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

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Background: Sphingosine-1-phosphate (S1P), which is produced by 2 sphingosine kinase (SphK) 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 murine 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 murine model of ovalbumin (OVA)–induced asthma. C57BL/6 mice received intranasal delivery of SK1-I before sensitization and challenge with OVA or only before challenge.
Results: SK1-I inhibited antigen-dependent activation of human and murine mast cells and suppressed activation of nuclear factor κB (NF-κB), a master transcription factor that regulates the expression of proinflammatory cytokines. SK1-I treatment of mice sensitized to OVA in the absence of adjuvant, in which mast cell–dependent allergic inflammation develops, significantly reduced OVA-induced AHR to methacholine; decreased numbers of eosinophils and levels of the cytokines IL-4, IL-5, IL-6, IL-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-κB 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-κB pathway. (J Allergy Clin Immunol 2013;131:501-11.)

Key words: *Sphingosine-1-phosphate, sphingosine kinase, mast cells, nuclear factor κB, airway hyperresponsiveness, asthma*

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 the numbers of which are increased in the airways of asthmatic patients, and they 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 increased 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 5 specific S1P receptors, denoted $S1P_{1\text{-}5}$.[4] However, recent studies demonstrated that S1P also has important intracellular actions required for activation of the transcription factor nuclear factor κB (NF-κB), which is important in inflammatory and immune responses.[7,8]

Cross-linking of FcεRI on mast cells activates sphingosine kinase (SphK) 1[9-11] and possibly also SphK2,[12,13] leading to rapid increases in levels of 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 FcεRI-mediated mast cell activation,[9-11,15] 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 used 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.

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Abbreviations used
AHR: Airway hyperresponsiveness
BAL: Bronchoalveolar lavage
BMMC: Bone marrow–derived mast cell
IκBα: Inhibitor of nuclear factor κB
IKK: IκB kinase
NF-κB: Nuclear factor κB
OVA: Ovalbumin
S1P: Sphingosine-1-phosphate
SphK: Sphingosine kinase

METHODS

Human skin and murine bone marrow–derived mast cells

Human skin mast cells and murine bone marrow–derived mast cells (BMMCs) were isolated and cultured, as previously described,[19] and were more than 95% pure. Human mast cells and BMMCs were sensitized overnight with 1 μg/mL or 0.5 μg/mL dinitrophenyl-specific murine IgE produced as described previously,[20] washed to remove unbound IgE, and then stimulated with 30 or 20 ng/mL dinitrophenyl-HSA (antigen), respectively.[15] Degranulation was measured by using β-hexosaminidase assays[15] or based on histamine release, as determined by means of ELISA (Neogen Corp, Lexington, Ky). Cytokine and chemokine release was measured by means of ELISA.[15]

Mice

Female C57BL/6 mice and mast cell–deficient $Kit^{W\text{-}sh/W\text{-}sh}$ 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 Virginia Commonwealth University's Institutional Animal Care and Use Committee.

Induction of allergic inflammation and AHR

Allergic airway inflammation and AHR were induced by repeated ovalbumin (OVA) immunization without alum, followed by challenge with OVA or PBS, as previously described,[17,21] with some modifications. Briefly, 8-week-old C57BL/6 mice were sensitized by intraperitoneal injection of 100 μL of PBS or OVA (50 μg) on days 1, 3, 5, and 7. Mice were challenged with intranasal injection of 20 μL of PBS or OVA (200 μg) on days 22, 25, and 28. Mice were assessed for AHR and airway inflammation 24 hours after the last intranasal challenge. SK1-I (5 mg/kg in PBS) or vehicle (PBS) was administered intranasally 1 hour before OVA sensitization and challenge (SK1-I group 1) or before OVA challenge only (SK1-I group 2).

Measurement of airway reactivity

Mice were anesthetized. After tracheotomy, the mice were ventilated and measurements of baseline lung function were made with the flexiVent apparatus (Scireq, Montreal, Quebec, Canada), as previously described.[22] Mice were then exposed to aerosols containing acetyl-β-methylcholine chloride, and resistance, compliance, Newtonian resistance, and tissue damping were measured with flexiVent software, version 5.3, as previously described.[22]

BAL fluid collection

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

Lung histology

After 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-μm sections were stained with hematoxylin and eosin or periodic acid–Schiff. A Nikon ECLIPSE E800M microscope (Nikon, Tokyo, Japan) equipped with a Diagnostic Instruments SPOT RT CCD camera (SPOT Imaging Solutions, Sterling Heights, Mich) was used to photograph the sections. Total lung inflammation was assessed as the severity of peribronchial and perivascular infiltration and was scored semiquantitatively, as previously described,[23] for the following features: 0, normal; 1, few cells; 2, rings of inflammatory cells 1 cell layer deep; 3, rings of inflammatory cells 2 to 4 cells deep; and 4, rings of inflammatory cells 4 or more cells deep.

Paraffin-embedded lung sections (5 μm) were stained with 0.1% methylene blue and examined with a bright-field microscope. Nondegranulated mast cells contained characteristic dark blue–purple 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 granules.[24]

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

Cytokine and chemokine measurements

The following cytokines and chemokines were measured with a Bio-Plex Array Reader (LUMINEX 100; Bio-Rad Laboratories, Hercules, Calif) with a custom murine Bio-Plex panel (Bio-Rad): IL-4, IL-5, IL-6, IL-13, eotaxin, IFN-γ, CCL2/monocyte chemoattractant protein 1, and TNF-α.

Mucin secretion

Mucin in BAL fluid was measured by using a previously described ELISA with murine Muc5AC-specific antibody (Pierce, Rockford, Ill).[25]

Mass spectrometry

Lipids were extracted from lung tissues and sera, and S1P, dihydro-S1P, and SK1-I were measured by using liquid chromatography–electrospray ionization–tandem mass spectrometry (4000 QTRAP; AB Sciex, Foster City, Calif), as described previously.[26]

Statistical analysis

Statistical significance was determined with the Student *t* test for unpaired samples. *In vitro* experiments were repeated at least 3 times in triplicate, with consistent results. *In vivo* experiments were repeated 4 times, and each experimental group consisted of at least 7 mice.

