RESEARCH ARTICLE

Sphingomyelin maintains the cutaneous barrier via regulation of the STAT3 pathway

Mariko Komuro | Masaki Nagane | Tomoki Fukuyama | Xiaolin Luo | Shinobu Hiraki | Masakatsu Miyanabe | Miyuki Ishikawa | Chiaki Niwa | Hironobu Murakami | Mariko Okamoto | Tadashi Yamashita

1Laboratory of Biochemistry, School of Veterinary Medicine, Azabu University, Sagamihara, Japan
2Center for Human and Animal Symbiosis Science, Azabu University, Sagamihara, Japan
3Laboratory of Pharmacology, School of Veterinary Medicine, Azabu University, Sagamihara, Japan
4Genuine R&D Co., Ltd., Fukuoka, Japan
5Laboratory of Animal Health 2, School of Veterinary Medicine, Azabu University, Sagamihara, Japan
6Laboratory of Veterinary Immunology, School of Veterinary Medicine, Azabu University, Sagamihara, Japan

Correspondence
Tadashi Yamashita, Laboratory of Biochemistry, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Sagamihara, Kanagawa 252-2472, Japan.
Email: yamashita@azabu-u.ac.jp

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Abstract
Epidermal tissues play vital roles in maintaining homeostasis and preventing the dysregulation of the cutaneous barrier. Sphingomyelin (SM), a sphingolipid synthesized by sphingomyelin synthase (SMS) 1 and 2, is involved in signal transduction via modulation of lipid-raft functions. Though the implications of SMS on inflammatory diseases have been reported, its role in dermatitis has not been clarified. In this study, we investigated the role of SM in the cutaneous barrier using a dermatitis model established by employing Sgms1 and 2 deficient mice. SM deficiency impaired the cutaneous inflammation and upregulated signal transducer and activator of transcription 3 (STAT3) phosphorylation in epithelial tissues. Furthermore, using mouse embryonic fibroblast cells, the sensitivity of STAT3 to Interleukin-6 stimulation was increased in Sgms-deficient cells. Using tofacitinib, a clinical JAK inhibitor, the study showed that SM deficiency might participate in STAT3 phosphorylation via JAK activation. Overall, these results demonstrate that SM is essential for maintaining the cutaneous barrier via the STAT3 pathway, suggesting SM could be a potential therapeutic target for dermatitis treatment.

KEYWORDS
cutaneous barrier, sphingomyelin, STAT3

Abbreviations: AD, atopic dermatitis; Df, dermatophagoides farinae; JAK, Janus kinase; SMS, sphingomyelin synthase; SM, sphingomyelin; STAT3, signal transducer and activator of transcription 3; TDI, Toluene-2,4-diisocyanate.

Mariko Komuro and Masaki Nagane contributed equally to this work.

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1 | INTRODUCTION

The epidermal tissue maintains the homeostasis in the cutaneous barrier and prevents the penetration of undesired substances, including several allergens, into the skin from the environment.1 Excessive immunological reactions in response to the external aggressors play an important role in developing cutaneous inflammation, such as atopic dermatitis (AD), which suggests that cutaneous barrier dysfunction and excessive immune responses are highly relevant to each other.

Lipid rafts, specific microdomains on the plasma membrane enriched in sphingomyelin (SM) and cholesterol, are thought to be important signaling platforms.2,3 SM, one of the essential sphingolipids, constitutes nearly 85% of all sphingolipids and 10%–20% of all cellular membrane phospholipids.4 SM is widely distributed in the myelin of neural tissues, lung surfactants, and the epidermis of the skin, in addition to lipid membranes in higher animals. Recently, several studies have shown that SM regulates signal transduction via modulation of lipid-raft functions.5–7 The SM signal transduction pathway is induced by various cytokines such as tumor necrosis factor α (TNFα), interferon γ (IFNγ), and interleukin 1 (IL-1) family, and functions as a major player in the signaling pathways of apoptosis, cell differentiation, stress responses, inducers of cell damage, and cell cycle arrest.

Sphingomyelin synthase (SMS) synthesizes SM by transferring the phosphatidyl head group, phosphatidylcholine, onto the primary hydroxyl of ceramide.6 SMS exists in two isoforms, SMS1 and SMS2 (encoded by SGMS1 and SGMS2, respectively), which have been cloned and characterized by their cellular localization.8 They are abundant in the Golgi apparatus and plasma membrane,9 and they are known to contribute to the maintenance of intracellular SM levels.10,11 It has been shown that SMS is associated with inflammation, such as pneumonia and colitis11,12; however, its role in dermatitis has not been elucidated.

To date, several investigations have revealed that ceramide, a common substrate of sphingolipids in lipid membranes, maintains the cutaneous barrier and regulates cutaneous inflammation13–15; however, the involvement of other sphingolipids has not been elucidated. Recently, it has been reported that lipid rafts modulate the activation of the signal transducer and activator of the transcription 3 (STAT3) signaling pathway.16 STAT3 is involved in multiple processes, including early development, cellular proliferation, survival, and differentiation,17,18 and is also highly expressed in the skin of AD and psoriasis.19,20 STAT3 is activated by many extracellular signaling molecules such as cytokines, growth factors, and hormones.21 Moreover, the activation of STAT3 is accomplished by its phosphorylation on a tyrosine residue, leading to nuclear translocation and transcriptional regulation of target genes.

Based on this background information, we hypothesized that SM deficiency would impair the cutaneous barrier via the STAT3 pathway. Therefore, this study was aimed to investigate the effects of SMS deficiency on cutaneous inflammation, such as severe dermatitis, cutaneous barrier dysfunction, and epidermal thickness. Additionally, we also investigated its underlying mechanisms.

