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A specific sphingosine kinase 1 inhibitor attenuates airway hyperresponsiveness and inflammation in a mast cell-dependent mouse model of allergic asthma

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Abstract

Background—Sphingosine-1-phosphate (S1P) produced by two sphingosine kinase isoenzymes, SphK1 and SphK2, has been implicated in IgE-mediated mast cell responses. However, studies of allergic inflammation in isotype-specific SphK knockout mice have not clarified their contribution and the role that S1P plays *in vivo* in a mast cell and IgE-dependent mouse model of allergic asthma has not yet been examined.

Objective—We used an isoenzyme-specific SphK1 inhibitor, SK1-I, to investigate the contributions of S1P and SphK1 to mast cell dependent airway hyperresponsiveness (AHR) and airway inflammation in mice.

Methods—Allergic airway inflammation and AHR were examined in a mast cell-dependent mouse model of ovalbumin (OVA)-induced asthma. C57BL/6 mice received intranasal delivery of SK1-I prior to sensitization and challenge with OVA or only prior to challenge.

Results—SK1-I inhibited antigen-dependent activation of human and murine mast cells and suppressed activation of NF- κ B, a master transcription factor that regulates expression of pro-inflammatory cytokines. SK1-I treatment of mice sensitized to OVA in the absence of adjuvant, which develop mast cell-dependent allergic inflammation, significantly reduced OVA-induced AHR to methacholine; decreased numbers of eosinophils and levels of the cytokines IL-4, 5, 6, 13, IFN- γ , and TNF- α and the chemokines eotaxin, and CCL2 in bronchoalveolar lavage fluid; and decreased pulmonary inflammation as well as activation of NF- κ B in the lungs.

CONCLUSION—S1P and SphK1 play important roles in mast cell-dependent, OVA-induced allergic inflammation and AHR, in part by regulating the NF- κ B pathway.

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Keywords

sphingosine-1-phosphate; sphingosine kinase; mast cells; NF- κ B; airway hyperresponsiveness; asthma

INTRODUCTION

Allergic asthma is a complex disease characterized by airway inflammation and airway hyperresponsiveness (AHR) that is becoming increasingly widespread in developed nations ¹. Mast cells are key effector cells that are increased in airways of asthmatics and can contribute to multiple features of allergic inflammation by secreting a vast array of inflammatory mediators that exacerbate vasodilation and vascular permeability, airway smooth muscle contraction, mucus secretion, and immune cell recruitment ².

Sphingosine-1-phosphate (S1P) is a new addition to the growing list of inflammatory mediators secreted by activated mast cells that is now emerging as a regulator of multiple aspects of both innate and adaptive immunity ^{3, 4}. S1P aggravates antigen-induced airway inflammation in mice ⁵ and its levels are elevated in the bronchoalveolar lavage (BAL) fluid of allergen challenged patients with allergic asthma ⁶. The majority of actions of S1P in innate and adaptive immunity are mediated by five specific S1P receptors, denoted S1P₁₋₅ ⁴. However, recent studies demonstrated that S1P also has important intracellular actions required for activation of the transcription factor NF- κ B important in inflammatory and immune responses ^{7, 8}.

Crosslinking of the high affinity IgE receptor (Fc ϵ RI) on mast cells activates sphingosine kinase 1 (SphK1) ⁹⁻¹¹ and possibly also SphK2 ^{12, 13} leading to rapid increases in intracellular S1P and its subsequent secretion ^{10, 12}. Although it has long been recognized that SphKs are involved in mast cell activation ¹⁴, the importance of each of the SphK isoenzymes is still a matter of debate. Whereas silencing of SphK1 but not SphK2 impaired Fc ϵ RI-mediated mast cell activation ^{9-11, 15}, in sharp contrast, calcium influx, cytokine production, and degranulation were abrogated in mast cells derived from *Sphk2* and not from *Sphk1* knockout mice ¹³. Furthermore, studies of allergic responses in isotype-specific SphK knockout mice have also yielded conflicting results ¹⁶. In the present study, we utilized a mast cell- and IgE-dependent murine model of chronic asthma ^{17, 18} to investigate the role that SphK1 and S1P play *in vivo* in mast cell-mediated allergic responses.