RESULTS

A specific SphK1 inhibitor attenuates activation of human mast cells

Although many studies using small interfering RNA to downregulate SphK1 indicate that S1P formed by its activation is pivotal in IgE-mediated mast cell degranulation and secretion of proinflammatory 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 FcεRI cross-linking with antigen in a

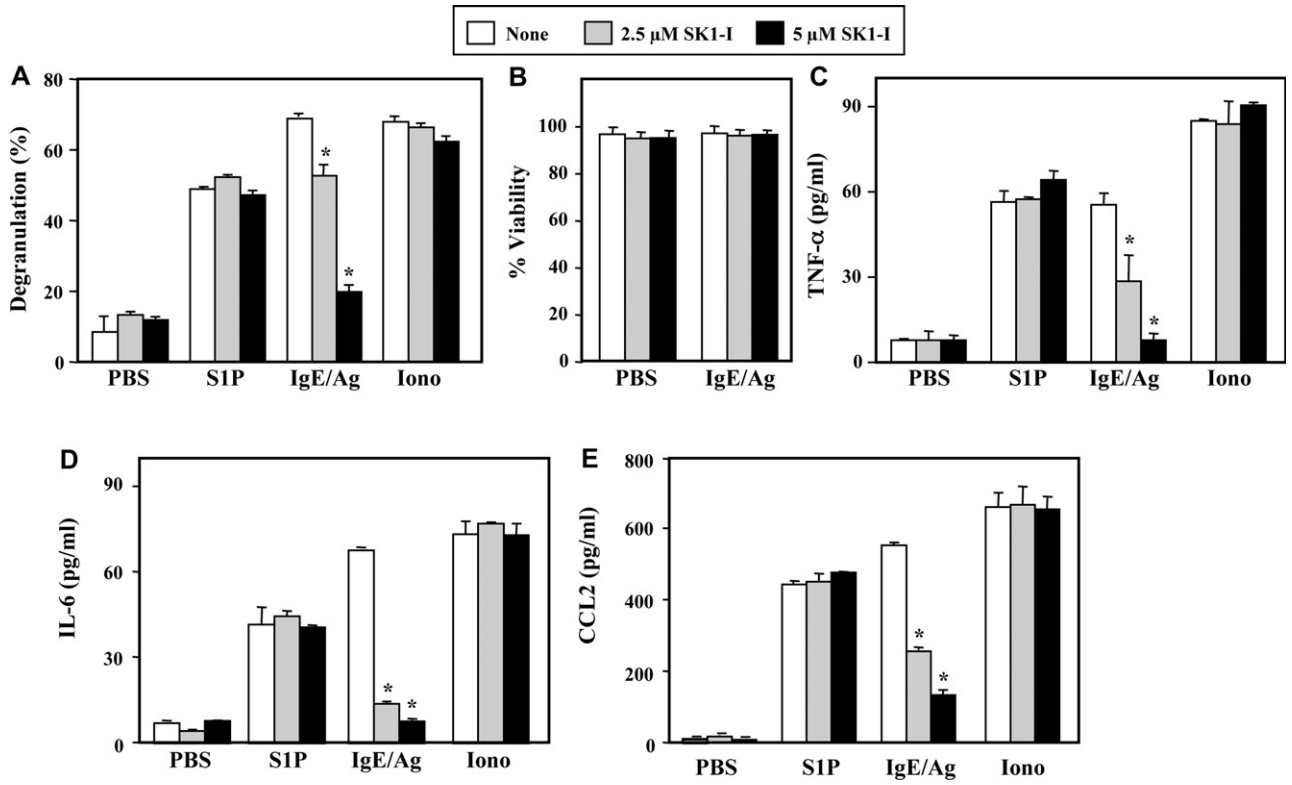

FIG 1. Inhibition of SphK1 reduces activation of human mast cells. IgE-sensitized skin-derived mast cells were treated without *(open bars)* or with (*gray bars*, 2.5 μmol/L; *black bars*, 5 μmol/L) SKI-1 and then stimulated with vehicle, 30 ng/mL antigen *(IgE/Ag)*, 100 nmol/L S1P, or 1 μmol/L ionomycin *(Iono)*. **A** and **B,** Degranulation was determined based on β-hexosaminidase release (Fig 1, *A*), and viability was determined by means of trypan blue exclusion after 24 hours (Fig 1, *B*). **C-E,** Secretion of TNF-α (Fig 1, *C*), IL-6 (Fig 1, *D*), and CCL2 (Fig 1, *E*) was measured by means of ELISA. Data are means ± SDs of triplicate determinations. **P* < .01. Similar results were obtained by using cells from 2 different donors.

dose-dependent manner (Fig 1, *A*). These concentrations of SK1-I had no effect on mast cell viability (Fig 1, *B*) or degranulation induced by ionomycin (Fig 1, *A*). 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 1, *A*). In addition, SK1-I significantly reduced antigen-induced secretion of the cytokines TNF-α (Fig 1, *C*) and IL-6 (Fig 1, *D*), whereas the secretion of these cytokines in response to S1P or ionomycin was unaffected. Similarly, SK1-I only reduced antigen-induced but not S1P- or ionomycin-induced secretion of CCL2/monocyte chemoattractant protein 1 (Fig 1, *E*), 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-κB activation

We also investigated the effects of inhibition of SphK1 on functions of murine BMMCs. Similar to human mast cells, treatment of murine mast cells with SK1-I greatly reduced their degranulation (Fig 2, *A*) without affecting viability (Fig 2, *B*). Moreover, SK1-I also significantly reduced secretion of the cytokines TNF-α (Fig 2, *C*), IL-6 (Fig 2, *D*), and IL-13 (Fig 2, *E*) and the chemokine macrophage inflammatory protein 1α (Fig 2, *F*). 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-κB, which regulates expression of many important proinflammatory cytokines.[7] Because IL-6 and TNF-α production in mast cells is dependent on NF-κB,[29,30] it was of interest to determine whether the inhibitory effect of SKI-1 on production of these cytokines was related to NF-κB activation. To test this, we analyzed phosphorylation of the inhibitor of NF-κB (IκBα) because the common pathway leading to NF-κB activation requires its phosphorylation by phosphorylated IκB kinase (IKK) and degradation. This is a key step in release of NF-κB subunits (p50 and p65), which then translocate from the cytosol to the nucleus and initiate cytokine gene transcription. Treatment of mast cells with SKI-1 greatly diminished the phosphorylation of IKK and IκBα triggered in response to FcεRI ligation without affecting extracellular signal–regulated kinase activation (Fig 2, *G*), suggesting that SphK1 is also important for NF-κB activation downstream of FcεRI cross-linking.