2 | MATERIALS AND METHODS

2.1 | Animal experiments

All animal experiments were performed according to the established guidelines of the “Law for the Care and Welfare of Animals in Japan” and approved by the Animal Experiment Committee of Azabu University (Approval No. 180316–5). Eight to 10 weeks old, C57BL/6N mice acquired from Charles River Laboratories (Kanagawa, Japan) were used for the study. Mice were housed in plastic cages in an air-conditioned room at 24°C under a 12 h light-dark cycle (light on at 7:00 a.m.) with food and water available ad libitum under Specific Pathogen Free conditions. At the end of the experiment, all animals were euthanized by cervical dislocation under 2% isoflurane anesthesia. Sgms1- and Sgms2-knock out (KO) mice were generated following the previously established protocols.22,23

2.2 | A mouse model of dermatitis

A mouse model of dermatitis was generated based on a previously described method with minor modifications.24 Briefly, at least 24 h before the first elicitation, hair of the rostral back region of all mice was depilated with an electric clipper and shaver. Barrier disruption was performed by 150 µl of 4% sodium dodecyl sulfate (Cat. No. 194–13985, Wako Pure Chemical Industries) treatment on the shaved dorsal skin 3 h before elicitation. Elicitation was performed by topical application of 50 mg Dermatophagoides farinae (DF) ointment (Biostir AD, Biostir Inc., Ohsaka, Japan) or ointment base (hydrophilic petrolatum) onto the shaved dorsal skin. The same procedure was repeated twice a week for two weeks. Crude sphingomyelin fraction in butter (BSM; GENUINE R&D, Fukuoka, Japan) based on hydrophilic petrolatum (contains 10% as sphingomyelin) was applied to the shaved dorsal skin every other day for one week, followed by DF application twice a week. The amount of SM applied to the dorsal skin of the
SM-treated group was calculated based on the SM content in the C57BL/6N wild-type (WT) mice back skin.25

The Toluene-2,4-diisocyanate (TDI)-induced AD model was established based on the previously described method with minor modification.26 In brief, the back of the mice was shaved with an electric clipper and shaver. On the day after shaving, 50 μl of TDI (0.5% dissolved in acetone, Cat. No. 584-84-9, Tokyo Chemical Industry CO., LTD., Tokyo, Japan) was applied to the shaved dorsal skin and ears, respectively. Then, the same procedure was repeated twice a week for three weeks. Nontreatment (0 day) mice were collected immediately after hair removal.

The erythema/hemorrhage and scaling/dryness symptoms were scored twice a week as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) based on the macroscopic criteria following the previously described method with minor modifications24 (Tables 1 and 2). A total dermatitis score indicating clinical severity was defined as the sum of the individual scores (maximum score 6). Transepidermal water loss (TEWL) of Df-treated skin was measured in non-treatment, 4 and 7 days after Df-challenge using VAPO SCAN AS-VT100RS (Aschi Techno Lab, Tokyo, Japan) according to the manufacturer’s guidelines.27

### 2.3 Histological analysis

Histological analysis was performed as previously described.28 Briefly, skin tissues were excised and fixed with 4% buffered formaldehyde, embedded in paraffin, and sectioned at 5-μm thickness. The sectioned tissues were then stained by hematoxylin and eosin-stained (H&E) to measure the thickness of the epidermis. For the parameters, the average of 10 fields was estimated and used as the representative values. The number of mast cells was estimated by Toluidine blue staining. The number of mast cells was counted for 10 fields per mice (×200 magnification, single-blinded).

For immunohistochemistry, slides were subjected to antigen retrieval by Immunosaver (Nissin EM, Tokyo, Japan). Primary antibodies and Histofine Simple Stain MAX PO kit (for mouse antibodies; Cat. No. 414321, for rabbit antibody; Cat. No. 414341, Nichirei Biosciences, Tokyo, Japan) were used to visualize the protein expression in tissue. The following antibodies were used as primary antibodies: SMS1 (Cat. No. 19050-1-AP, ProteinTech Group, Inc., Chicago, IL, USA) (1:500), SMS2 (Cat. No. orb4557, Biorbyt, Berkeley, CA) (1:200), phospho-STAT3 (Cat. No. ab65541, Abcam, Cambridge, UK) (1:500), and Ki67 (Cat. No. ACR 325, Biocare Medical Inc., Concord, CA, USA) (1:300). Immunostaining was performed following the manufacturer’s instructions. Images were acquired using a BZ-X700 microscope (Keyence, Osaka, Japan), and the percentage of phospho-STAT3- and Ki67-positive cells were calculated.

