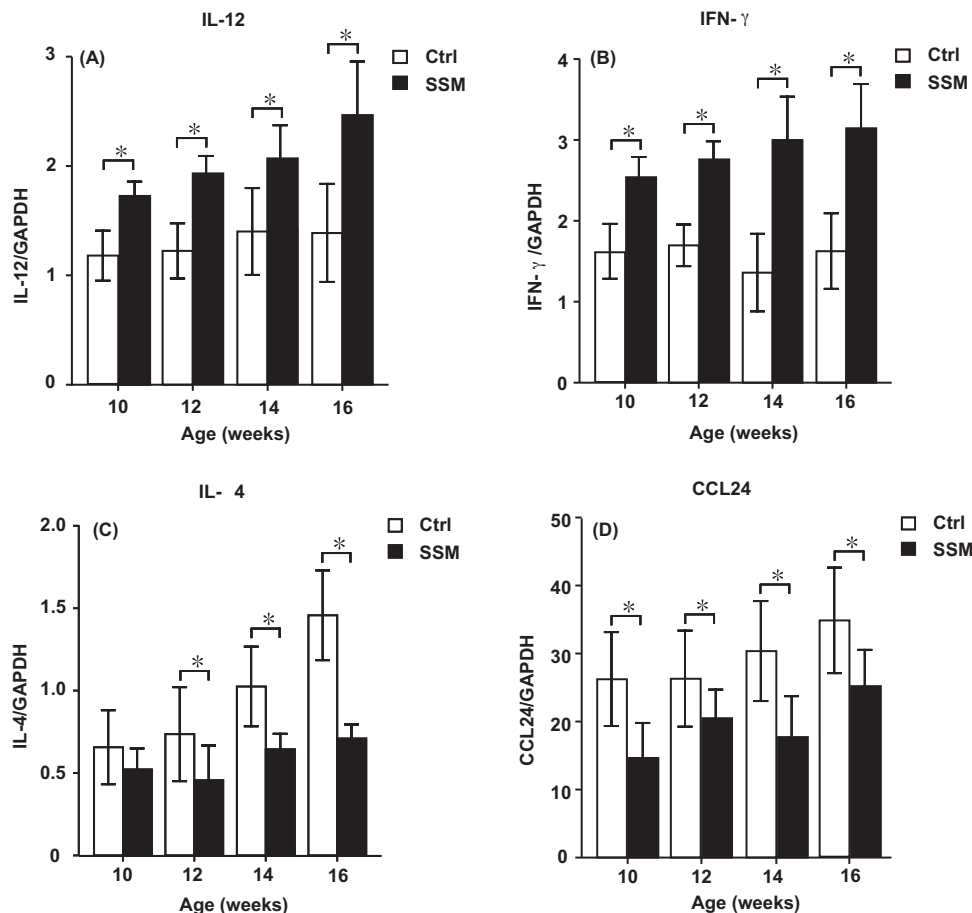


Fig. 3. Th1 and Th2 cytokine expression in Ds-Nh mice with or without SSM treatment. Total RNA was isolated from facial skin lesion biopsies obtained from Ds-Nh mice with or without SSM treatments at 10, 12, 14, and 16 weeks of age, and quantitative RT-PCR was performed. (A) Th1 cytokines. IL-12 and IFN- γ mRNA levels were significantly higher in the Ds-Nh mice treated with SSM than in the control mice at the age of 16 weeks. (B) Th2 cytokines. SSM treatment significantly inhibited the production of IL-4 and CCL24 mRNA at age 12, 14, and 16 weeks. Data represent means $\pm$ SEM, * P < 0.05 (Mann-Whitney two-sided U test).

