

The Role of Sphingosine Kinase in a Murine Model of Allergic Asthma¹

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Asthma is an allergic disease characterized by chronic airway eosinophilia and pulmonary infiltration of lymphocytes, particularly of the Th2 subtype, macrophages and mast cells. Previous studies have shown a pivotal role for sphingosine kinase (SphK) on various proinflammatory cells, such as lymphocyte and eosinophil migration and mast cell degranulation. We therefore examined the roles of SphK in a murine model of allergic asthma. In mice previously sensitized to OVA, i.p. administration of *N,N*-dimethylsphingosine (DMS), a potent SphK inhibitor, significantly reduced the total inflammatory cell infiltrate and eosinophilia and the IL-4, IL-5, and eotaxin levels in bronchoalveolar lavage fluid in response to inhaled OVA challenge. In addition, DMS significantly suppressed OVA-induced inflammatory infiltrates and mucus production in the lungs, and airway hyperresponsiveness to methacholine in a dose-dependent manner. OVA-induced lymphocyte proliferation and IL-4 and IL-5 secretion were reduced in thoracic lymph node cultures from DMS-treated mice. Moreover, similar reduction in inflammatory infiltrates, bronchoalveolar lavage, IL-4, IL-5, eotaxin, and serum OVA-specific IgE levels was observed in mice with SphK1 knock-down via small interfering RNA approach. Together, these data demonstrate the therapeutic potential of SphK modulation in allergic airways disease. *The Journal of Immunology*, 2008, 180: 4323–4329.

Sphingolipids are ubiquitously expressed in all eukaryotic cell membranes. Over the past few years, it has become clear that sphingolipids are sources of important signaling molecules in addition to their role as structural components of the membranes. In particular, sphingolipid metabolites such as ceramide and sphingosine 1-phosphate (S1P),⁴ have emerged as a new class of potent bioactive messengers involved in an array of cellular processes, including cell differentiation, proliferation, and apoptosis (1, 2). Recently, interest in S1P focused on two distinct cellular roles, namely its function as an intracellular second messenger or extracellularly as a specific and high affinity ligand for a family of G protein-coupled receptors previously known as the endothelial differentiation gene family. To date, five S1P receptors in the endothelial differentiation gene family have been identified, including EDG-1, EDG-3, EDG-5, EDG-6, and EDG-8, now collectively known as S1P_{1–5} receptors (3–5).

As an intracellular second messenger, S1P was found to play a role in calcium signaling and mobilization, cell proliferation, and survival. Activation of various plasma membrane receptors, such as the platelet-derived growth factor receptor (6, 7), FcεRI and FcγRI Ag receptors (8–10), the fMLP receptor (11), the C5a receptor (12, 13), and TNF-α receptor (14), leads to rapid increase in intracellular S1P level via sphingosine kinase (SphK) stimulation. Inhibition of SphK stimulation strongly reduced or even prevented cellular events triggered by these receptors, such as receptor-stimulated DNA synthesis, Ca²⁺ mobilization, and vesicular trafficking (6–11). We have previously shown that inhibition of SphK activity, by *N,N*-dimethylsphingosine (DMS), a potent SphK inhibitor, leads to reduced Ca²⁺ mobilization, enzyme release, chemotaxis, cytokine and chemokine production in human neutrophils, monocytes, and macrophages (12, 13). Moreover, by using a specific antisense knock-down approach, SphK1, one of the two cloned human SphK isoforms, was found to be a critical regulator in TNF-α-mediated proinflammatory responses in human monocytes (14).

Asthma is a chronic allergic disorder characterized by airway hyperresponsiveness (AHR), inflammatory infiltrates in the bronchial walls containing eosinophils, and elevated serum IgE levels. Lung biopsy of patients with asthma have revealed pulmonary infiltration of lymphocytes, particularly of the Th2 type, and macrophages and mast cells. These cells, together with the resident airway cells, interact with one another to initiate and perpetuate allergic airway inflammation (15). Th2 cytokines such as IL-4, IL-5, and IL-13 play a critical role in promoting airway inflammation and are required for the development of airway eosinophilia and IgE production (16–18). Eotaxin, a C-C chemokine, is highly expressed in asthmatic airway epithelium and is pivotal for the recruitment of eosinophils (19–21). Thus, it has been proposed that airway eosinophilia together with various inflammatory cytokines mentioned may contribute to eventual AHR in asthma.