### 2.4 Reverse transcription-quantitative polymerase chain reaction analysis

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was performed as previously described with minor modifications.29 Skin tissue was lysed by TRIzol Reagent (Cat. No. 15596026, Thermo

| Score | Severity | Description |
|-------|----------|-------------|
| 0     | None     | No erythema or hemorrhage |
| 1     | Mild     | Erythema is localized, but hemorrhage with excoriations is not presented |
| 2     | Moderate | Erythema is disseminated, but hemorrhage with excoriations is not presented |
| 3     | Severe   | Erythema is overall, or hemorrhage with excoriations is presented |

| Score | Severity | Description |
|-------|----------|-------------|
| 0     | None     | No scaling or dryness |
| 1     | Mild     | Scaling or dryness is localized with slight exfoliating |
| 2     | Moderate | Scaling or dryness is disseminated with apparent exfoliating |
| 3     | Severe   | Scaling or dryness is overall with apparent exfoliating |
Fisher Scientific) using a bead beater-type homogenizer (µT-12, Taitec Corporation, Saitama, Japan) at 3200 rpm for 120 s with two steel beads (φ5 mm). After the phase separation by chloroform, total RNA dissolved in colorless supernatant was purified using a Tissue Total RNA Mini Kit (Cat. No. FATRK 001; Favorgen Biotech Corporation, Ping-Tung, Taiwan). Briefly, the same volume of 70% ethanol was added to the supernatant and applied to the spin column. Spin-columns were washed using wash buffer 1 and wash buffer 2 and then eluted with RNase-free water. Five hundred nanograms of total RNA were reverse-transcribed using ReverTra Ace qPCR RT Master Mix with gDNA remover (Cat. No. FSQ-301, TOYOBO, Osaka, Japan). The real-time PCR analysis was performed using a LightCycler96 System and a FastStart Essential DNA Green Master reaction mix (Cat. No. 06402712001). The sequences of the primers used for RT-qPCR are shown in Table 3. Quantitative PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. The fluorescence intensity was measured at the end of every extension cycle. Using β-2-microglobulin as internal control, relative mRNA quantities were obtained by E-method.

### 2.5 | Flowcytometry

Auricular lymph nodes (LNs) cells were corrected and washed by FACS buffer (phosphate-buffered saline [PBS] containing 1% FBS). Then, one-million cells were stained with monoclonal anti-mouse antibodies against FITC-IgE (1:100 in FACS buffer, Cat. No. 2634530, Sony Biotechnology, Tokyo, Japan), PE-CD19 (1:100 in FACS buffer, Cat. No. 130-111-229, Miltenyi Biotec K.K., Tokyo, Japan), FITC-MHC classII (1:100 in FACS buffer, Cat. No. 130-112-229, Miltenyi Biotec K.K.), PE-CD11c (1:100 in FACS buffer, Cat. No. 130-110-701, Miltenyi Biotec K.K.), FITC-CD3e (1:100 in FACS buffer, Cat. No. 11-0032-82, Thermo Fisher Scientific) and PE-CD62L (1:100 in FACS buffer, Cat. No. 553151, BD Biosciences, CA, USA) for 30 min at 4°C in the dark. Cells were washed with FACS buffer, resuspended at 1 × 10^6 cells per tube in PBS (500 µl), and analyzed using Cell Analyzer EC800 (Sony Biotechnology) and FlowJo software (BD Biosciences, CA, USA).

### 2.6 | ELISA

For cytokine evaluation, single-cell suspensions of LNs (5 × 10^5 cells/well) were incubated with mouse Dynabeads T-activator (Thermo Fisher Scientific, Inc.) for 24 or 96 h. IFNγ and TNFα concentrations in the supernatant were evaluated using ELISA according to the manufacturer protocols (R&D Systems).

### 2.7 | Cell culture

The human immortalized epidermal keratinocyte HaCaT cell was obtained from Cell Line Service (Cat. No. 300493, Eppelheim, Germany), and was maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS. Mouse embryonic fibroblast (MEF) derived from WT and Sgms1-KO mice were immortalized by SV40 transfection. Mouse MEFs were incubated with Dulbecco’s Modified Eagle Medium (Cat. No. 044–29765, Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FBS, sodium pyruvate solution (Cat. No. 190–14881, Wako

| Gene                                      | Forward primer sequence (5′-3′)            | Reverse primer sequence (5′-3′)          |
|-------------------------------------------|-------------------------------------------|-----------------------------------------|
| Mouse β-2-microglobulin                   | TTTCTGGTGCTTGCTCTCAGT                    | GTTACGATATGTCGGCTTCC                    |
| Mouse Sgms1                               | ATAGTTGGCACGCTGATACC                    | AAAGAGCTTCGGAGGACAG                     |
| Mouse Sgms2                               | GAGAAGCTTCAGGCAAAAT                      | GACCAAGTGATACCAGA                      |
| Mouse Filagrin                            | TCGGAAAGGACCAACTACAGG                    | CATTCTTGAGTCCCTACA                      |
| Mouse Il25                                | GGGAGCTATGAGTTGGACAGG                    | CTTCTTGATCTTGATGCA                      |
| Mouse Il33                                | TGGAGAAATGAGATTTGATTTG                   | TCTGGATCTTGGATGAGAG                     |
| Mouse Tslp                                | CGACACAGCATGGTCTTTCCT                    | ATTCGATGAAAGGAAC                      |
| Mouse Il4                                 | GAACAGGATGCCAAGGGGAGGAGG                 | TGAAGGCCTACACCGAG                      |
| Mouse Il13                                | GAGGCCACACTCACAACAGAC                   | AACACAGGGCTACACAGAC                    |
| Mouse Il6                                 | TGTGGAATTGTGGAGAGAGAG                   | AATGCAGAAAGCAAC                      |
| Mouse Il1β                                | CTGAGACAGGATGTTGTCG                      | TCACCAACTAATATGCTAGG                   |
| Mouse Ifnγ                                | TACCTTCCTTACGACCAACAGCA                 | GACTCTTCTTTCGGCTTC                     |
| Mouse Tnfa                                | CCCAAAGGATGAGAAGG                      | CACTTGGTGTTGGCTACAG                    |
Pure Chemical Industries), and MEM Non-essential Amino Acids Solution (Cat. No. 139–15651, Wako Pure Chemical Industries). The cells were cultured with 5% CO2 at 37°C. Tofacitinib (0.1 μM dissolved in DMSO, Cat. No. CP-690550, Pfizer Global Research and Development, Groton, CT) were pre-treated for 1 h before 10 ng/ml IL-6 treatment for 5 min. Since it is known that SM added to the extracellular can permeate the cell membrane and exert its function intracellularly,6,31,32 MEFs were treated with or without 5 μM of SM (Cat. No. NS220103, Nagara Science Co., Ltd., Gifu, Japan) and BSM (crude fraction of sphingomyelin from butter, Genuine R&D, Fukuoka, Japan), and then with IL-6.