SK1-I treatment reduces development of mast cell–dependent AHR to methacholine

Having established that SK1-I 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 murine models of allergic responses all used OVA antigen sensitization with

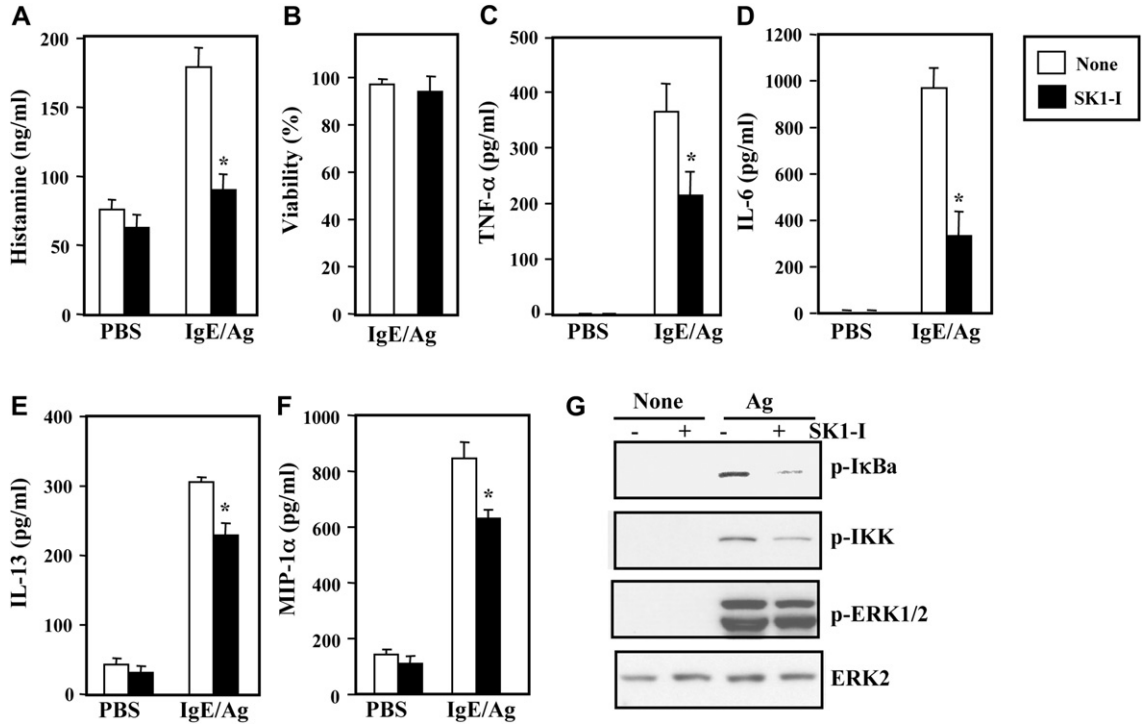

FIG 2. Inhibition of SphK1 reduces murine mast cell activation. Sensitized BMMCs were treated without *(open bars)* or with (*black bars*, 10 μmol/L) SKI-1 and then stimulated with vehicle (PBS) or with 20 ng/mL antigen *(IgE/Ag)*. **A** and **B,** Secretion of histamine was measured by means of ELISA (Fig 2, *A*), and viability was determined by using trypan blue exclusion after 24 hours (Fig 2, *B*). **C-F,** Secretion of TNF-α (Fig 2, *C*), IL-6 (Fig 2, *D*), IL-13 (Fig 2, *E*), and macrophage inflammatory protein 1α (*MIP-1α*; Fig 2, *F*) was measured by using ELISA. Data are means ± SDs of triplicate determinations. **P* < .01. **G,** IgE-sensitized BMMCs were treated without or with antigen for 5 minutes, and cell lysates were immunoblotted with antibodies against phosphorylated IκBα, IKK, and extracellular signal–regulated kinase *(ERK)* 1/2 and total ERK2 as a loading control.

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 SK1-I on 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 intraperitoneally and challenged with OVA intranasally, as shown in Fig 3, *A*. AHR to methacholine was significantly increased in OVA-challenged mice compared with that seen in mice challenged with PBS only (Fig 3, *B*). In agreement with previous studies,[17,21] mast cell–deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice exhibited much weaker bronchial hyperreactivity responses to aerosolized methacholine compared with congenic control animals (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). Because SK1-I is water soluble, it was administered intranasally in PBS and had no effect on AHR to methacholine in unsensitized mice (Fig 3, *B*). However, administration of SK1-I intranasally 1 hour before both sensitization and challenge (SK1-I group 1) significantly reduced AHR to methacholine. Lung resistance (Fig 3, *B*), lung compliance, the ease with which lungs can be extended (Fig 3, *C*), Newtonian resistance (a measure of central airway resistance; Fig 3, *D*), and tissue damping (Fig 3, *E*) were all significantly attenuated compared with values seen in OVA-sensitized mice. Next, it was of interest to examine whether later SK1-I treatment only during the challenge phase (SK1-I group 2) would be able to attenuate AHR induced by OVA. Interestingly, although this treatment significantly reduced lung resistance (Fig 3, *B*), Newtonian resistance (Fig 3, *D*), and tissue damping (Fig 3, *E*), lung compliance was significantly different in OVA-sensitized mice at lower but not higher doses of methacholine (Fig 3, *C*). These data suggest that SphK1 inhibition can attenuate the development of AHR.