S1P promotes monocytes and lymphocyte activation and migration (22–25), and mast cell degranulation and chemotaxis (8, 9, 26). Local administration of S1P causes inflammation coupled

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⁴ Abbreviations used in this paper: S1P, sphingosine 1-phosphate; AHR, airway hyperresponsiveness; i.n., intranasally; BAL, bronchoalveolar lavage; DMS, *N,N*-dimethylsphingosine; siRNA, small interfering RNA; SphK, sphingosine kinase; PAS, periodic acid-Schiff; R_L, pulmonary resistance; C_{dyn}, dynamic compliance; ASM, airway smooth muscle.

with eosinophil recruitment in a rat-paw model, which can be inhibited by anti-CCR3 Ab (27). Moreover, elevated levels of S1P in bronchoalveolar lavage (BAL) fluid were recovered from allergic asthma patients after ragweed Ag challenge and may play a role in both acute bronchoconstriction and airway remodeling through its direct action on airway smooth muscle (ASM) cells (28, 29). In a murine model of asthma, S1P causes a dose-dependent contraction of bronchi and triggers AHR in OVA-sensitized mice, and is coupled to an enhanced expression of SphK1, SphK2, and S1P₂ and S1P₃ receptors (30). In addition, S1P has been proposed to be a key regulator of Th2 lymphocyte trafficking in an OVA-induced food allergy model in mice (31). Murine allergic asthma represents an ideal model to explore the diverse inflammatory effects of SphK blockade. In the present study, we investigated whether inhibition of SphK activity, either by DMS or specific targeting of SphK1 by the small interfering RNA (siRNA) approach, may possess immunomodulatory effects in such a model. In this study, we show that both DMS and SphK siRNA can effectively suppress eosinophilic airway inflammation and Th2 cytokine and chemokine secretion, and markedly attenuate OVA-induced AHR in sensitized animals. These data identify SphK as a potential therapeutic target in allergic asthma.

Materials and Methods

Preparation of DMS and siRNA

DMS (Cayman Chemicals) was prepared as a 50 mg/ml stock in ethanol and diluted to the appropriate concentration in sterile PBS before use. The siRNA sense and antisense strands were purchased from Qiagen with the following sequences: SphK1, sense 5'-GGGCAAGGCUCUGCAGCUC-dTdT-3', antisense 3'-GAGCUCGACAGCCUUGCCC-dTdT-5'; and scramble control, sense 5'-GACUCCAUGGACUGGCAU-dTdT-3', antisense 3'-AUGCCAGUCCAUGGAGUC-dTdT-3'.

Sensitization and challenge with OVA

Female BALB/c mice at 8- to 10-wk-old were obtained from the Laboratory Animals Centre, National University of Singapore. Animal experiments were conducted according to the Institutional Guidelines for Animal Care and Use Committee for National University of Singapore. Mice were immunized with fraction V OVA (100 µg; Sigma-Aldrich) in an alum suspension (2% Inject Alum; Pierce) in a volume of 200 µl by i.p. injection on days 0 and 14. On day 14, mice were anesthetized and 100 µg of OVA in 50 µl of PBS administered intranasally (i.n.). Mice were again anesthetized before being challenged with 50 µg of OVA in 50 µl of PBS on each of days 25–27. Control mice were given PBS in place of OVA in both the i.n. sensitization and challenge stages of the protocol.

Treatment protocols

DMS (200 or 400 µg/kg) was given by i.p. injection 30 min before the OVA challenge on days 25–27. Control mice received PBS supplement with ethanol as carrier control. Similarly, siRNA against SphK1 or scramble control (5 µg/animal; equivalent to 200 µg/kg) was given i.n. on days 21 and 23 and subsequently 1 h before each OVA challenge on days 25–27.

BAL process

BAL was performed 24 h after the last OVA challenge. Mice were anesthetized and thoracic cavity was opened by careful dissection. The trachea was then exposed, and a small transverse incision made just below the level of the larynx. BAL was then performed using two doses of 0.5 ml of PBS, ensuring that both lungs inflated during the lavage process and that there was no leakage of lavage fluid from the trachea. The lavage samples from each mouse were pooled and kept on ice until processing. BAL fluid was centrifuged at 400 × g for 5 min, and the supernatant was removed and stored at –70°C until assay of cytokines. BAL cell number was determined using a hemocytometer. Cytospin preparations were made using a Cytospin (Thermo Shandon), then were stained with a modified Wright stain. Differential cell counting was performed using standard morphological criteria in which 300 cells were counted per slide.