2.8 | RNA interference

RNA interference was performed as previously described.28 Briefly, siRNA-mediated knockdown of SGMS1 and SGMS2 was performed using Opti-mem® (Product no. 31985-070, Thermo Fisher Scientific), lipofectamine RNAiMAX (Thermo Fisher Scientific), and siRNA. The siRNAs of Human SGMS1 (sense; CCUGUGGACACUGGUUAA, anti-sense; UUAACCAGAGCUCCUAAAG), Human SGMS2 (sense; GGACUAUAUCCAAAUUGCU, anti-sense; AGCAAUUGGAAUAGUCC), and negative control siRNA (Cat. No. SIC001-10NMOL) was obtained from Sigma–Aldrich. HaCat cells (2 × 105 cells per 6-cm dish) were transfected with siRNA complex (siRNA: 10 nM, RNAiMax: 4.4 μl per 6-cm dish) for 72 h and harvested for western blot. In some experiments, cells were treated with IL-6 (0.1 and 10 ng/ml) from 72 to 84 h after siRNA transfection.

2.9 | SDS-PAGE and western blotting

Cells were collected and lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, and protease inhibitor cocktail) following sonication (on: 30 s, off: 30 s, 5 cycles) using a BIORUPTOR II (BM Equipment Co., Ltd., Tokyo, Japan). Lysates were centrifuged at 15 000xg for 15 min at 4°C, and the supernatants were collected as protein samples. The protein samples were quantified by the Bradford method to adjust the protein concentration. Laemmlı’s sample buffer (0.1875 M Tris-HCl [pH 6.8], 15% β-mercaptoethanol, 6% SDS, 30% glycerol, and 0.006% bromophenol blue) was added to the supernatant, and samples were boiled for 3 min. Twenty micrograms of protein were separated by 9% SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA, USA). Transfer conditions were 100 V in Towbin buffer (25 mM Tris, 192 mM glycine, 1% SDS, and 20% methanol) for 60 min at 4°C. The membrane was probed with specific antibodies diluted with TBST (10 mM Tris-HCl [pH 7.4], 0.1 M NaCl, and 0.1% Tween-20) containing 5% bovine serum albumin or milk overnight at 4°C. After probing with horseradish peroxidase (HRP)-conjugated secondary antibodies, bound antibodies were detected with Immobilon Western HRP substrate. Densitometry was performed using ImageJ software (NIH, Bethesda, MD, USA). The following antibodies were used for western blotting and immunostaining: anti-STAT3 (Cat. No. ab155671) (1:5000) and anti-phospho STAT3 (Cat. No. ab65541) (1:5000), SMS1 (Cat. No. 19050-1-AP, ProteinTech Group, Inc.) (1:1000), SMS2 (Cat. No. orb4557, Biorbyt) (1:2000), and HRP-conjugated secondary antibodies, goat anti-rabbit IgG(H+L) (Cat. No. #A16110, Thermo Fisher Scientific) (1:5000). Immobilon Western HRP substrate was purchased from Merck Millipore (Billerica, MA, USA).

2.10 | Cell-cycle analysis

To analyze cell-cycle perturbation, DNA was stained with propidium iodide and the cells were analyzed by flow cytometry.33 Cells were incubated with BSM and IL-6 for 48 h. We resuspended 1 × 106 cells in PBS and fixed them in ice-cold 70% ethanol for at least 6 h. Fixed cells were washed in PBS twice, and incubated with propidium iodide staining solution (50 μg/ml propidium iodide, 5 μg/ml RNase I, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2PO4, and 1.47 mM KH2PO4) at 37°C for 30 min. The DNA content of at least 10 000 cells/sample was analyzed using an EC800 Analyzer (Sony Biotechnology, Tokyo, Japan).

2.11 | Patients’ data analysis

RNA-seq raw data were obtained by Gene Expression Omnibus under accession code GSE121212. Data sets were classified as AD patients (with lesions and without lesions) and healthy controls. Clinical data and RNA sequences are described elsewhere.34 Reads Per Kilobase of exon per method was used to normalize the RNA-seq data set.

2.12 | Statistical analysis

The data are expressed as mean ± SD. The differences between the groups were analyzed by one or two-way
analysis of variance, followed by Dunnett’s multiple comparison test. Statistical significance was estimated at 5% and 1% levels of probability. The data were analyzed using Prism 9 (GraphPad Software, San Diego, CA, USA).

3  |  RESULTS

3.1  |  Depletion of SM enhances Df-induced dermatitis

To investigate the role of SM on the cutaneous barrier, Df-induced dermatitis was evaluated on the dorsal skin of WT, Sgms1, and Sgms2-KO mice. Df ointment barely induced dermatitis in WT mice; however, Sgms1-KO and Sgms2-KO mice showed a significant increase in cutaneous inflammation on 4–7 days post-Df application (Figure 1A). The clinical dermatitis score analysis showed that WT mice were resistant to the Df-challenge, whereas the Sgms1-KO and Sgms2-KO mice were sensitive (Figure 1B).

