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

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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 (SIP), have emerged as a new class of potent bioactive messengers involved in an array of cellular processes, including cell differentiation, proliferation, and apoptosis. Recently, interest in SIP 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 SIP 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 SIP1-5 receptors.

As an intracellular second messenger, SIP 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 iMLP receptor (11), the C5a receptor (12, 13), and TNF-α receptor (14), leads to rapid increase in intracellular SIP 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.

SIP promotes monocytes and lymphocyte activation and migration (22–25), and mast cell degranulation and chemotaxis (8, 9, 26). Local administration of SIP causes inflammation coupled
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 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’-GGCAGAGGCUCCAGCUC-gdTdT-3’; antisense 3’-GAGCUGCAGAGCCUUGCC-dTdT-5’; SphK2, sense 5’-GACCUUAGGACUGCAU-dTdT-3’; antisense 3’-AUUGCGGACUAAUGGAG-dTdT-5’. 

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% Imject 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 by counting (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 ± SEM.

Cell culture

Thoracic lymph nodes 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%.

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 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 ± SEM.

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

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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 μg/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 μl/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 (Rl) 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.

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 μg/kg, produced a significant, dose-dependent reduction in BAL total cell count, and eosinophil and lymphocyte count (Fig. 1, A–C). DMS at 400 μg/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 μg/kg) markedly attenuated OVA-induced inflammatory infiltrates as compared with infiltrates found in PBS carrier control (Fig. 2D). DMS recipients at 200 μg/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 production was observed.
production and goblet hyperplasia were evident in mice treated with DMS (400 \mu 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 controls. *p < 0.05, determined by Student’s t test. Data are expressed as mean ± SD of triplicate cultures of pooled lymph node cell suspensions.

**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 g/kg i.p., in a dose-dependent manner (Fig. 4, A–C). In addition, OVA-specific IFN-\(\gamma\) was found to be slightly elevated in mice treated with 400 \mu 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-\(\gamma\) 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 (\(R_l\)) 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 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 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.
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) 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 IgG2 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. S1P 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 S1P 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. S1P 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 S1P 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 S1P 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 cell 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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