METHODS

Human skin and murine bone marrow derived mast cells

Human skin mast cells and murine bone marrow derived mast cells (BMMC) were isolated and cultured as described ¹⁹ and were more than 95% pure. Human mast cells and BMMC were sensitized overnight with 1 μ g/ml or 0.5 μ g/ml dinitrophenyl (DNP)-specific mouse IgE produced as described previously ²⁰, washed to remove unbound IgE, and then stimulated with 30 or 20 ng/ml DNP-HSA (Ag), respectively ¹⁵. Degranulation was measured by β -hexosaminidase assays ¹⁵ or by histamine release determined by ELISA (Neogen Corporation, Lexington, KY). Cytokine and chemokine release were measured by ELISAs ¹⁵.

Mice

Female C57BL/6 mice and mast cell-deficient Kit^{W-sh/W-sh} mice on the C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME) and kept in the animal care facilities at Virginia Commonwealth University under standard temperature,

humidity, and timed light conditions, and were provided with mouse chow and water *ad libitum*. All experiments were performed in compliance with the “Guide for the Care and Use of Laboratory Animals” of the Institute of Laboratory Animal Resources, National Research Council, published by the National Academy Press (revised 1996), and with approval from the VCU institutional animal care and use committee.

Induction of allergic inflammation and AHR

Allergic airway inflammation and AHR were induced by repeated OVA immunization without alum, followed by challenge with OVA or PBS as previously described^{17, 21}, with some modifications. Briefly, eight-week old C57BL/6 mice were sensitized by intraperitoneal (i.p.) injection of 100 μ l PBS or OVA (50 μ g) on days 1, 3, 5, and 7. Mice were challenged by intranasal (i.n.) injection of 20 μ l PBS or OVA (200 μ g) on days 22, 25, and 28. Mice were assessed for airway hyperresponsiveness (AHR) and airway inflammation 24 hours after the last i.n. challenge. SK1-I (5 mg/kg in PBS) or vehicle (PBS) was administered i.n. 1 hour prior to OVA sensitization and challenge (SK1-I group 1) or prior to OVA challenge only (SK1-I group 2).

Measurement of airway reactivity

Mice were anesthetized and after tracheotomies, were ventilated and measurements of baseline lung function were made with the FlexiVent apparatus (Scireq, Montreal, QC, Canada) as described²². Mice were then exposed to aerosols containing acetyl- β -methylcholine chloride and resistance (R), compliance (C), Newtonian resistance (Rn), and tissue damping (TD) were measured using the FlexiVent software version 5.3 as described²².

BAL fluid collection

BAL fluid was collected by lavaging the lungs twice with PBS (0.75 ml). Cells and supernatants were collected by centrifugation and cells resuspended in 100 μ l PBS. Total cell numbers were determined, cytopsin specimens prepared and stained with Diff-Quik (Siemens Healthcare Diagnostics, Deerfield, IL), and proportions of different cell types quantified by counting of at least 150 cells per cytopsin.

Lung histology

Following lavage, lungs were inflated through the trachea, removed, and fixed in 10% neutral buffered formalin. The formalin-fixed tissues were embedded in paraffin and 5 μ m sections stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS). A Nikon ECLIPSE E800M microscope equipped with a Diagnostic Instruments Spot RT CCD camera was used to photograph the sections. Total lung inflammation was assessed as the severity of peribronchial and perivascular infiltration and was scored semi-quantitatively as described²³ for the following features: 0, normal; 1, few cells; 2, rings of inflammatory cells 1 cell layer deep; 3, rings of inflammatory cells 2–4 cells deep; 4, rings of inflammatory cells 4 cells deep.

Paraffin lung sections (5 μ m) were stained with 0.1% methylene blue and examined with a bright-field microscope. Non-degranulated mast cells contained characteristic dark blue-purple color granules and degranulated mast cells were identified by the appearance of large purple granules outside of mast cells with a lower content of condensed intracellular granules²⁴.

In some experiments, lung sections were stained with anti-phospho-p65 antibody (Ser276, Abcam, Cambridge, MA) and visualized with a Dako LSAB+ kit (Dako North America, Carpinteria, CA).

Cytokine and chemokine measurements

The following cytokines and chemokines were measured with a Bioplex Array Reader (LUMINEX 100, Bio-Rad, Hercules, CA) using a custom mouse Bioplex panel (Bio-Rad): IL-4, IL-5, IL-6, IL-13, eotaxin, IFN- γ , CCL2/MCP-1, and TNF- α .