In non-treatment WT mice, the immunohistochemical analysis showed that both SMS1 and SMS2 were highly expressed in the epidermis tissue. In contrast, we did not observe the expression of SMS1 or SMS2 on each KO mouse (Figure S1A). We observed that Df-treatment did not alter genes expression of Sgms1 and Sgms2 and the expression of Sgms1 was higher than that of Sgms2 in both time points (Figure S1B).

To assess the cutaneous barrier, we determined TEWL. Consistent with the symptoms of cutaneous inflammation, the value of TEWL was increased in Sgms1-KO and Sgms2-KO mice compared to WT mice at 4 and 7 days after Df-challenge (Figure 1C). Moreover, the expression level of filaggrin, a major structural protein in the stratum corneum of the epidermis, was also increased in Sgms1-KO and Sgms2-KO mice 7 days after Df-challenge (Figure 1D). These results suggested that SM deficiency is associated with barrier dysfunction in the skin. Therefore, we performed the histological evaluation of cutaneous sections from WT, Sgms1-KO, and Sgms2-KO mice treated with Df ointment for 7 days. The epidermis of the skin lesions in Sgms1-KO and Sgms2-KO mice was significantly thickened compared to WT mice (Figure 1E,F).

Next, we examined whether Sgms-deficient mice could upregulate skin inflammation. The gene expression of cytokines, which play key initiators of inflammation in epithelial tissues (IL-25, IL-33, and Thymic stromal lymphopoietin [TSLP]) or associated with cutaneous inflammation (IL-4 and IL-13) tended to increase in Sgms1-KO and Sgms2-KO mice, which was clearly in TSLP, IL-4 and IL-13 (Figure S2A–E).

Although Sgms1/2-KO mice have shown severe cutaneous inflammation, we observe that immune cell infiltration was low in the Df-induced model. We then, investigate the effect of SMS1 and SMS2 on the skin immune response using the TDI-induced dermatitis model. Sgms1-KO mice showed severe inflammation compared to WT and Sgms2-KO mice (Figure S3A). Also, Sgms1-KO mice showed a significant increase in inflammation score (Figure S3B), TEWL (Figure S3C), epidermal thickness (Figures S3D,E), and immune cell infiltration (Figure S3G) in comparison to WT and Sgms2-KO mice. Moreover, 21 days after TDI treatment, Sgms1-KO mice showed increased filaggrin expression compared to untreated mice, whereas the expression of it was decreased compared to WT and Sgms2-KO mice (Figure S3F).

We examined whether Sgms-deficient mice could upregulate the immune reaction caused by TDI-induced allergic dermatitis. After TDI application, immune cells in LNs were analyzed by flow cytometry. The population of IgE-positive B cells and dendritic cells was increased in Sgms1-KO mice (Figure S4A). The levels of IFNγ and TNFα were increased in Sgms1-KO mice after CD3/CD28 stimulation (Figure S4B). Moreover, Sgms1-KO mice showed increased infiltration of mast cells in the dermis of dermatitis lesions (Figure S4C).

3.2  |  Depletion of SM increase epidermal STAT3 phosphorylation

Recent studies have reported the importance of the STAT3 pathway in developing AD and cutaneous inflammation. Therefore, to investigate the role of SM in the STAT3 pathway, we evaluated the STAT3 phosphorylation levels in Sgms1-KO and Sgms2-KO mice after Df-ointment application. Seven days after Df treatment, the immunohistochemical analysis demonstrated an increased number of phosphorylated STAT3 (p-STAT3)-positive epidermal cells in Sgms1-KO and Sgms2-KO mice, which was more pronounced in Sgms1-KO mice (Figure 2A). Additionally, the quantitative results for p-STAT3-positive epidermal cells also revealed similar results in Sgms1-KO and Sgms2-KO mice (Figure 2B). The increase in p-STAT3-positive cells was also observed in the allergic dermatitis model induced by TDI treatment, which was clearly in Sgms1-KO mice (Figure S5). Therefore, we examined the functionality of p-STAT3 using one of the most cited markers for cell proliferation, Ki67. The proliferation of basal keratinocytes in Sgms1-KO and Sgms2-KO mice skin after Df treatment was monitored by immunohistochemical analysis. Sgms1-KO and Sgms2-KO mice showed
**FIGURE 1**  *Sgms* knockout leads to progressive cutaneous inflammation in the Df-induced dermatitis model. Mice were administered Df ointment to induce dermatitis. Representative images of wild-type, *Sgms1-KO*, and *Sgms2-KO* mice at 0 (non-treatment), 4, and 7 days after Df treatment (A), and clinical dermatitis score. Scale bar = 1 cm (B). Analysis of transepidermal water loss of untreated and Df-treated mice after 7 days of the treatment (*n* = 3–4 mice/group) (C). Gene expression of filaggrin of untreated and Df-treated mice after 7 days of the treatment (*n* = 4–5 mice/group) (D). Histopathology was examined by H&E staining in untreated and Df-treated mice after 7 days of the treatment. Scale bar = 100 µm (*n* = 3 mice/group) (E). Epidermal thickness was analyzed by measuring 10 points per mouse (*n* = 3 mice/group) (F). Plots and bars are presented as the mean ± SD. *p < .05, **p < .01
**FIGURE 2**  *Sgms* knockout leads to epidermal STAT3 phosphorylation and cell proliferation in Df-induced dermatitis model. Epidermal tissue treated with Df ointment was analyzed histologically. Histopathology was examined by immunohistochemistry with p-STAT3 in untreated and Df-treated mice after 7 days of the treatment. Scale bar = 100 µm (A). p-STAT3 positive epidermal cells were analyzed by counting the number of p-STAT3 positive nuclei in 100 nuclei per mouse, and the percentage was calculated (B). Immunohistochemistry with Ki67 in untreated, and Df-treated mice after 7 days of the treatment. Scale bar = 100 µm (C). Ki67-positive epidermal cells were analyzed by counting the number of Ki67-positive nuclei in 100 nuclei per mouse, and the percentage was calculated (D). Plots and bars are presented as the mean ± SD (n = 3 mice/group). **p < .01**
increased Ki67-positive cells 7 days after Df application, which was prominent in Sgms1-KO mice (Figure 2C,D). In addition, the gene expression levels of IL-6, IL-1β, IFNγ, and TNFα, which are known to the downstream of STAT3 in inflammation,38-40 were also increased in Sgms1-KO and Sgms2-KO mice (Figure S2F–I).