SK1-I reduces cellular infiltration, goblet cell hyperplasia, and pulmonary eosinophilia

As expected, OVA-challenged mice displayed extensive inflammatory infiltrates into both the peribronchial and perivascular areas of the lung (Fig 4, *A*), whereas cellular infiltrates were nearly absent in OVA-challenged mast cell–deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice (see Fig E1, *B*, in this article's Online Repository) or in PBS-challenged mice, as evidenced by hematoxylin and eosin staining (Fig 4, *A*), as well as by means of semiquantitative inflammatory scoring (Fig 4, *C*). Interestingly, treatment with SK1-I during sensitization and challenge markedly attenuated OVA-induced inflammatory infiltrates. Moreover, even treatment with SK1-I only during challenge also significantly reduced infiltration of inflammatory cells (Fig 4, *A*). Similarly, increases in mucus production and goblet cell hyperplasia were evident by means of periodic acid–Schiff staining only in OVA-challenged mice, which was greatly reduced by SK1-I treatment throughout and to a lesser

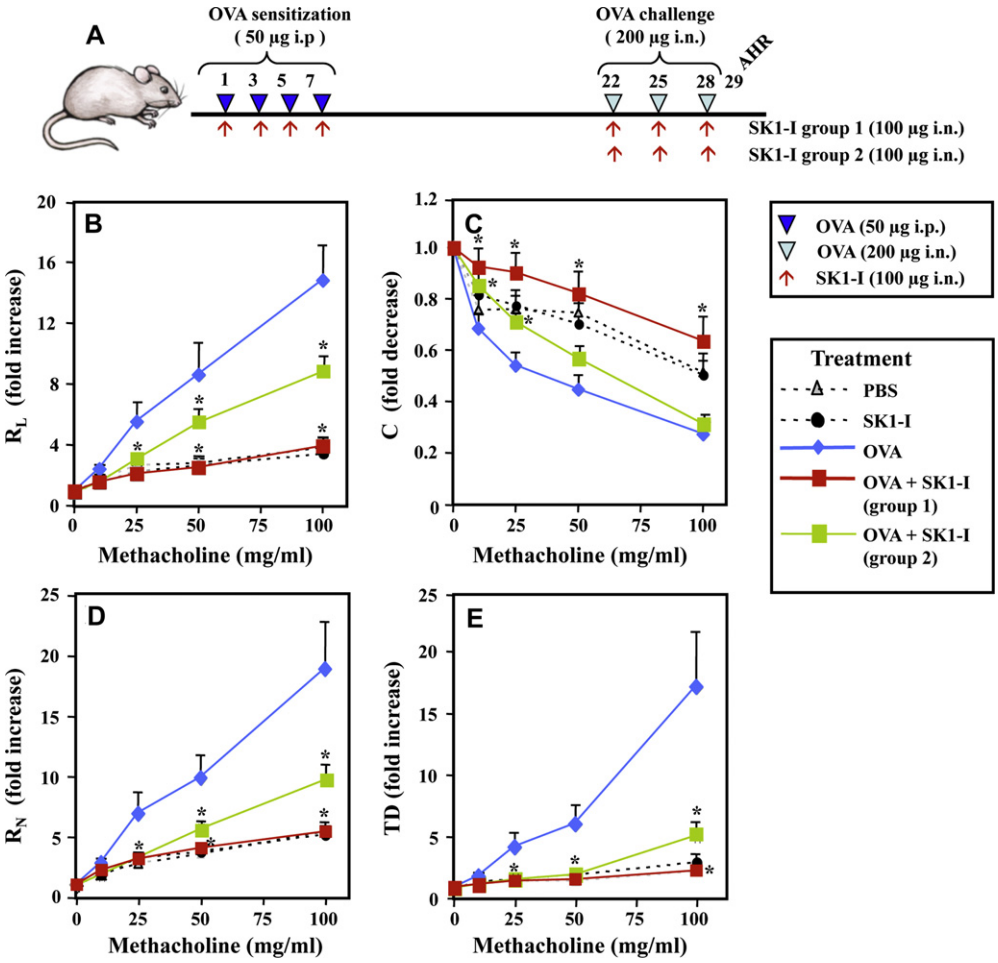

FIG 3. Effect of SK1-I on development of OVA-induced mast cell–dependent AHR. **A,** Mast cell– and IgE-dependent allergic asthma model. Mice were sensitized and challenged with OVA administered intraperitoneally (50 μg) or intranasally (200 μg), respectively, on the indicated days. PBS or SK1-I (100 μg) was administered intranasally 1 hour before OVA sensitization and challenge (group 1) or only before 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 intranasal OVA or PBS challenge. **B-E,** Lung resistance (R_L; Fig 3, *B*), compliance (*C*; Fig 3, *C*), Newtonian resistance (R_N; Fig 3, *D*), and tissue damping (*TD*; Fig 3, *E*). *$P < .05$ compared with OVA-challenged mice. Data are means ± SEMs from at least 7 mice in each group and are shown as fold changes.

but significant extent in mice treated only during the challenge, and fewer of the smaller bronchioles were positively stained (Fig 4, *B*). Mucin released into bronchial lavage fluid was also increased by means of OVA challenge and decreased by SK1-I treatments (Fig 4, *D*), as measured with a mucin-specific ELISA.[25] OVA challenge also induced significant increases in the numbers of lung mast cells (Fig 4, *E*), the majority of which were degranulated (Fig 4, *F*). Both mast cell numbers and their degranulation were greatly reduced by treatment with SK1-I (Fig 4, *E* and *F*).

Consistent with these histologic findings and in agreement with a recent study,[34] OVA challenge significantly increased the infiltration of inflammatory cells, especially eosinophils and neutrophils (Fig 5). Treatment with SK1-1 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 the progression of mast cell–dependent airway inflammation.