Serum collection

Blood was collected by cardiac puncture immediately after the thoracic cavity was opened and before BAL was performed. Blood was allowed to

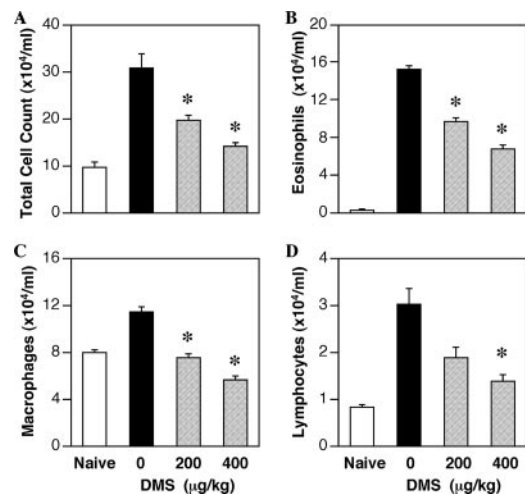


FIGURE 1. Effects of DMS on BAL fluid cell and differential cell count. BALB/c mice were sensitized with OVA and then i.n. challenged with OVA on three consecutive days (days 25–27). Mice were treated daily 30 min before each OVA challenge with increasing doses of DMS, 200 µg/kg, $n = 15$; 400 µg/kg, $n = 15$; or PBS/ethanol as carrier control ($n = 14$). Naive mice ($n = 12$) were given PBS in place of OVA in both the i.n. sensitization and challenge stages. BAL cell counts were performed on day 28. Treatment with DMS resulted in a significant reduction in total cell count (A) and in eosinophilia (B), macrophage (C), and lymphocyte (D) numbers when compared with PBS controls. *, $p < 0.05$, by Mann-Whitney U test. Data are expressed as mean $\pm$ SEM.

clot, then was centrifuged, and aliquots of serum were stored at –70°C before analysis by ELISA for serum IgG.

Lung histology

After BAL sampling had been completed, the lungs were removed from the thoracic cavity by careful dissection. The lungs were inflated with 1 ml of 10% neutral-buffered Formalin and then fixed in 10% neutral-buffered Formalin for 72 h. After fixation, the left lung was dissected free and embedded in paraffin, and 6-µm sections were cut. Sections were then stained with H&E. Total lung inflammation was defined as the sum of the peribronchial plus perivascular scores. The severity of peribronchial and perivascular inflammation was graded semiquantitatively for the following features: 0, normal; 1, few cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells of >4 cells deep. Mucus production and goblet cell hyperplasia was examined by periodic acid-Schiff (PAS) staining as described previously (32). The numerical scores for the abundance of PAS-positive mucus-containing cell in each airway were determined as follows: 0, <0.5% PAS-positive cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, >75%.

Cell culture

Thoracic lymph nodes were removed from the lungs and passed through cell strainers (BD Biosciences) to prepare a single-cell suspension. Cells were cultured at 2×10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES buffer, and 10% heat-inactivated FCS (all from Invitrogen Life Technologies). Cells were cultured with 200 µg/ml OVA for 72 h, and the supernatants from parallel triplicate cultures were stored at –70°C until analysis of cytokine concentrations by ELISA. Proliferation assays were performed in triplicate in 96-well plates as described for 96 h and measured by Alamar Blue according to the manufacturer's recommendations (Serotec).

ELISA and Western blot analysis

Murine IL-4, IL-5, IFN- γ (BD Biosciences), and eotaxin (R&D Systems) in BAL fluid and culture supernatants were assayed by ELISA using paired Abs according to the manufacturer's instructions. The lower limit of detection for IL-4, IL-5, and eotaxin was 10 pg/ml, and that for IFN- γ was 40 pg/ml. Serum OVA-specific IgG1, IgG2a, and IgE titers were measured by ELISA as previously described (33), with modification of the dilution of