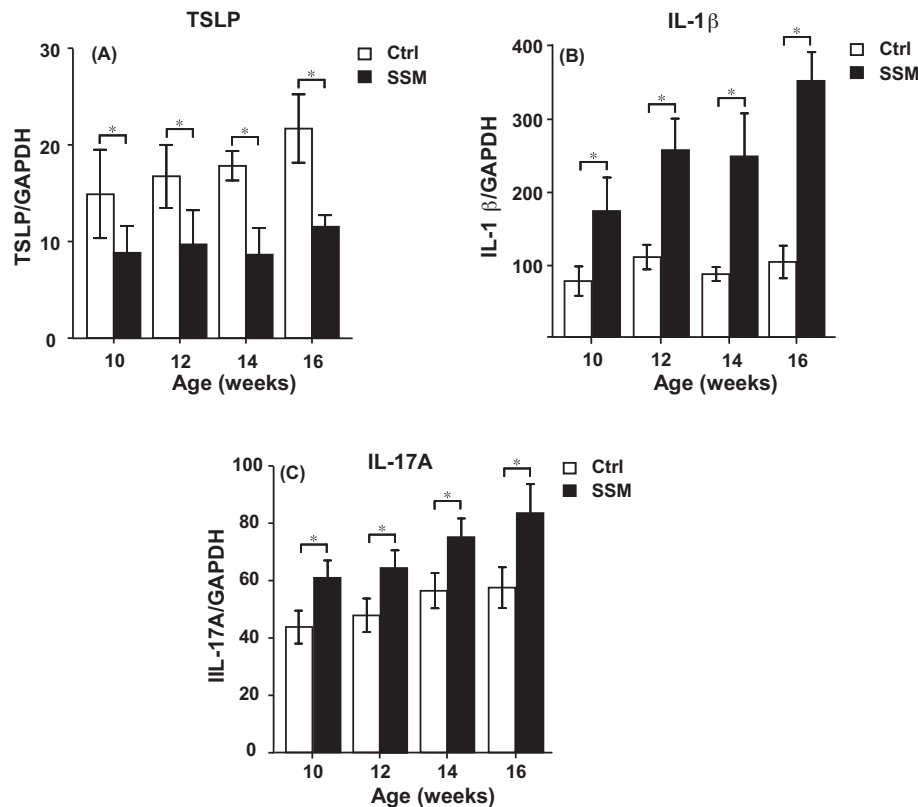


Fig. 4. IL17A and IL-1β mRNA expression was induced, while TSLP mRNA expression was reduced in Ds-Nh mice treated with SSM as compared to control mice. Total RNA was isolated from facial skin biopsies obtained from conventionally housed Ds-Nh mice with or without SSM treatments at 10, 12, 14, and 16 weeks of age, and quantitative RT-PCR was performed. Data represent means ± SEM, *P < 0.05 (Mann-Whitney two-sided U test).

markers of DC maturation, were enhanced by treatment with SSM (Fig. 5B). These data demonstrate that SSM increases co-stimulatory molecule expression on DCs, suggesting that SSM may modulate Th cell differentiation in lesional skin tissues.

4. Discussion

Ds-Nh mice develop spontaneous AD-like skin lesions that are associated with *Staphylococcus aureus* infection [3,7–11]. In the current study, we examined whether SSM could inhibit expression of Th2 cytokines and prevent the development of AD-like skin lesions in Ds-Nh mice, and found that SSM inhibited AD-like skin lesions histologically with low IgE and high IgG2a serum levels. In addition, SSM enhanced Th1 and Th17 cytokines and suppressed Th2-related cytokines. Moreover, SSM enhanced the maturation of DCs. These results suggest that SSM attenuates AD-like skin lesions by shifting from Th2 to Th1/Th17 conditions.

Recent studies have suggested that the Bacillus Calmette-Guerin (BCG) vaccine may be useful as an adjuvant for the treatment of allergic diseases [23–27]. Yoneyama et al. demonstrated that successful BCG vaccination inhibits the development of AD and asthma in children [27]. Cavallo et al. indicated that the BCG vaccine reduces preexistent Th2 responses in allergic rhinitis patients [23]. Of interest, total IgE levels significantly decrease following BCG vaccination. Therefore, the BCG vaccination may be valuable for modulating the allergic profile of AD. However, the mechanism by which BCG vaccination improves symptoms in AD patients remains unknown. More recently, Yokoi et al. reported that the BCG vaccination can alter the function of thymic stromal lymphoprotein (TSLP)-activated DCs by inducing DC production of IL-12 and down-regulation of OX40L [28]. BCG vaccination also

activates DCs to produce additional inflammatory cytokines such as TNF-α and IL-1β [25,26].