Mucin secretion

Mucin in BAL fluid was measured using a previously described ELISA with mouse Muc5AC-specific antibody (Pierce, Rockford, IL) ²⁵.

Mass spectrometry

Lipids were extracted from lung tissues and serum, and S1P, dihydro-S1P, and SK1-I were measured by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS; 4000 QTRAP; AB Sciex, Foster City, CA), as described previously ²⁶.

Statistical analysis

Statistical significance was determined with the Student's t test for unpaired samples. In vitro experiments were repeated at least three times in triplicate with consistent results. In vivo experiments were repeated four times and each experimental group consisted of at least seven mice.

RESULTS

A specific SphK1 inhibitor attenuates activation of human mast cells

Although many studies using siRNA to downregulate SphK1 indicate that S1P formed by its activation is pivotal in IgE-mediated mast cell degranulation and secretion of pro-inflammatory cytokines ^{9–11, 15}, others using mast cells derived from knockout mice concluded that SphK2 rather than SphK1 is indispensable for these mast cell functions ¹³. To clarify this controversy, we examined the effect of a specific SphK1 inhibitor, SK1-I, which does not affect SphK2 activity ²⁷, on mast cell functions. SK1-I drastically inhibited degranulation of human skin-derived mast cells triggered by Fc ϵ R1 crosslinking with antigen in a dose-dependent manner (Fig. 1A). These concentrations of SK1-I had no effect on mast cell viability (Fig. 1B) or degranulation induced by ionomycin (Fig. 1A). In agreement with our previous results ¹⁵, S1P potently induced degranulation of human mast cells, which as expected, was not altered by inhibition of SphK1 (Fig. 1A). In addition, SK1-I significantly reduced Ag-induced secretion of the cytokines, TNF- α (Fig. 1C) and IL-6 (Fig. 1D), whereas the secretion of these cytokines in response to S1P or ionomycin was unaffected. Similarly, SK1-I only reduced Ag-induced but not S1P- or ionomycin-induced secretion of CCL2/MCP-1 (Fig. 1E), an important chemokine that plays a major role in allergic asthma ²⁸.

Inhibition of SphK1 in murine mast cells reduces IgE-mediated degranulation, cytokine release, and NF- κ B activation

We also investigated the effects of inhibition of SphK1 on functions of murine bone marrow-derived mast cells. Similar to human mast cells, treatment of murine mast cells with SK1-I greatly reduced their degranulation (Fig. 2A) without affecting viability (Fig. 2B). Moreover, SK1-I also significantly reduced secretion of the cytokines TNF- α (Fig. 2C) and IL-6 (Fig. 2D), and IL-13 (Fig. 2E) and the chemokine MIP-1 α (Fig. 2F). Altogether, these data substantiate that SphK1 is generally important for mast cell functions.

We recently showed that S1P formed by SphK1 plays a critical role in TNF- α -induced activation of the master transcription factor NF- κ B which regulates expression of many

important pro-inflammatory cytokines⁷. As IL-6 and TNF- α production in mast cells is dependent on NF- κ B^{29,30}, it was of interest to determine whether the inhibitory effect of SKI-1 on production of these cytokines was related to NF- κ B activation. To test this, we analyzed phosphorylation of the inhibitor of NF- κ B (I κ B α), as the common pathway leading to NF- κ B activation requires its phosphorylation by phosphorylated I κ B kinase (IKK) and degradation. This is a key step in release of NF- κ B subunits (p50, p65), which then translocate from the cytosol to the nucleus and initiate cytokine gene transcription. Treatment of mast cells with SKI-1 greatly diminished phosphorylation of IKK and I κ B α triggered in response to Fc ϵ RI ligation, without affecting ERK activation (Fig. 2G), suggesting that SphK1 is also important for NF- κ B activation downstream of Fc ϵ RI crosslinking.