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

It has previously been shown that S1P levels are increased in the BAL fluid of asthmatic patients after ragweed challenge.[6] Therefore it was of interest to determine changes in S1P levels after OVA challenge and the effects of SphK1 inhibition. Indeed, levels of S1P were significantly increased in both lung tissues and sera after OVA challenge (Fig 6, *A* and *C*), whereas levels of dihydro-S1P, which is present at much lower levels, were increased only in the serum (Fig 6, *C*). Consistent with its effect on lung inflammatory responses, administration of SK1-I during sensitization and challenge markedly reduced these increases in S1P levels in both the lung and the circulation (Fig 6, *A* and *C*), whereas treatment with SK1-I only during the OVA challenge

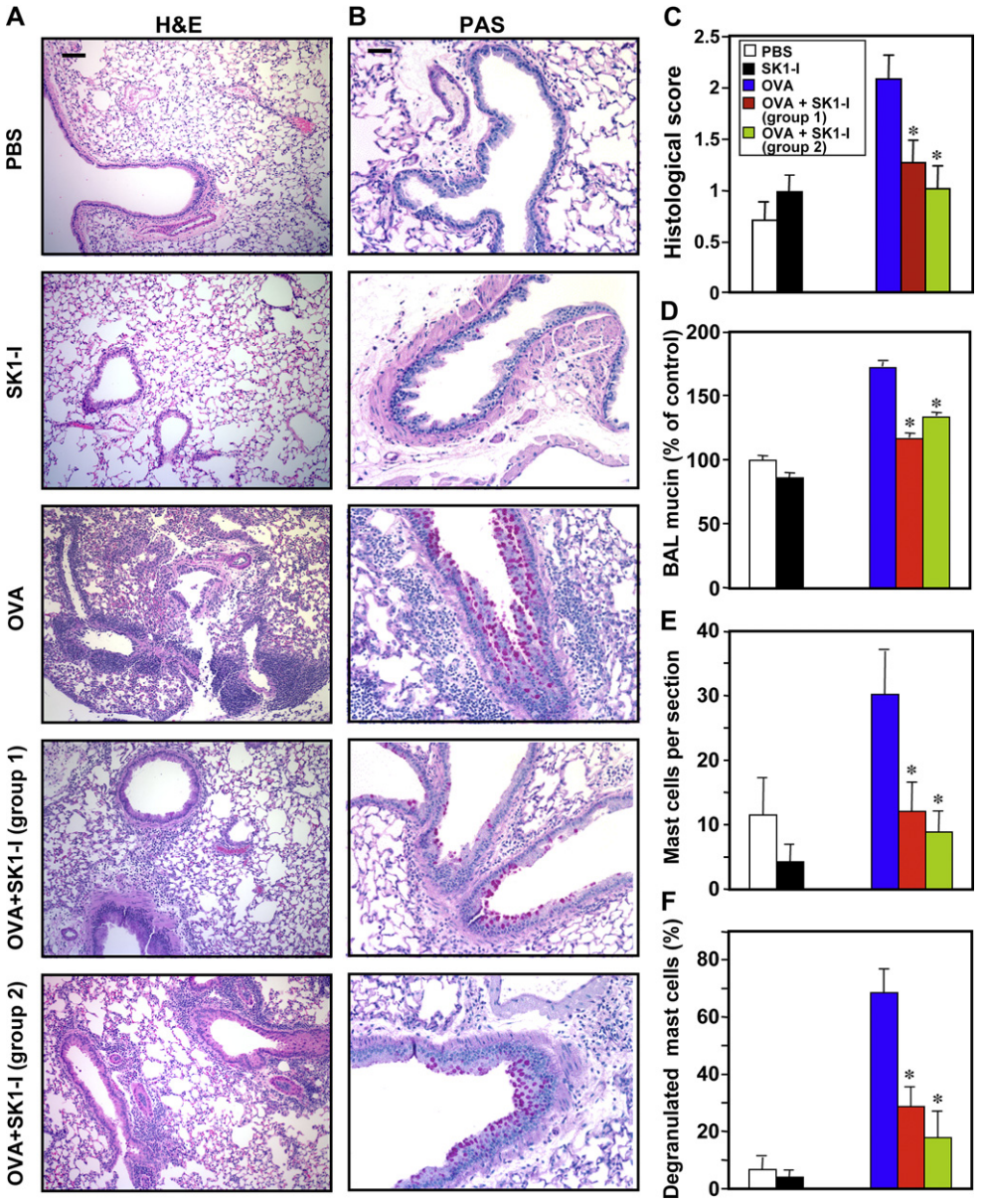

FIG 4. Inhibition of SphK1 attenuates airway immune cell infiltration and mucus secretion. Mice were sensitized, challenged, and treated as described in Fig 3. **A** and **B,** At day 29, lung sections were fixed and stained with hematoxylin and eosin (*H&E*; Fig 4, *A*; *scale bar* = 100 μm) or periodic acid/Alcian blue/Schiff (*PAS*; Fig 4, *B*) and photographed under light microscopy at ×100 magnification (*scale bar* = 50 μm). Prominent infiltrates of inflammatory cells are present in OVA-sensitized and OVA-challenged mice but not in SK1-I–treated mice. **C,** Peribronchial and perivascular inflammation were scored as described in the Methods section. **D,** Mucin levels in BAL fluid were measured by using a mucin-specific ELISA. **E** and **F,** Total (Fig 4, *E*) and degranulated (Fig 4, *F*) mast cells in lung sections were determined as described in the Methods section. Data are means ± SEMs. **P* < .05 compared with OVA-challenged mice.

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 6, *B*) but also in the serum (Fig 6, *D*). Surprisingly, however, SK1-I levels in the lungs and sera of group 1 animals were greater than those in 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 6, *B* and *D*).