SSM is produced by tubercle bacillus extracts [11]. Therefore, SSM is similar to components of the BCG vaccine. Our results suggest that SSM activated DCs and increased the number of DCs. The activated DCs enhanced IL-12 expression, but suppressed TSLP expression, resulting in a shift from a Th2 to a Th1 response. Thus, SSM suppressed the formation of AD-like skin lesions by a similar underlying mechanism as does the BCG vaccine. Therefore, SSM may serve as a useful adjuvant for the suppression of skin lesions in murine models of AD.

Immunohistochemical analysis indicated that the number of CD209⁺ cells, which are mainly DCs, was higher in the lesional dermis of Ds-Nh mice treated with SSM than in the control mice. SSM treatment activated DCs as indicated by increased levels of CD80, CD86, and MHC class II mRNA in bone marrow-derived DCs following treatment with SSM. These results suggest that SSM act on DCs to modulate the cutaneous immune responses. In patients with AD, TSLP is expressed by keratinocytes [27]. TSLP expression activates DCs without inducing Th1 cytokines [29]. In this study, high levels of TSLP mRNA were observed in 10- to 16-week-old control Ds-Nh mice. Therefore, increased TSLP expression may have stimulated DCs, resulting in inflammatory Th2 responses in control Ds-Nh mice. In contrast, TSLP mRNA levels were not elevated in Ds-Nh mice treated with SSM. IL-4 and CCL24 mRNA were expressed at much higher levels in Ds-Nh mice treated with SSM than in control Ds-Nh mice. In this study, we have demonstrated that SSM modulated DC functions. But since TSLP is expressed on keratinocytes, keratinocyte might also be a target of SSM, which will be evaluated in the future.

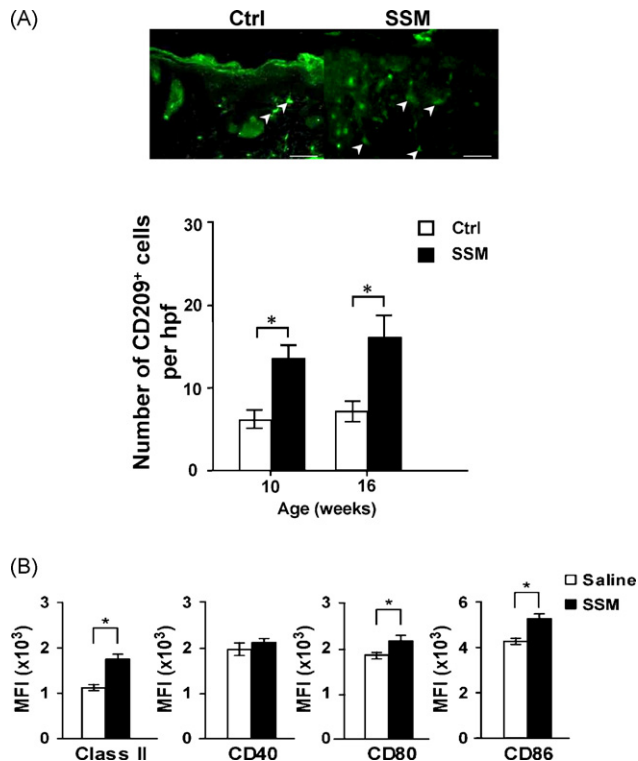


Fig. 5. The number of CD209⁺ DCs was elevated in Ds-Nh mice following treatment with SSM and SSM treatment enhanced BMDC maturation. (A) Immunohistochemical analysis of CD209⁺ DCs in the dermis of facial lesions of Ds-Nh mice with or without SSM treatments. Arrows indicated the CD209⁺ DCs. The number of DCs was enumerated from five high-power fields (HPFs) of each immunostained section. Data are expressed as the number of DCs per HPF. Scale bar = 100 μ m. Statistical analysis was performed using two-sided Mann-Whitney *U* tests. (B) Bone marrow cells from C57BL/6J Slc (B6) mice were cultured for 5 days, treated with saline or SSM for 2 additional days, and subjected to flow cytometric analysis. The mean fluorescence intensities (MFIs) of MHC class II, CD40, CD80, and CD86, indicators of DC maturation, are shown. Data are means $\pm$ SD and are representative of three independent experiments with similar results.

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