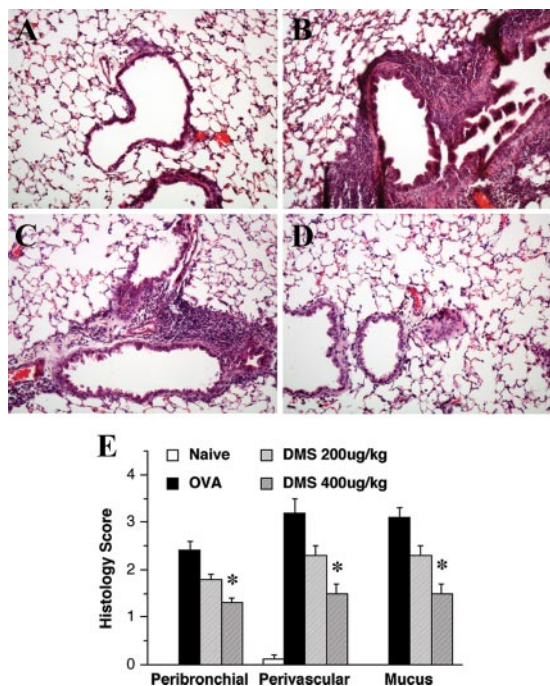


FIGURE 2. Histological evidence of decreased lung inflammation and mucus production in mice treated with DMS. Lung tissues were fixed 24 h after the last OVA challenge, sectioned, and stained with H&E for histological assessment or with PAS for mucus production. Original magnification is $\times 200$. A representative H&E section from each group of mice is shown. A, Naive mouse with PBS challenge. B, OVA-challenged mouse with peribronchial and perivascular inflammatory infiltrates, together with eosinophils and mucosal hyperplasia. C, OVA-challenged mouse treated with 200 μ g/kg DMS. D, OVA-challenged mouse treated with 400 μ g/kg DMS; a reduction in inflammation is seen compared with B. E, Histological appearances were scored for the presence of peribronchial and perivascular inflammation, mucus production, and goblet cell hyperplasia. Naive mice ($n = 7$); OVA PBS-treated mice ($n = 9$); DMS recipients, 200 μ g/kg ($n = 10$); and DMS recipients, 400 μ g/kg ($n = 10$) are shown. Data are mean $\pm$ SEM. *, $p < 0.05$ vs PBS, determined by Mann-Whitney U test.

sera as required. Western blot analysis for SphK1 and SphK2 levels was conducted in PBMC freshly isolated from mice treated with either SphK1 or scrambled control siRNA (5 μ g/animal/day). Mice were sacrificed 24 h after the last treatment and cell lysates were separated by 10% SDS-PAGE, transferred onto membrane, and probed with anti-SphK1, anti-SphK2, or control Abs as previously described (14).

Measurements of AHR

Mouse airway responsiveness to methacholine (Sigma-Aldrich) was measured using an invasive system previously described (34). Briefly, mice were anesthetized and tracheotomy was performed. The internal jugular vein was cannulated and connected to a microsyringe for i.v. methacholine administration. The mouse was then placed in a whole-body plethysmograph chamber (Buxco Electronics) and ventilated mechanically by a rodent ventilator (Hugo Sachs Elektronik; Harvard Apparatus) at a tidal volume of 200 μ l/ breath and a respiratory rate of 150/min. Airflow changes in the sealed chamber and pressure changes in the airway were analyzed by the BioSystem XA software (Buxco Electronics). The highest value of pulmonary resistance (R_L) and the lowest value of dynamic compliance (Cdyn) for each methacholine dose were chosen and expressed as a percentage of the respective basal values in response to PBS.

Statistical analysis

BAL total/differential cell counts and histological scores were analyzed with the nonparametric Mann-Whitney U test. Cytokine and OVA-specific Ab levels, and AHR response were compared by Student's t test.

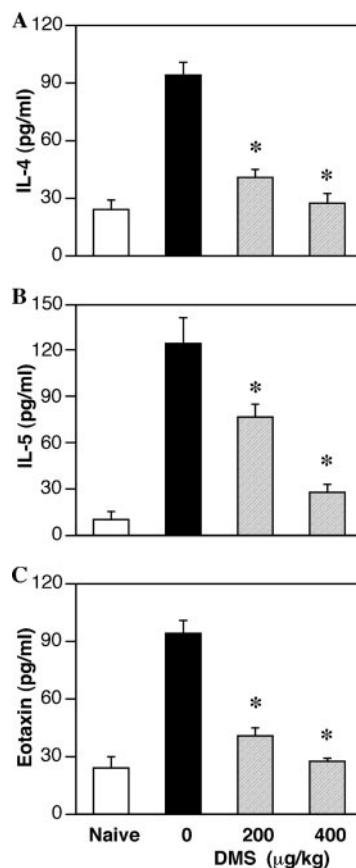


FIGURE 3. DMS reduces IL-4, IL-5, and eotaxin levels in BAL fluid. BAL fluids were collected 24 h after the last OVA challenge, and levels of IL-4 (A), IL-5 (B), and eotaxin (C) were determined by ELISA. Data are expressed as mean $\pm$ SEM of individual measurements ($n = 9$). *, $p < 0.05$ vs PBS, determined by Student's t test.

Results

Effects of DMS on OVA-induced airway inflammation

To determine the role of SphK in OVA-induced airway inflammation, mice were given DMS by i.p. injection 30 min before i.n. OVA challenges (days 25–27). DMS is a potent inhibitor of SphK and has been used in a number of in vitro and in vivo studies (8–13, 35). BAL was collected 24 h after the last OVA challenge, and total and differential cell counts were performed. The i.n. OVA challenge induced significant increase in total cell, eosinophil, macrophage, and lymphocyte counts ($p < 0.05$) as compared with saline control (Fig. 1). DMS at both 200 and 400 μ g/kg, produced a significant, dose-dependent reduction in BAL total cell count, and eosinophil and lymphocyte count (Fig. 1, A–C). DMS at 400 μ g/kg was also associated with a significant reduction in BAL lymphocyte count (Fig. 1D).