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

As noted in Fig 2, SphK1 is required for optimal NF-κB activation and proinflammatory cytokine production on FcεRI triggering of mast cells. Therefore it was of interest to examine the status of NF-κB activation *in vivo* in mast cell–dependent allergic responses. The classical pathway of activation of NF-κB involves physical dissociation of p65-p50 subunits from IκBα and subsequent nuclear translocation. However, it is also well established that regulation of transcriptional activity of NF-κB also requires

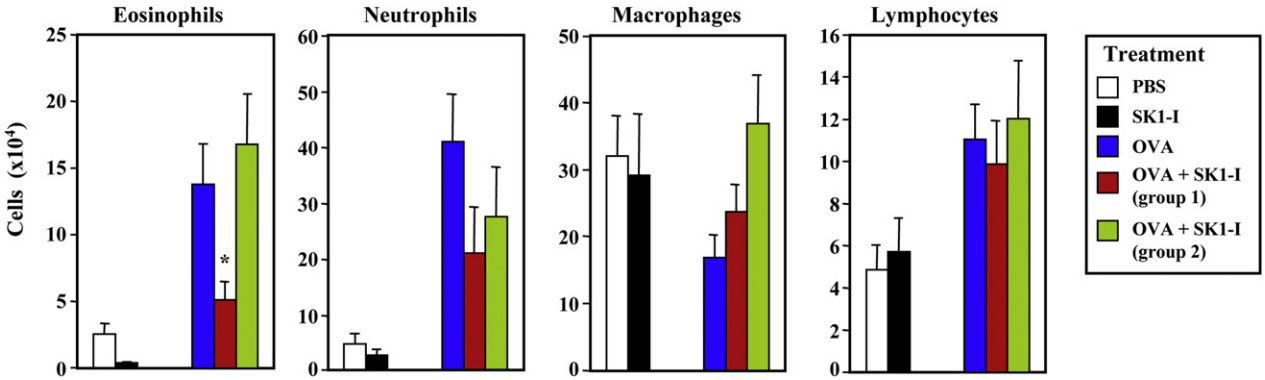

FIG 5. SK1-I treatment reduces airway eosinophilia. Mice were sensitized, challenged, and treated as described in Fig 3. BAL fluid was collected on day 29, and the numbers of eosinophils, neutrophils, macrophages, and lymphocytes were determined. *$P < .05$ compared with OVA-challenged mice. Data are means ± SEMs from at least 7 mice in each group.

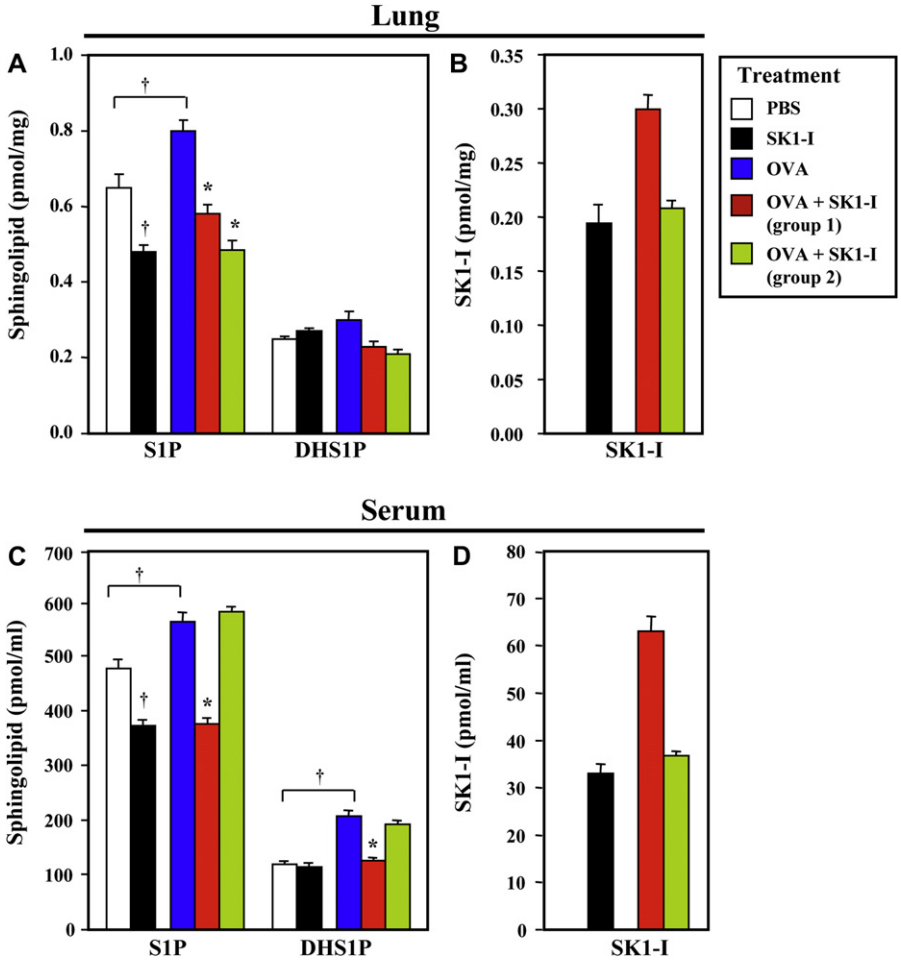

FIG 6. Effect of SK1-I on S1P levels in lungs and sera. Mice were sensitized, challenged, and treated as described in Fig 3. On day 29, lung tissue (**A** and **B**) and sera (**C** and **D**) were collected, and levels of S1P, dihydro-S1P *(DHS1P)*, and SK1-I were determined by using liquid chromatography–electrospray ionization–tandem mass spectrometry. †$P < .05$ compared with PBS-treated mice. *$P < .05$ compared with OVA-challenged mice.

phosphorylation of p65.[35] 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 IκBα.[35] In agreement with previous studies,[36] very little pulmonary staining of phospho-p65 (Ser276) was detected in unchallenged animals,