3.3 | Depletion of SM promotes the JAK/STAT3 pathway activated by IL-6

To further investigate the molecular mechanism of STAT3 phosphorylation in Sgms-deficient mice, we generated immortalized MEF derived from WT and Sgms1-KO mice. Western blot analysis indicated that STAT3 was significantly phosphorylated in Sgms1-KO MEF at 0.01 and 0.1 ng/ml of IL-6, whereas STAT3 of WT MEF was barely phosphorylated under these low concentrations of IL-6 (Figure S6A). Then, we treated low (0.1 ng/ml) and high (10 ng/ml) concentration of IL-6 (Figure 3A,B). While STAT3 was remarkably phosphorylated in Sgms1-KO in low IL-6 treatment, we did not observe a significant difference among WT and Sgms1-KO in a high concentration of IL-6.

To confirm the involvement of SMS1/2 in the STAT3 phosphorylation in keratinocytes, we employed the RNA interference of SGMS1 and SGMS2 in Human keratinocyte HaCaT cells. At 72 h after siRNA transfection, western blot analysis showed the downregulation of SMS1 and SMS2 expression (Figure S7A,B). SGMS1 downregulation enhanced STAT3 phosphorylation in low concentrations (0.1 ng/ml) of IL-6, whereas SGMS2 downregulation was barely enhanced STAT3 phosphorylation under that of IL-6.

Janus kinase (JAK), an upstream protein to STAT3, has been widely implicated in inflammations involving dermatitis, particularly AD.41 Therefore, we tested the hypothesis that inhibition of JAK would inhibit the phosphorylation of STAT3 using tofacitinib, a potent inhibitor of JAK1, JAK2, JAK3, and TYK2, which is currently under development as a therapeutic agent against inflammatory and autoimmune diseases.42,43 To examine differences of JAK function between WT and Sgms1-KO MEF under conditions of equal STAT3 phosphorylation, we treated MEFs with 10 ng/ml IL-6 after 0.1 μM tofacitinib pre-treatment. Tofacitinib attenuated the STAT3 phosphorylation in both WT and Sgms1-KO MEFs (Figure 3C), suggesting the potential role of SMS1/2 in regulating the JAK/STAT3 pathway.

3.4 | SM controls the JAK/STAT3 pathway activated by IL-6

To confirm whether SM, the product of SMS1 and SMS2, can regulate JAK/STAT signaling, we examined the phosphorylation of STAT3 by treating SM and BSM pretreated cells with IL-6. Since 0.1 ng/ml IL-6 significantly increased STAT3 phosphorylation in Sgms1-KO MEF (Figures S6A and 3A,B), we treated the MEF with 0.1 ng/ml IL-6 in the downstream experiments. WST assay showed IC50 of SM and BSM were > 100 μM; we used 5 μM in this experiment. SM and BSM pretreatment suppressed STAT3 phosphorylation induced by IL-6 treatment in Sgms1-KO mice (Figures 3D and S6B,C). Consistent with this result, STAT3 phosphorylation was also decreased in the SM-treated HaCaT cell (Figure S7C).

It has been reported that STAT3 is involved in cellular proliferation44; therefore, as a next step, we examined the effects of SM on cell proliferation. It was observed that IL-6 treatment significantly increased cell proliferation consistent with STAT3 phosphorylation in Sgms1-KO MEFs, which was downregulated by SM and BSM treatment (Figures 3E and S6D). However, BSM-treated MEF did not change the cell cycle distribution (Figure S6E), which suggests that SM slows down cellular proliferation via STAT3 suppression.

3.5 | SM prevented cutaneous barrier dysfunction by Df-induced dermatitis in Sgms-KO mice

To examine the involvement of SM in dermatitis, gene expression of SGMS1/2 was analyzed in AD patients in silico. The expression levels of SGMS1 were significantly reduced in the skin of AD patients (Figure 4A,B). Also, inflammatory cytokines (IL-5, IL-6, and IL-13) and chemokines (C-C motif chemokine [CCL]17 and CCL22) were increased in the skin of AD patients (Figure 4C–G).