SKI-1 treatment reduces development of mast cell dependent airway hyperresponsiveness to methacholine

Having established that SKI-1 inhibits *in vitro* mast cell activation, it was next important to examine the effects of SphK1 inhibition on mast cell functions and allergic responses *in vivo*. Previous studies of the role of SphK1 in mouse models of allergic responses all utilized ovalbumin (OVA) antigen sensitization with alum as an adjuvant^{23,31,32}. However, mast cells are not essential for the development of allergic airway inflammation with this type of protocol^{17,21,33}. Therefore, we examined the effects of SKI-1 in the development of antigen-induced AHR in mice after OVA sensitization without alum, a chronic allergic asthma model that is significantly mast cell-dependent^{17,21}. In this protocol, mice were sensitized with OVA i.p. and challenged with OVA i.n. as shown in Fig. 3A. AHR to methacholine was significantly increased in OVA challenged mice compared to mice challenged with PBS only (Fig. 3B). In agreement with previous studies^{17,21}, mast cell-deficient Kit^{W-sh/W-sh} mice exhibited much weaker bronchial hyperreactivity responses to aerosolized methacholine, compared to congenic controls (see this article's Fig E1A, in the Online Repository at www.jacionline.org). Because SKI-1 is water soluble, it was administered i.n. in PBS and had no effect on AHR to methacholine in unsensitized mice (Fig. 3B). However, administration of SKI-1 i.n. one hour prior to both sensitization and challenge (SKI-1 group 1), significantly reduced AHR to methacholine. Lung resistance (Fig. 3B), lung compliance, the ease with which lungs can be extended (Fig. 3C), Newtonian resistance, a measure of central airway resistance (Fig. 3D), and tissue damping (Fig. 3E) were all significantly attenuated compared to OVA-sensitized mice. Next it was of interest to examine whether later SKI-1 treatment only during the challenge phase (SKI-1 group 2) would be able to attenuate AHR induced by OVA. Interestingly, although this treatment significantly reduced lung resistance (Fig. 3B), Newtonian resistance (Fig. 3D), and tissue damping (Fig. 3E), lung compliance was significantly different in OVA-sensitized mice at lower but not higher doses of methacholine (Fig. 3C). These data suggest that SphK1 inhibition can attenuate development of AHR.

SKI-1 reduces cellular infiltration, goblet cell hyperplasia, and pulmonary eosinophilia

As expected, OVA challenged mice displayed extensive inflammatory infiltrates into both peribronchial and perivascular areas of the lung (Fig 4A), whereas cellular infiltrates were nearly absent in OVA challenged mast cell-deficient Kit^{W-sh/W-sh} mice (see this article's Fig E1B, in the Online Repository at www.jacionline.org) or in PBS challenged mice as evidenced by H&E staining (Fig. 4A), as well as by semi-quantitative inflammatory scoring (Fig. 4C). Interestingly, treatment with SKI-1 during sensitization and challenge markedly attenuated OVA-induced inflammatory infiltrates. Moreover, even treatment with SKI-1 only during challenge also significantly reduced infiltration of inflammatory cells (Fig. 4A). Similarly, increases in mucus production and goblet hyperplasia were evident by periodic acid-Schiff (PAS) staining only in OVA challenged mice, which was greatly reduced by

SK1-I treatment throughout and to a lesser but significant extent in mice treated only during the challenge and fewer of the smaller bronchioles were positively stained (Fig. 4B). Mucin released into bronchial lavage fluid was also increased by OVA challenge and decreased by SK1-I treatments (Fig. 4D), as measured by a mucin-specific ELISA²⁵. OVA challenge also induced significant increases in the numbers of lung mast cells (Fig. 4E), the majority of which were degranulated (Fig. 4F). Both mast cell numbers and their degranulation was greatly reduced by treatments with SK1-I (Fig. 4E,F).

Consistent with these histology findings and in agreement with a recent study³⁴, OVA-challenge significantly increased infiltration of inflammatory cells, especially eosinophils and neutrophils (Fig. 5). Treatment with SK1-I throughout sensitization and challenge drastically reduced eosinophilia, whereas treatment with SK1-I only during the challenge had no significant effect on eosinophil infiltration (Fig. 5). Taken together, these results indicate that SphK1 and S1P play an important role in progression of mast cell-dependent airway inflammation.