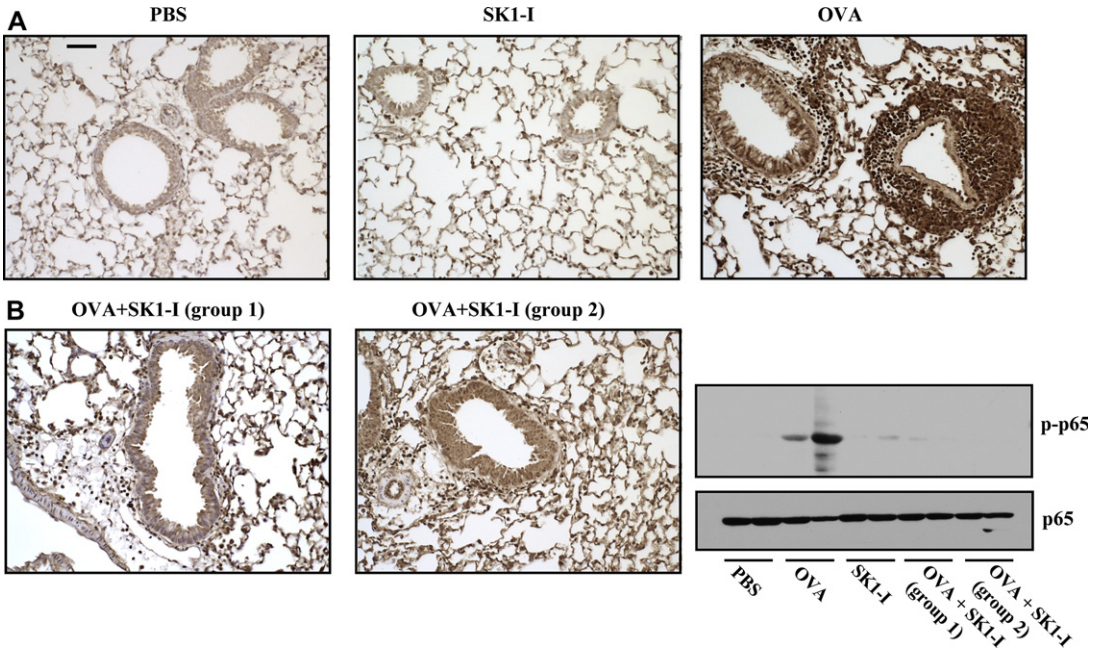

FIG 7. Inhibition of SphK1 attenuates activation of NF-κB in the lungs of OVA-challenged mice. Mice were sensitized, challenged, and treated as described in Fig 3. **A,** At day 29, lung sections were fixed and stained with anti-p65 (phospho-Ser276) antibody and photographed under light microscopy at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

whereas staining was strikingly increased after OVA challenge (Fig 7, *A*), 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 7, *A*). The increase in 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), which is known to be important for its transcriptional activity, which was markedly decreased by SK1-I treatment (Fig 7, *B*).

Inhibition of SphK1 decreases cytokine and chemokine levels

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 by Kalesnikoff and Galli[37]), levels of cytokines, including the T_H2-type cytokines IL-4 and IL-13, which are implicated in the induction of AHR associated with allergic inflammation in the lungs, and IL-5, which contributes to eosinophilia, and levels of the chemokines eotaxin and CCL2, which are also involved in eosinophil and diverse types of inflammatory cell recruitment, respectively, were all significantly increased 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 cytokine levels were greatly diminished in both SK1-I treatment groups. Similarly, levels of the NF-κB–regulated pleiotropic cytokines IL-6 and TNF-α, the release of which from activated mast cells is dependent on SphK1,[11,15,23] were increased in the OVA-sensitized mice and were also reduced by treatment with SK1-I. As was observed by others,[34] levels of the T_H1-type cytokine IFN-γ were slightly but significantly increased after 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/antigen-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 FcεRI-mediated mast cell activation.

SphK1, but not SphK2, plays a critical role in IgE/antigen-induced degranulation, migration toward antigen, and CCL2 secretion from human mast cells, as determined by specifically downregulating their expression.[15] Nevertheless, both isoenzymes were required for efficient TNF-α secretion.[15] Surprisingly, however, BMMCs derived from SphK1 knockout mice had normal responses, whereas silencing SphK1 markedly impaired BMMC functions,[11] leading to the suggestion that this is due to a compensation mechanism during the development of mice with a deletion of this important gene.[11]

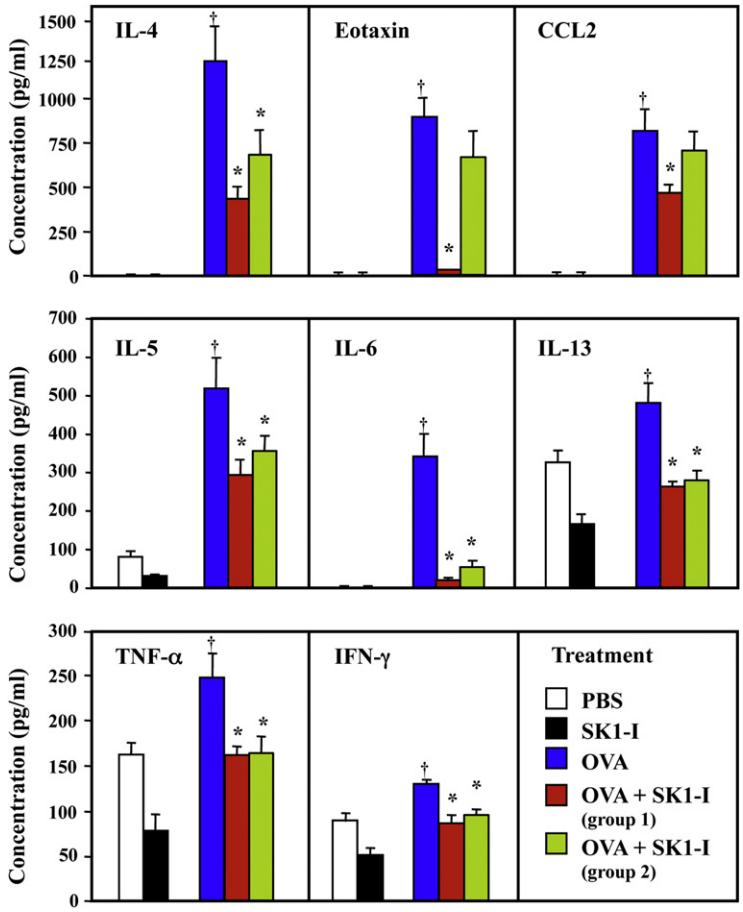

FIG 8. SK1-I reduces OVA challenge–induced pulmonary cytokines and chemokines. Mice were sensitized, challenged, and treated as described in Fig 3. On day 29, BAL fluid was collected, and levels of the indicated cytokines and chemokines were measured by using the Bio-Plex assay. Data are means ± SEMs from at least 7 mice in each group. **P* < .05 compared with OVA-challenged mice. †*P* < .05 compared with PBS-treated mice.