DMS suppresses inflammatory infiltration and mucus production

Lung tissue was removed 24 h after the last OVA challenge for histological examination. OVA challenged mice displayed extensive inflammatory infiltrates into both peribronchiolar and perivascular connective tissues as compared with infiltrates in saline challenged mice (Fig. 2, A and B). DMS (400 μ g/kg) markedly attenuated OVA-induced inflammatory infiltrates as compared with infiltrates found in PBS carrier control (Fig. 2D). DMS recipients at 200 μ g/kg were associated with a mild but nonsignificant reduction in inflammatory scores ($p > 0.05$) (Fig. 2C). Furthermore, by PAS staining, significant reduction in mucus

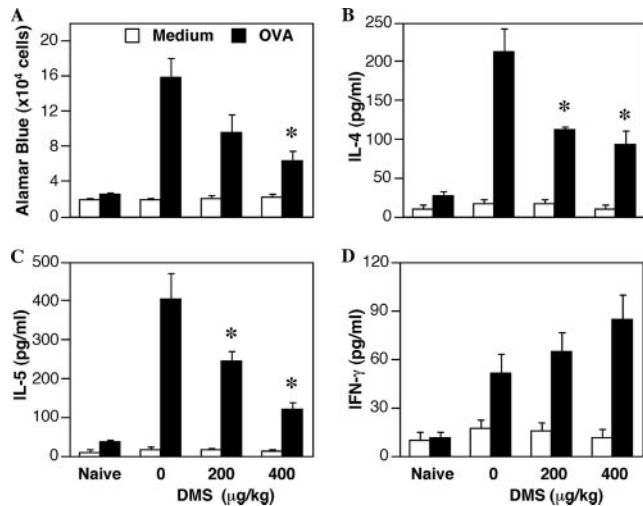


FIGURE 4. Reduced in vitro OVA-specific responses in mice treated with DMS. Thoracic lymph node cells ($n = 5$ mice/group) were harvested from mice on day 28 and cultured for up to 96 h with medium alone or OVA (200 $\mu\text{g/ml}$). A, T cell proliferation was assayed by Alamar Blue after 96 h. Cytokine concentrations of IL-4 (B), IL-5 at 72 h (C), and IFN- γ at 96 h (D) in culture supernatant were determined by ELISA. Significant suppression in OVA-induced cytokine production and proliferation was observed in lymph node cultures removed from DMS-treated mice compared with PBS carrier controls. *, $p < 0.05$, determined by Student's t test. Data are expressed as mean $\pm$ SD of triplicate cultures of pooled lymph node cell suspensions.

production and goblet hyperplasia were evident in mice treated with DMS (400 $\mu\text{g/kg}$) than evidenced in OVA control mice (Fig. 2E).

DMS treatment reduces Th2 cytokine levels in BAL

To determine the levels of cytokines in vivo, BAL fluid samples were collected 24 h after the last OVA challenge. The i.p. administration of DMS produced a dose-dependent reduction in the levels of IL-4, IL-5, and eotaxin in BAL fluid when compared with PBS carrier control (Fig. 3). IFN- γ was not detectable. Furthermore, OVA-specific serum IgG1, IgG2a, and IgE were not significantly different (data not shown).

DMS suppresses OVA-specific responses in vitro

From these observations, it seemed likely that the polarity of OVA-specific responses had been modified by transient DMS treatment during the i.n. Ag exposure. We therefore examined the OVA-specific immune responses in thoracic lymph node cultures to assess whether DMS treatment directly influenced lymphocyte function. The OVA-specific proliferation and production of IL-4 and IL-5 were significantly reduced in DMS recipients, either 200 or 400 $\mu\text{g/kg}$ i.p., in a dose-dependent manner (Fig. 4, A–C). In addition, OVA-specific IFN- γ was found to be slightly elevated in mice treated with 400 $\mu\text{g/kg}$ DMS (Fig. 4D). Finally, immune modulation by DMS in vivo was Ag-specific because Con A-induced production of IL-4, IL-5, and IFN- γ in parallel cultures was not affected (data not shown).

DMS treatment reduces lung resistance in vivo

We next investigated the effect of DMS on the development of AHR in response to increasing concentrations of methacholine. Sensitized BALB/c mice challenged with 1% OVA aerosol for 20 min daily for 3 consecutive days developed AHR to methacholine. Airway responsiveness was determined by pulmonary resistance