SK1-I reduces S1P levels in lung and in circulation in mast cell dependent airway inflammation

It has previously been shown that S1P levels are increased in BAL fluid of asthmatics after ragweed challenge⁶. Therefore, it was of interest to determine changes in S1P levels following OVA challenge and the effects of SphK1 inhibition. Indeed, levels of S1P were significantly increased in both lung and serum after OVA challenge (Fig. 6A,C), while dihydro-S1P, which is present at much lower levels, was elevated only in the serum (Fig. 6C). Consistent with its effect on lung inflammatory responses, administration of SK1-I during sensitization and challenge markedly reduced these elevations of S1P in both the lung and the circulation (Fig. 6A,C), whereas treatment with SK1-I only during the OVA challenge was less efficacious and only reduced S1P levels in the lung. Administration of SK1-I intranasally during both sensitization and challenge resulted in higher levels of SK1-I than when administered only during the challenge, not only in the lung (Fig. 6B) but also in the serum (Fig. 6D). Surprisingly, however, SK1-I levels in the lungs and serum of group 1 animals were greater than non- OVA treated mice even though the amount of SK1-I administered was identical, suggesting that either OVA administration or the inflammation itself increased retention or uptake of SK1-I (Fig. 6B, D).

Inhibition of SphK1 decreases NF- κ B activation in lungs of OVA challenged mice

As noted in Fig. 2, SphK1 is required for optimal NF- κ B activation and pro-inflammatory cytokine production upon Fc ϵ RI triggering of mast cells. Therefore, it was of interest to examine the status of NF- κ B activation *in vivo* in mast cell dependent allergic responses. The classical pathway of activation of NF- κ B involves physical dissociation of p65-p50 subunits from I κ B α and subsequent nuclear translocation. However, it is also well established that regulation of transcriptional activity of NF- κ B also requires phosphorylation of p65³⁵. For example, phosphorylation of Ser276 enhances its transactivation potential and DNA-binding activity and phosphorylation of Ser536 also enhances its transactivation potential and decreases affinity to I κ B α ³⁵. In agreement with previous studies³⁶, very little pulmonary staining of phospho-p65 (Ser276) was detected in unchallenged animals while staining was strikingly increased after OVA challenge (FIG 7A), particularly in airway epithelial cells and in the infiltrated inflammatory cells that were nearly absent in unchallenged mice treated with PBS or SK1-I (Fig. 7A). The increase of phospho-p65 staining was dramatically reduced in OVA challenged mice treated with SK1-I. Similarly, immunoblotting demonstrated that OVA challenge induced phosphorylation of p65 (serine 536), known to be important for its transcriptional activity, which was markedly decreased by SK1-I treatment (Fig. 7B).

Inhibition of SphK1 decreases cytokines and chemokines

Because inhibition of SphK1 has been shown to greatly reduce production of cytokines and chemokines secreted from activated mast cells^{10, 11, 15, 23, 31}, we next examined the effect of SK1-I administration on relevant chemokine and cytokine levels in the BAL fluid. In agreement with previous studies (reviewed in³⁷), cytokines including T_H2-type IL-4 and IL-13, which have been implicated in the induction of AHR associated with allergic inflammation in the lungs, IL-5 that contributes to eosinophilia, the chemokines eotaxin and CCL2 which are also involved in eosinophil and diverse types of inflammatory cell recruitment, respectively, were all significantly elevated in OVA challenged mice (Fig. 8). Although the chemokines were only significantly reduced by treatment with SK1-I throughout OVA sensitization and challenge (group 1), the increases in cytokines were greatly diminished in both SK1-I treatment groups. Similarly, levels of the NF- κ B regulated pleiotropic cytokines IL-6 and TNF- α , whose release from activated mast cells is dependent on SphK1^{11, 15, 23}, were elevated in the OVA sensitized mice and were also reduced by treatment with SK1-I. As was observed by others³⁴, levels of the T_H1-type cytokine IFN- γ were slightly but significantly elevated following OVA challenge and were reduced by treatment with SK1-I. Taken together, these data demonstrate that SphK1 is involved in the regulation of numerous cytokines and chemokines and thus helps to perpetuate pulmonary inflammation.

DISCUSSION

S1P has emerged as an important regulator of mast cell effector functions and pathogenesis of allergic disease^{4, 16}. S1P produced in IgE/Ag-stimulated mast cells is involved in their degranulation, cytokine and chemokine production, and chemotaxis. Here we have shown that specifically inhibiting SphK1 with SK1-I effectively attenuated degranulation of both human and murine mast cells and also inhibited secretion of cytokines and chemokines that contribute to the pathophysiology of allergic disease. Our results suggest that SphK1 is the key SphK isoenzyme involved in Fc ϵ RI mediated mast cell activation.