To further investigate whether SM is a therapeutic target for dermatitis, we applied BSM to the dorsal skin of Sgms1-KO and Sgms2-KO mice for one week before the Df-challenge. From Figures 3 and Figures S4B–D, there was no difference between SM and BSM in the effect on STAT3 regulation. Therefore, BSM, water-soluble and less damaging to the skin, was treated to these mice. As shown in Figure 5A, BSM suppressed cutaneous inflammation in Sgms1-KO and Sgms2-KO mice. Clinical dermatitis score was also reduced in BSM-treated mice compared to the control mice (Figure 5B). Moreover, the BSM treatment prevented the increase in TEWL in cutaneous inflammation (Figure 5C) as well as the increase in epidermal thickness (Figure 5D,E). Finally, dermatitis was suppressed and the number of phospho-STAT3-expressing cells in epidermal tissues was reduced after BSM treatment (Figure 5F,G). These results indicated that SM plays an important role in maintaining the cutaneous barrier and preventing dermatitis via STAT3 suppression.
**FIGURE 3**  *Sgms* knockout enhanced STAT3 phosphorylation by IL-6 in mouse embryonic fibroblast (MEF). MEF derived from wild-type, and *Sgms1-KO* mice were treated with IL-6 to induce inflammation. Expression of p-STAT3 treated with 0.1 ng/ml of IL-6 for the indicated times (*n* = 3/group) (A). Expression of p-STAT3 treated with 10 ng/ml of IL-6 for the indicated times (*n* = 3/group) (B). Expression of p-STAT3 treated with 0.1 µM tofacitinib for 1 h before treatment with 10 ng/ml IL-6 for 5 min (*n* = 3/group) (C). Expression of p-STAT3 treated with 5 µM SM for 12 h before 0.1 ng/ml IL-6 treatment for 12 h (*n* = 4–6/group) (D). Growth rates in 0.1 ng/ml IL-6 and 5 µM SM treatment for 72 h (*n* = 3/group) (E). The relative band intensity of phosphorylated proteins was normalized to that of total proteins. Plots and bars are presented as the mean ± SD. *p < .05, **p < .01
FIGURE 4  SGMS expression is diminished in atopic dermatitis. The expression level of SGMS, inflammatory cytokines, and chemokines in the skin of patients with AD were analyzed. Expression of SGMS1 (A), SGMS2 (B) IL5 (C), IL6 (D), IL13 (E), CCL17 (F), and CCL22 (G) in the skin lesions of AD patients. Plots and bars are presented as the mean ± SD (CTRL n = 38 and AD patients n = 27). *p < .05, **p < .01
Skin, the largest organ of the body, contains a strong barrier preventing the penetration of external aggressors, such as bacteria, fungi, and chemicals, and the dysfunction of the cutaneous barrier has been implicated in chronic inflammatory skin diseases, such as AD. In the present study, we demonstrated that SM, a sphingolipid expressed in the plasma membrane, plays a key role in maintaining the cutaneous barrier. It was shown that SM treatment ameliorated the STAT3 hyper-phosphorylation in Sgms-deficient mice, indicating the potential of SM in
FIGURE 5 BSM prevented cutaneous barrier dysfunction in the Df-induced dermatitis model. Mice were treated with 10% BSM solution for 7 days before Df ointment. Representative images of Sgms1-KO and Sgms2-KO mice in non-treatment, Df treatment for 7 days, and BSM treatment for 7 days before Df ointment (A), and clinical dermatitis score (Sgms1-KO mice n = 3–4/group and Sgms2-KO mice n = 4–7/group) (B). Scale bar = 1 cm. Analysis of transepidermal water loss in non-treated (Sgms1-KO mice n = 3 and Sgms2-KO mice n = 3), Df-treated (for 7 days) (Sgms1-KO mice n = 3 and Sgms2-KO mice n = 5), and BSM-treated (for 7 days before Df ointment) (Sgms1-KO mice n = 3 and Sgms2-KO mice n = 4) mice (C). Histopathological examination by H&E staining in non-treated, Df-treated (for 7 days), and BSM-treated (for 7 days before Df ointment) mice. Scale bar = 100 µm (D). Epidermal thickness was analyzed by measuring 10 points per mouse (n = 3 mice/group) (E). Histopathology was examined by immunohistochemistry with p-STAT3 in non-treated, Df-treated (for 7 days), and BSM-treated (for 7 days before Df ointment) mice. Scale bar = 100 µm (F). Phospho-STAT3 (p-STAT3)-positive epidermal cells were analyzed by counting the number of p-STAT3-positive nuclei in 100 nuclei per mouse; the percentage was calculated (n = 3 mice/group) (G). Plots and bars are presented as the mean ± SD. *p < .05, **p < .01

preventing the cutaneous barrier dysfunction and reducing the aggravation of dermatitis.

The stratum corneum consists of corneocytes surrounded by multilaminar lipid membranes that prevent excessive water loss from the body and entrance of undesired substances and plays a pivotal role in forming a proper physiological cutaneous barrier. Several investigators have reported that the skin of patients with AD has a decreased proportion of ceramide, a major intercellular lipid in the stratum corneum.46,47 SM, produced from ceramide via SMS, is an essential lipid enriched in the plasma membranes of animal cells, where it regulates membrane properties and many intracellular signaling processes.48,49 Sgms1 and Sgms2 double KO mice exhibited viviparous lethality (data not shown); therefore, in this study, Sgms1-KO, and Sgms2-KO mice were employed.