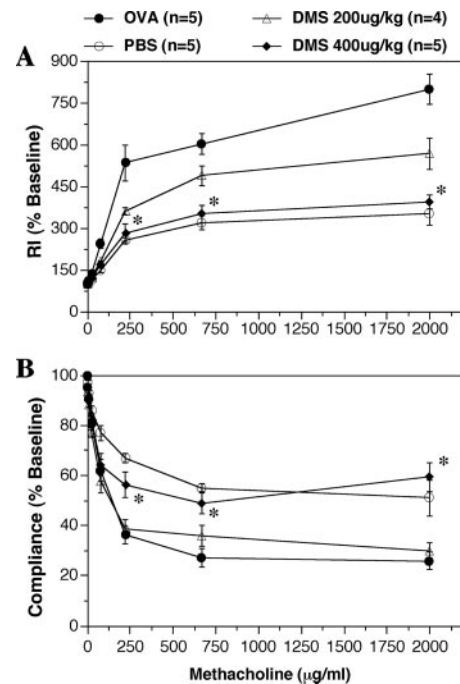


FIGURE 5. DMS dose-dependently reduces airway hyperreactivity in OVA-challenged mice. Airway responsiveness of mechanically ventilated mice in response to i.v. methacholine was measured 24 h after last OVA challenge with pretreatment of PBS ($n = 5$ mice), 200 $\mu\text{g/kg}$ ($n = 4$ mice), 400 $\mu\text{g/kg}$ ($n = 5$ mice), or naive PBS controls ($n = 5$ mice) as described in *Materials and Methods*. AHR is expressed as a percentage of change from baseline level of lung resistance (R_L) (A) and dynamic compliance (C_{dyn}) (B). Results for baseline R_L and C_{dyn} are 2.183 cm $\text{H}_2\text{O/ml/second}$ and 0.023 ml/cm H_2O , respectively. Data are expressed as mean $\pm$ SEM of individual measurements. *, $p < 0.05$ vs PBS, determined by Student's t test.

(R_L) and compliance (C_{dyn}). R_L is defined as the pressure driving respiration divided by flow. C_{dyn} refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. OVA-challenged mice developed AHR that was typically reflected by high R_L and low C_{dyn} (Fig. 5). DMS significantly suppressed methacholine-induced AHR in a dose-dependent manner, with 400 $\mu\text{g/kg}$ DMS exhibiting a greater reduction in R_L and increase in C_{dyn} , suggesting OVA-mediated pathology in vivo was modified (Fig. 5).

Treatment with SphK1 siRNA suppresses eosinophilic airway inflammation

Further experiments were to verify that the inhibitory effects of DMS from these observations were mediated by direct SphK inhibition rather than nonspecific off-target side effects (36), and to determine whether SphK1 inhibition could influence OVA-induced airway inflammation. BALB/c mice were given i.n. 200 $\mu\text{g/kg}$ SphK1 siRNA or scrambled siRNA as control on days 21 and 23, and subsequently 1 h before each i.n. OVA challenge (days 25–27). In accordance to the previous DMS results, SphK1 siRNA-treated animals exhibited a significant reduction in BAL total cell count and eosinophilia (Fig. 6, A and B) as compared with counts in control mice that received scrambled siRNA. In addition, a moderate but nonsignificant reduction in macrophage and lymphocyte counts was observed ($p > 0.05$) in SphK1 siRNA-treated mice (Fig. 6, C and D). Finally, to confirm the specificity of the observed inhibition, we examined the protein expression of SphK1 and SphK2 in both lung lysates and PBMCs pre- and posttreatment. Fig. 6E shows SphK1 protein expression in the lung lysates