SphK1 but not SphK2 plays a critical role in IgE/Ag-induced degranulation, migration toward antigen, and CCL2 secretion from human mast cells, as determined by specifically down-regulating their expression¹⁵. Nevertheless, both isoenzymes were required for efficient TNF- α secretion¹⁵. Surprisingly however, BMMCs derived from SphK1 knockout mice had normal responses, whereas silencing SphK1 markedly impaired BMMC functions¹¹, leading to the suggestion that this is due to a compensation mechanism during development of mice with a deletion of this important gene¹¹.

TNF- α and IL-6 expression in response to Fc ϵ RI ligation is strictly dependent on NF- κ B³⁸. Indeed, we found that inhibition of SphK1 nearly abrogated Ag-induced phosphorylation of both IKK and I κ B α , key players in the NF- κ B pathway, and secretion of these pro-inflammatory cytokines, suggesting SphK1 is also important for NF- κ B activation downstream of Fc ϵ RI crosslinking. Similar effects were obtained by downregulating SphK1, but not SphK2, in BMMC¹¹.

S1P levels are elevated in human asthmatics⁶ and recent studies have implicated S1P and SphK1 in the pathogenesis of chronic asthma based on animal models of allergic airway inflammation^{5, 23, 31, 32, 39, 40}. In mice, the administration of S1P aggravates Ag-induced airway inflammation⁵ and bronchial hyperresponsiveness⁴⁰. Conversely, treatment of OVA challenged mice with a pan SphK inhibitor or with siRNA targeted to SphK1 reduced pulmonary infiltration of inflammatory cells, eosinophilia, cytokine and chemokine secretion, and AHR^{23, 39}. Similarly, SphK1 deficiency in mice decreased allergen-induced airway inflammation³¹. Moreover, treatment of OVA challenged mice with SKI-II, another

SphK1 inhibitor, ameliorated the development of bronchial smooth muscle hyperresponsiveness, yet had no effect on other features of airway inflammation³². Some of these apparent controversies regarding the roles of S1P and SphK1 in various features of mouse models of asthma probably reflect the pleiotropic actions of S1P in immune regulation and its diverse roles in many types of immune cells in addition to the regulation of the effector functions of mast cells. Furthermore, the majority of these reports utilized an allergic model with alum as an adjuvant, which can itself enhance T_H2 responses and thus mask important functions of mast cells⁴¹. Due to this deficiency, Galli and colleagues have developed a murine model of allergic asthma that is strictly mast cell-dependent^{17, 21} and displays many features of severe asthma in humans, including inflammatory responses in the lung and increases in mast cells, eosinophils, and neutrophils, and mucus production³⁴. Using this model of chronic asthma, we found that SphK1 inhibition suppressed development of AHR, chronic inflammation including infiltration of eosinophils, and airway epithelial goblet cell hyperplasia. Interestingly, the concentration of SK1-I in the lung was 10-fold higher than in the circulation when SK1-I was administered intra-nasally rather than systemically. Although several lines of evidence indicate that while mast cells are likely to be the major target of SK1-I in the asthma models, other cell types can also be affected by this inhibitor. First, we confirmed that in the mouse allergic asthma model that we used, the airway inflammation and responsiveness were mast cell dependent. Second, SK1-I inhibits activation of mast cells, the key effector cells in this model. Thirdly, there was a marked increase in mast cell numbers and their degranulation in the lungs of OVA sensitized and challenged mice, which was suppressed by intra-nasal treatment with SK1-I. This suppressive action of SK1-I is consistent with previous findings that SphK1 expression is enhanced by OVA challenge⁴², particularly around bronchial epithelial walls³⁹, and that S1P is important for recruitment of inflammatory cells, including mast cells, lymphocytes, and eosinophils to sites of inflammation (reviewed in^{3, 4}) and has also been implicated in production of MUC5AC and in goblet cell hyperplasia^{39, 43}. Interestingly, we found that SphK2 deleted mice have exacerbated inflammatory responses and exhibit much greater bronchial hyperreactivity responses to aerosolized methacholine due to compensatory increases in pulmonary expression of SphK1 and increased levels of S1P in lung and serum (data not shown). This agrees with the notion that exposure of mast cell progenitors to higher levels of S1P in the cellular environment can change the differentiated phenotype and enhance the responsiveness of mast cells¹⁶. The observation that deletion of SphK2 did not prevent the pathology in the mast cell-dependent model of allergic asthma further supports the importance of SphK1. Because S1P regulates a wide range of processes in many types of immune cells and SK1-I could affect other cell types in the lung in addition to mast cells, future studies with mast cell-specific SphK1 knockout mice will be needed to conclusively demonstrate the importance of S1P produced by mast cells to immune and allergic responses.