Our study found exacerbation of dermatitis in Sgms-deficient mice, which was more pronounced in Sgms1-KO mice than Sgms2-KO (Figures 1E,F and S3G). The difference in the dermatitis response between Sgms1-KO and Sgms2-KO could be due to the difference in SM content level in tissue type. SMS1 expresses almost all the tissues, whereas SMS2 varies expression depending on the tissue, and is known to be abundant in liver, small intestine, colon, and kidney but not in the immune cell.50 As shown in Figures S3 and S4, dermatitis was exacerbated only in Sgms1-KO mice with extensive infiltration of immune cells. Several studies have reported that SM1 produces 80% of the cellular SM and SM2 produces 20% and reduction of SM content in Sgms1- or Sgms2-deficient mice. In addition, the total tissue SM in Sgms1-KO mice was reported to be significantly lower than that of Sgms2-KO mice.10,11,51,52 Moreover, induction of dermatitis hardly altered the gene expressions of Sgms1 and Sgms2 (Figure S1B). These results suggest that both Sgms1/2 are constitutively expressed in skin tissues and that SM levels are involved in the maintenance of skin barrier function.

Aberrant lipid organization in the lipids increases TEWL in AD skin.47,53 Consistent with these reports, our data showed that TEWL levels were increased in Sgms-deficient mice together with cutaneous inflammation (Figures 1C and S3C). It has been reported that changes in the components of the lipid membrane in the epidermis disrupt the cutaneous barrier,54 which strongly suggests that the components of the lipid membrane affect the cutaneous barrier and subsequent pathology of dermatitis. Additionally, dietary supplementation with a concentrate of milk phospholipids, including SM, has been shown to significantly increase the hydration levels of the skin.55 Furthermore, SMS has been shown to be associated with inflammation, including pneumonia and colitis.11,12 Consistent with these reports, in our experiments, the Sgms deficient mice were unable to maintain their membrane properties leading to the onset of cutaneous barrier dysfunction and dermatitis development. IL-25, IL-33, and TSLP are the cytokines released from the epidermis when the cutaneous barrier is damaged and activates immune cells such as mast cells, dendritic cells, and Th2 cells.56–60 The levels of mast cell and Th2 cytokines, IL-4 and IL-13, were also increased along with inflammation (Figures S2D,E and S4B). In addition, Sgms1-KO mice sometimes showed inflammatory responses even before the induction of dermatitis. As shown in Figure S2A–C, the mRNA levels of IL-25, IL-33, and TSLP tend to increase in Sgms-deficient mice both in non-treated and Df-treated mice, which was clearly in TSLP. These data suggested that the slightest stimulation (such as depilation by hair remover) initiates dermatitis in SM-deficient skin. Further investigation with cutaneous-specific conditional Sgms1 and Sgms2 double KO mice is necessary.

Filaggrin is a key component of the stratum corneum for maintaining a normal cutaneous barrier. Decreased expression of filaggrin in the skin and loss of function mutants of the filaggrin gene have been described in AD,61,62 whereas the increased filaggrin expression in human and murine keratinocytes attenuated the development of AD-like cutaneous inflammation.63 The filaggrin expression of spontaneous AD mouse models such as NC/Nga should be suppressed as the symptom progressed. Mechanism of dermatitis development and the recovery process of C57BL/6N mice and the spontaneous AD model might be
different, therefore, further analysis has to be performed in the future study.

The activation of STAT3 signaling occurs in the skin of AD patients, wherein phosphorylated STAT3 in keratinocytes has been shown to play an important role in the onset of AD by promoting the expression of inflammatory cytokines. In this study, hyper-phosphorylation of STAT3 in the epidermis was observed in Sgms-deficient mice with dermatitis (Figures 2A,B and S5); in addition, STAT3-downstream genes were also increased (Figure S2F–I). In addition to the mouse dermatitis model, we performed in vitro experiments using Sgms1-KO MEF, which had a more robust dermatitis phenotype than Sgms2-KO mice and lower expression in patient data. Consistent with our findings in mouse experiments, the phosphorylated STAT3 was also increased in MEFs derived from Sgms-deficient mice treated with a low concentration of IL-6 (Figure 3A), indicating that the cellular sensitivity to IL-6 was increased by Sgms deficiency. These findings are consistent with the findings of a recent study demonstrating that the phosphorylated STAT3 levels reflect inflammation severity.66

The experiment with MEF indicated that Sgms deficiency might participate in STAT3 phosphorylation via effects on JAK activation. JAK has been reported as a signal transducer for STAT3 in various inflammatory processes, including dermatitis. In this study, tofacitinib, a clinical JAK inhibitor, inhibited STAT3 phosphorylation induced by IL-6, indicating that JAK functions upstream of STAT3 in the present condition. It has been reported that tofacitinib directly enhances the expression of structural proteins in the epidermis, such as filaggrin, and is expected to effectively reduce inflammation and improve the cutaneous barrier via the JAK/STAT pathway in AD. In summary, the present study demonstrates that the cutaneous barrier dysfunction in Sgms-KO mice sensitizes the inflammation to epicutaneous antigen. In addition to the skin inflammation triggered by the entrance of undesired substances, excessive phosphorylation of STAT3 in the cytoplasm causes the development of dermatitis. Our results show that SM is essential for maintaining the cutaneous barrier and is regulated by the JAK/STAT pathway. Overall, these findings strongly suggest that SM could be an important therapeutic target in treating dermatitis (Figure 6).

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BSM was manufactured by Genuine R&D Co., Ltd. (Masakatsu Miyanabe, Shinobu Hiraki, Xiaolin Luo). Other authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Mariko Komuro, Masaki Nagane and Tomoki Fukuyama. The first draft of the manuscript was written by Mariko Komuro and Masaki Nagane and Tomoki Fukuyama commented on the previous versions of the manuscript. All authors read and approved the final manuscript. Conceptualization: Tadashi Yamashita. Methodology: Mariko Komuro, Masaki Nagane, Tomoki
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