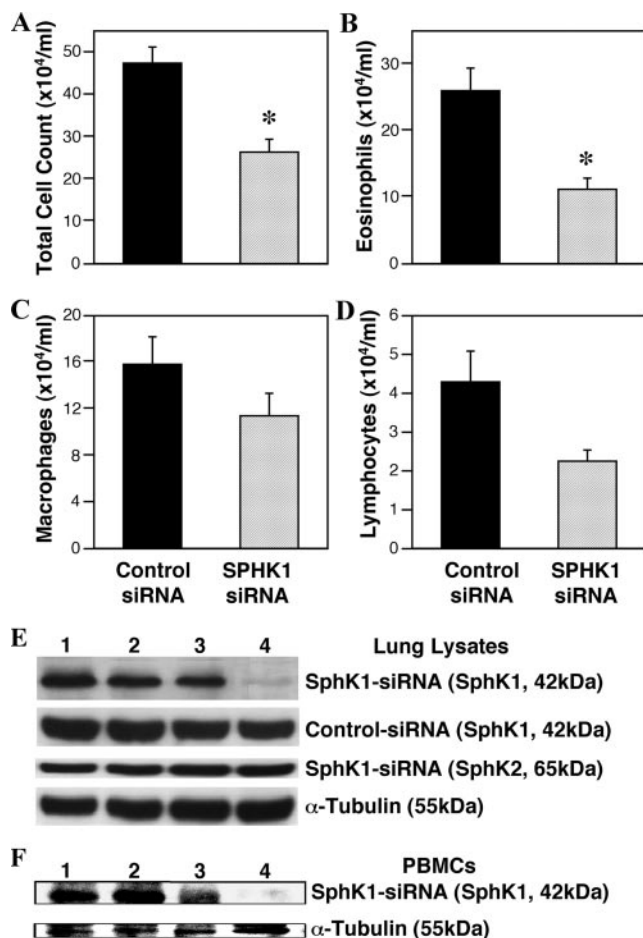


FIGURE 6. Total BAL cellularity and differential cell count after treatment with SphK1 siRNA. BALB/c mice were sensitized with OVA and then challenged with i.n. OVA as described in *Materials and Methods*. Mice were treated i.n. on days 21 and 23 and subsequently 1 h before each OVA challenge on days 25–27 with 200 $\mu\text{g/kg}$ SphK1 siRNA ($n = 5$ mice) or scramble control ($n = 5$ mice). BAL cell counts were performed on day 28. Treatment with SphK1 siRNA resulted in a significant reduction in total cell count (A) and eosinophilia (B) compared with treatment with control siRNA. There was no significant difference in macrophage (C) or lymphocyte (D) numbers. *, $p < 0.05$, determined by Mann-Whitney U test. Data are expressed as mean $\pm$ SEM. Western blot analysis of SphK1 expression in lung lysates (E) and PBMCs (F) of BALB/c mice before and after siRNA treatment. Untreated control (lane 1), daily 200 $\mu\text{g/kg}$ SphK1 siRNA treatment for 1 day (lane 2) or 2 days (lane 3), and three consecutive daily treatments (lane 4) are represented. Equal loading of proteins was confirmed using α -tubulin as an internal control.

of BALB/c mice was effectively blocked by three-daily administration of SphK1 siRNA, whereas SphK2 remains unaffected, suggesting such inhibition is dependent of SphK1 rather than SphK2. Similarly, SphK1 protein expression in PBMCs of these animals was effectively inhibited by SphK1 siRNA treatment (Fig. 6F). Equal loadings of proteins were confirmed using α -tubulin as an internal control.

SphK1 siRNA treatment reduces Th2 cytokine levels in BAL and serum IgE levels

Finally, we sought evidence whether targeting SphK1 with specific siRNA could modify OVA-specific response in vivo. BAL fluid and serum samples were collected 24 h after the last OVA challenge. The i.n. administration of SphK1 siRNA significantly reduced levels of IL-4, IL-5, and eotaxin in BAL fluid (Fig. 7, A–C)

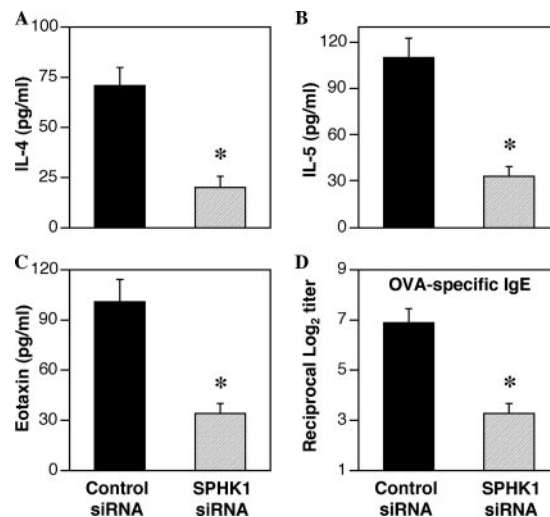


FIGURE 7. BAL, IL-4, IL-5, eotaxin, and serum OVA-specific IgE levels in mice treated with SphK1 siRNA. BAL fluids were collected 24 h after the last OVA challenge and levels of IL-4 (A), IL-5 (B), and eotaxin (C) as well as serum anti-OVA IgE Ab levels (D) were determined by ELISA. Data are expressed as mean $\pm$ SEM of individual measurements ($n = 5$ mice). *, $p < 0.05$ vs control siRNA treatment, as determined by Student's t test.

as compared with scrambled siRNA control. Moreover, serum levels of OVA-specific IgE were also significantly reduced in SphK1 siRNA-treated mice (Fig. 7D), whereas OVA-specific IgG1 and IgG2a were found to be similar (data not shown). These data together clearly demonstrate that SphK inhibition, either in the form of DMS administration or targeting of SphK1 via siRNA, can directly modulate the progression of airway inflammation.

Discussion

The prevalence of asthma is increasing in many developed nations. Consequently, there is an increased need for the development of new agents for the treatment of asthma, especially for patients who respond poorly to conventional therapy such as corticosteroids. Elevated levels of S1P in BAL fluid were recovered from allergic asthma patients after ragweed Ag challenge and may play a role in both acute bronchoconstriction and airway remodelling through its direct action on ASM cells (28, 29). In this study, we have demonstrated that blockade of SphK activity, either by DMS or by specific siRNA targeting of SphK1, can effectively suppress eosinophilic airway inflammation and Th2 cytokine and chemokine secretion, and markedly attenuate OVA-induced AHR in sensitized animals. These data indicate that SphK pathways may find therapeutic use in allergic asthma.