SK1-I also suppressed OVA-induced secretion of the T_H2 cytokines, IL-4, IL-5, and IL-13, and the chemokines, eotaxin and CCL2, which orchestrate the inflammatory response in chronic asthma. Furthermore, inhibition of SphK1 with SK1-I significantly suppressed the pro-inflammatory cytokines TNF- α and IL-6 that amplify inflammation, probably by inhibiting NF- κ B activation. In this regard, intracellular S1P produced by SphK1 has recently been implicated as a critical regulator of NF- κ B as a required cofactor for the K63-linked polyubiquitination of RIP1 by TRAF2⁷, a key step leading to activation of NF- κ B. NF- κ B may have an important role in asthma. Animals in which components of the NF- κ B pathway have been deleted or inactivated have reduced OVA-induced inflammation, AHR, and airway remodeling^{44, 45}. Human bronchial biopsies showed increased NF- κ B activation in asthmatic airway epithelial and inflammatory cells than control specimens⁴⁶. Because SK1-I markedly suppressed activation of NF- κ B in the lung and the NF- κ B-regulated cytokines, TNF- α and IL-6, it is possible that its profound attenuation of AHR and

inflammation in a mast-cell dependent model of allergic asthma are due to inhibition of this important inflammatory pathway.

Interestingly, SK1-I was effective even when only administered during the OVA challenge, which has therapeutic implications for treatment of allergic patients. It is likely that SK1-I was effective at reducing lung inflammation because it is relatively water-soluble and could be administered intranasally. This maintained SK1-I at a high enough concentration in the lungs to inhibit SphK1 and S1P production. Moreover, we demonstrated that inhalation of SK1-I not only prevented the OVA-induced increase in S1P levels in the lungs but also in the circulation. Therefore, modulating the production of S1P by specific targeting of SphK1 deserves consideration as a potential therapeutic approach to control chronic airway diseases and other mast cell-mediated allergic reactions.

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ABBREVIATIONS USED

Ag	antigen
AHR	airway hyperresponsiveness
BAL	bronchoalveolar lavage
C	compliance
FcεRI	high affinity receptor for IgE
i.n	intranasal
i.p	intraperitoneal
TD	tissue damping
IκBα	inhibitor of NF-κB
OVA	ovalbumin
R	lung resistance
R_n	Newtonian lung resistance
S1P	sphingosine-1-phosphate
SphK	sphingosine kinase

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Clinical implications

The findings that intranasal administration of the specific SphK1 inhibitor SK1-I reduced mast cell-dependent allergic inflammation and AHR associated with asthma support the therapeutic potential of SphK1 inhibitors for the treatment of allergic airway inflammation.

FIG. 1. Inhibition of SphK1 reduces activation of human mast cells

IgE sensitized skin-derived mast cells were treated without (open bars) or with SKI-1 (2.5 μ M, grey bars; 5 μ M, black bars) and then stimulated with vehicle, 30 ng/ml Ag (IgE/Ag), 100 nM SIP, or 1 μ M ionomycin. **A**, Degranulation was determined by β -hexosaminidase release and **B**, viability was determined by trypan blue exclusion after 24h. Secretion of **C**, TNF- α , **D**, IL-6, and **E**, CCL2 were measured by ELISA. Data are the means $\pm$ SD of triplicate determinations. * P <0.01. Similar results were obtained using cells from 2 different donors.