TNF-α and IL-6 expression in response to FcεRI ligation is strictly dependent on NF-κB.[38] Indeed, we found that inhibition of SphK1 nearly abrogated antigen-induced phosphorylation of both IKK and IκBα, key players in the NF-κB pathway, and secretion of these proinflammatory cytokines, suggesting SphK1 is also important for NF-κB activation downstream of FcεRI cross-linking. Similar effects were obtained by downregulating SphK1, but not SphK2, in BMMCs.[11]

S1P levels are increased in human asthmatic patients,[6] 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 antigen-induced airway inflammation[5] and bronchial hyperresponsiveness.[40] Conversely, treatment of OVA-challenged mice with a pan-SphK inhibitor or with small interfering RNA 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 inflammation.[31] 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 inflammation.[32] Some of these apparent controversies regarding the roles of S1P and SphK1 in various features of murine 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 used an allergic model with alum as an adjuvant, which can itself enhance T_H2 responses and thus mask important functions of mast cells.[41] Because of 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 human subjects, including inflammatory responses in the lung and increases in mast cell, eosinophil, and neutrophil counts and mucus production.[34] Using this model of chronic asthma, we found that SphK1 inhibition suppressed the development of AHR; chronic inflammation, including infiltration of eosinophils; and airway epithelial goblet cell hyperplasia. Interestingly, the concentration of SK1-I in the lungs was 10-fold higher than in the circulation when SK1-I was administered intranasally rather than systemically.

Several lines of evidence indicate that although mast cells are likely to be the major target of SK1-I in asthma models, other cell types can also be affected by this inhibitor. First, we confirmed that in our murine allergic asthma model, the airway

inflammation and responsiveness were mast cell dependent. Second, SK1-I inhibits activation of mast cells, the key effector cells in this model. Third, there was a marked increase in mast cell numbers and their degranulation in the lungs of OVA-sensitized and OVA-challenged mice, which was suppressed by intranasal treatment with SK1-I. This suppressive action of SK1-I is consistent with previous findings that SphK1 expression is enhanced by OVA challenge,[42] particularly around bronchial epithelial walls,[39] and that S1P is important for recruitment of inflammatory cells, including mast cells, lymphocytes, and eosinophils, to sites of inflammation[3,4] and has also been implicated in the production of MUC5AC and 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 because of compensatory increases in pulmonary expression of SphK1 and increased levels of S1P in lungs and sera (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 cells.[16] 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 proinflammatory cytokines TNF-α and IL-6, which amplify inflammation, probably by inhibiting NF-κB activation. In this regard intracellular S1P produced by SphK1 has recently been implicated as a critical regulator of NF-κB and as a required cofactor for the K63-linked polyubiquitination of receptor-interacting protein 1 and TNF receptor-associated factor 2,[7] a key step leading to activation of NF-κB. NF-κB might have an important role in asthma. Animals in which components of the NF-κB pathway have been deleted or inactivated have reduced OVA-induced inflammation, AHR, and airway remodeling.[44,45] Human bronchial biopsy specimens showed NF-κB activation in asthmatic airway epithelial and inflammatory cells that was increased from that seen in control specimens.[46] Because SK1-I markedly suppressed activation of NF-κB in the lung and the NF-κB–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 the 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 through 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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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.

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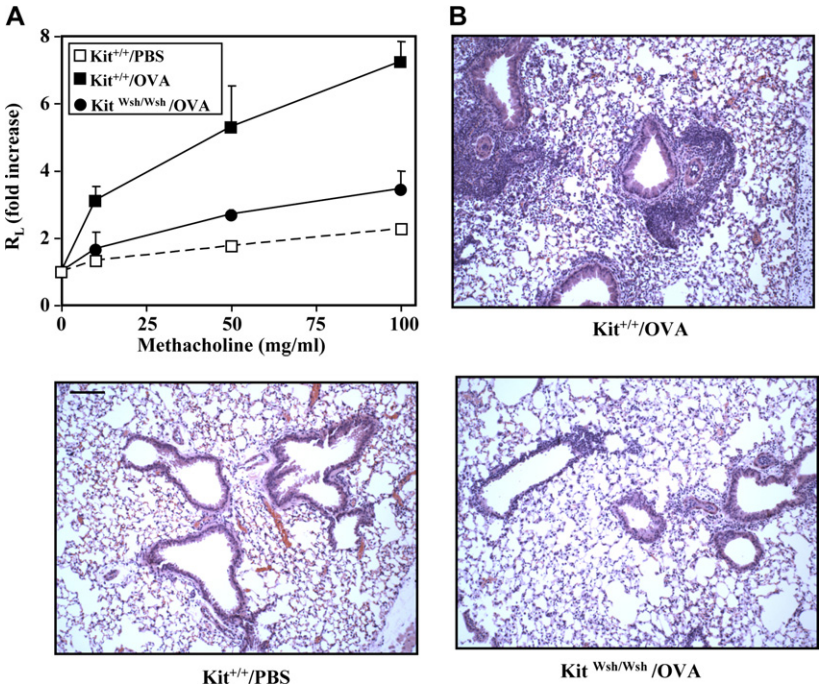

FIG E1. Airway inflammation and hyperreactivity to methacholine are reduced in OVA-sensitized and OVA-challenged mast cell–deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice. $Kit^{W\text{-}sh/W\text{-}sh}$ and wild-type congenic mice were sensitized and challenged with OVA or PBS as described in Fig 3. Airway responses to methacholine were measured with the flexiVent apparatus 24 hours after the last intranasal OVA or PBS challenge. **A,** Lung resistances *(R_L)* are shown as fold changes. **B,** Lung sections were fixed and stained with hematoxylin and eosin. *Scale bar* = 100 μm.