Sphingolipids have been identified as important signaling molecules in addition to their role as structural components of the membranes. In particular, sphingolipid metabolites such as S1P can promote monocytes and lymphocyte activation and migration (22–25, 31), and mast cells degranulation and chemotaxis (8, 9, 26, 31). Our data indicate that DMS has an inhibitory action on inflammatory cells infiltration into the lungs and mucus production, as revealed by histological examination, and by a significant drop in total cells and eosinophil counts in BAL fluid. This suppressive action of DMS on leukocyte migration is consistent with previous studies showing that SphK plays a role in chemotaxis of human peripheral blood neutrophils, macrophages, and eosinophils (12, 13, 27, 35). Inhibition of SphK may have a direct effect on cellular migratory machinery such as calcium mobilization and expression of adhesion molecules, including VCAM-1 and E-selectin (37).

Finally, indirect effect mediated through the suppression of chemokine production is also possible as reduced eotaxin BAL levels were observed in DMS-treated mice.

There is now clear evidence that Th2 cells play an important role in the pathogenesis of the allergic airway inflammation (15, 18). Eosinophil transmigration into the airways is a multistep process that is regulated by Th2 cytokines such as IL-4, IL-5, and IL-13, as well as specific chemokines like eotaxin and adhesion molecules such as VCAM-1 and selectins (38). Our present data show that the anti-inflammatory effect of DMS is at least in part mediated through a suppressive action on T lymphocytes, as OVA-specific cell proliferation, IL-4, and IL-5 productions were reduced in thoracic lymph node cultures from DMS-treated mice. A reduction in BAL fluid, IL-4, and IL-5 levels was also observed in these mice. Finally, the reduction in Th2 cytokine production in the thoracic lymph node cultures was accompanied by a slight increase in the production of IFN- γ .

In addition, the anti-inflammatory effects of SphK blockade may extend to resident ASM cells. Our results indicate that DMS significantly suppressed OVA-induced AHR to methacholine in a dose-dependent manner. SIP has been identified as an important factor in orchestrating both the acute asthmatic bronchoconstriction and the chronic features of airway remodeling (28, 29, 30). Using human ASM cells embedded in collagen matrices, Rosenfeldt et al. (29) have shown that SIP induced formation of stress fibers, contraction of individual human ASM cells, and stimulated myosin L chain phosphorylation in a Rho kinase-dependent manner. Thus, the suppression of AHR by DMS could in part due to the direct inhibition of ASM contraction. Moreover, IL-5 has been recognized to play an important role in AHR by recruiting and activating eosinophils, leading to release of proinflammatory products such as cysteinyl-leukotrienes and major basic protein, which are closely associated with AHR (39, 40). As a result, the observed alleviation of AHR may be also associated with the reduced tissue eosinophilia and Th2 cytokine levels via SphK pathway inhibition.

It is important to establish that the inhibitory effects of DMS from these observations were mediated by direct SphK inhibition rather than nonspecific off-target side effects (36), and to determine the efficacy of SphK1 in murine asthma using a highly specific siRNA. Our data showed that i.n. administration of SphK1 siRNA substantially reduced eosinophilic infiltration into the lungs of the asthmatic mice. Reduced levels of IL-4, IL-5, and eotaxin were also observed in the BAL fluid of SphK1 siRNA-treated animals. IgE-mediated mast cell activation and degranulation is a hallmark of allergic inflammation. Our data showed that serum levels of OVA-specific IgE were reduced by SphK1 siRNA. SIP is known to play a pivotal role in the regulation of lymphocyte emigration from organized lymphoid tissues such as lymph nodes and thymus. It is of interest that FTY720 phosphate, which binds to SIP receptors, caused the rapid disappearance of peritoneal B cells by inhibiting both their emigration from parathymic lymph nodes, and reduced peritoneal B cell-derived intestinal secretory IgA production (41). This finding suggests SIP may play an important role in regulating B cell trafficking and Ab production.

In conclusion, we demonstrate in this study inhibition of SphK is effective in reducing pulmonary inflammation and eosinophilia, AHR, BAL eotaxin, IL-4, IL-5, and serum OVA-specific IgE levels as well as in vitro Ag-specific inflammatory responses in a murine model of allergic asthma. This immunomodulatory effect is likely to occur through several different mechanisms. Elevated levels of SIP have been found in BAL fluid of patients with asthma and is regulated by the enzyme SphK. Recent data have suggested that SIP is involved in regulating ASM contraction, and inflammatory cells activity including mast cells, eosinophils, and T cells.

Taken together, these findings suggest SphK pathway may represent a novel target in the treatment of asthma.

Disclosures

The authors have no financial conflict of interest.

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