FIG. 2. Inhibition of SphK1 reduces murine mast cell activation

Sensitized BMMCs were treated without (open bars) or with SKI-1 (10 μ M, black bars) and then stimulated with vehicle (PBS) or with 20 ng/ml Ag (IgE/Ag). **A**, Secretion of histamine was measured by ELISA and **B**, viability was determined by trypan blue exclusion after 24h. Secretion of **C**, TNF- α , **D**, IL-6, **E**, IL-13, and **F**, MIP-1 α were measured by ELISA. Data are the means $\pm$ SD of triplicate determinations. * P <0.01. **G**, IgE-sensitized BMMCs were treated without or with Ag for 5 min and cell lysates were immunoblotted with antibodies against p-I κ B α , p-IKK, p-ERK1/2 and total ERK2 as a loading control.

FIG. 3. Effect of SK1-I on development of OVA-induced mast cell-dependent AHR

A, Mast cell and IgE dependent allergic asthma model. Mice were sensitized and challenged with OVA administered i.p. (50 µg) and i.n. (200 µg), respectively, on the indicated days. PBS or SK1-I (100 µg) were administered i.n. 1 hour prior to OVA sensitization and challenge (group 1) or only prior to the OVA challenge (group 2). Unsensitized mice received either PBS or SK1-I on days 1, 3, 5, 7, 22, 25, and 28. Airway responses to methacholine were measured with the Flexivent apparatus on day 29, 24 hours after the last i.n. OVA or PBS challenge. **B**, lung resistance, **C**, compliance, **D**, Newtonian resistance, and **E**, tissue damping. * $P < 0.05$, compared to OVA-challenged mice. Data are means $\pm$ SEM from at least 7 mice in each group and are shown as fold changes.

FIG. 4. Inhibition of SphK1 attenuates airway immune cell infiltration and mucus secretion
Mice were sensitized, challenged, and treated as described in Fig. 3. At day 29, lung sections were fixed and stained with **A**, hematoxylin and eosin (H&E), scale bar 100 μ m or **B**, periodic acid/alcian blue/Schiff (PAS) and photographed under light microscopy at 100x magnification. Scale bar 50 μ m. Prominent infiltrates of inflammatory cells are present in OVA-sensitized and challenged mice but not in SK1-I-treated mice. **C**, Peribronchial and perivascular inflammation were scored as described in Methods. **D**, Mucin in BAL fluid was measured by a mucin-specific ELISA. **E**, Total and **F**, degranulated mast cells in lung sections were determined as described in Methods. Data are means $\pm$ SEM. * $P < 0.05$, compared to OVA-challenged mice.

FIG. 5. SKI-1 treatment reduces airway eosinophilia

Mice were sensitized, challenged, and treated as described in Fig. 3. BAL fluid was collected on day 29 and the numbers of eosinophils, neutrophils, macrophages, and lymphocytes were determined. * $P < 0.05$, compared to OVA-challenged mice. Data are means $\pm$ SEM from at least 7 mice in each group.

FIG. 6. Effect of SK1-I on S1P levels in lung and serum

Mice were sensitized, challenged, and treated as described in Fig. 3. On day 29, lung **A, B**, and serum **C, D**, were collected and levels of S1P, dihydro-S1P (DHS1P), and SK1-I were determined by LC-ESI-MS/MS. † $P < 0.05$, compared to PBS-treated mice. * $P < 0.05$, compared to OVA-challenged mice.

FIG. 7. Inhibition of SphK1 attenuates activation of NF- κ B in the lungs of OVA challenge mice Mice were sensitized, challenged, and treated as described in Fig. 3. At day 29, lung sections were fixed and stained with anti-p65 (phospho-Ser276) antibody and photographed under light microscopy at 200x magnification. Scale bar 50 μ m. **B**, Lungs were homogenized and equal amounts of proteins were analyzed by immunoblotting with anti-p65 (phospho-Ser536) antibody. Blots were stripped and blotted with p65 antibody to demonstrate equal loading and transfer.

FIG. 8. SK1-I reduces OVA challenge induced pulmonary cytokines and chemokines

Mice were sensitized, challenged, and treated as described in Fig. 3. On day 29, BAL fluid was collected and levels of the indicated cytokines and chemokines were measured by Bioplex assay. Data are means $\pm$ SEM from at least 7 mice in each group. * $P < 0.05$, compared to OVA-challenged mice. † $P < 0.05$, compared to PBS treated mice.