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Usage of Sphingosine Kinase Isoforms in Mast Cells Is Species and/or Cell Type Determined

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FcεRI engagement in mast cells (MCs) induces the activation of two distinct sphingosine kinase isoforms (SphK1 and SphK2) to produce sphingosine-1-phosphate, a mediator essential for MC responses. Whereas embryonic-derived SphK2-null MCs showed impaired responses to Ag, RNA silencing studies on other MC types indicated a dominant role for SphK1. Given the known functional heterogeneity of MCs, we explored whether the reported differences in SphK1 or SphK2 usage could be reflective of phenotypic differences between MC populations. Using lentiviral-based short hairpin RNA to silence SphK1 or SphK2, we found that SphK2 is required for murine MC degranulation, calcium mobilization, and cytokine and leukotriene production, irrespective of the tissue from which the MC progenitors were derived, the stage of MC granule maturity, or the conditions used for differentiation. This finding was consistent with the lack of a full allergic response in SphK2-null mice challenged to undergo passive cutaneous anaphylaxis. A redundant role for both SphKs was uncovered, however, in chemotaxis toward Ag in all MC types tested and in TNF-α production in certain MC types. In contrast, human MC responses were dependent only on SphK1, associating with a more robust expression of this isoform and a more varied representation of SphK variants relative to murine MCs. The findings show that the function of SphK1 and SphK2 can be interchangeable in MCs; however, an important determinant of SphK isoform usage is the species of origin and an influencing factor, the tissue from which MCs may be derived and/or their differentiation state. The Journal of Immunology, 2013, 190: 2058–2067.

Two mammalian sphingosine kinase isoforms (SphK1 and SphK2) are responsible for the phosphorylation of sphingosine to generate sphingosine-1-phosphate (S1P) when cells are activated by a variety of stimuli (1). S1P is a pleiotropic lipid mediator of diverse biological functions, including the regulation of vascular permeability and vascular tone (2, 3), modulation of immune cell trafficking and function (4, 5), and regulation of numerous disease processes (6–10). S1P generated during activation of SphKs may bind and regulate its intracellular targets or, once transported out of the cells, may bind and engage its membrane receptors (S1PR1 through 5), thus mediating complex arrays of responses (reviewed in Ref. 9). In part, the mode of action of S1P depends on the location where it is produced, the regulation of its levels by enzymes involved in its degradation, and the coupling of its synthesis either to its export via lipid transporters or to specific signaling pathways (9, 11, 12). Each individual isoform of SphK may also contribute to the type of cellular actions S1P is able to elicit (12, 13). SphK1 and SphK2 share a high degree of structural homology but differ considerably in their overall sequence, tissue distribution, biochemical properties, as well as in the cellular functions they can mediate. Evidence exists for unique, redundant, or even opposing roles for SphK1 and 2 (12–14). This versatility may be attributed to their relative expression in the cell, their subcellular redistribution under a particular stimulus, or their impact on other bioactive sphingolipid metabolites. Furthermore, splicing variants for both isoforms have been described (14–18), although their specific function in cells is largely unknown. Although the function and mechanism of activation of SphK1 have been investigated in several systems, those for SphK2 remain largely unexplored. The emerging view, gathered from accumulated studies, suggests that there is a preferential use for one of the isoforms of SphK in a particular cell, stimulus, and type of response. Dominance of SphK1 function is most common and consistent among mammalian systems, whereas the SphK2 function is more variable, with an apparent dependence on where it may be localized in a given cell type.

MCs are key effector cells of allergic responses, characterized by the constitutive expression of the high-affinity IgE receptor, FcεRI, on their surface. Allergen-mediated cross-linking of FcεRI results in a cascade of signaling events that culminates in the secretion of preformed mediators and the production of a variety of cytokines and lipid mediators, all of which promote allergic and inflammatory responses in vivo (19, 20). Engagement of the IgE receptor by Ag in MCs induces the activation of both SphK1 and SphK2 and the production of S1P, which promotes the release of MC-derived mediators (21–26). S1P is also secreted by activated MCs in considerable amounts to the extracellular medium by the

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Abbreviations used in this article: BMMC, bone marrow–derived mast cell; CTMC, connective tissue–like mast cell; HuMC, human mast cell; LDMC, liver derived mast cell; LTB4, leukotriene B4; MC, mast cell; mMCP, murine mast cell protease; PCA, passive cutaneous anaphylaxis; PDMC, peritoneum–derived mast cell; qRT-PCR, quantitative RT-PCR; SA, streptavidin; SCF, stem cell factor; shRNA, short hairpin RNA; siRNA, silencing RNA; S1P, sphingosine-1-phosphate; SphK1, sphingosine kinase 1; SphK2, sphingosine kinase 2; WT, wild-type.

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ABCC-1 transporter (27). Because S1P has been found elevated at sites of inflammation in diseases in which MCs may play important roles (i.e., asthma and arthritis) (28, 29), it is possible that, during allergic and inflammatory processes, MCs produce S1P in the tissue environment, which can affect the pathological course of these diseases (10, 11). Furthermore, because the generation of S1P is intrinsically important for MC responses, an understanding of the specific role for each isoform of SphKs in early- or late-phase MC responses is essential and could provide novel therapeutic targets for specific diseases.

SphK isoform dominance in MC function is not completely understood. Evidence for SphK1 and SphK2, either individually or jointly, in MC effector functions has been reported (21, 26, 30–32). However, a number of discrepancies are found in these studies that may arise from differences in the experimental approaches, the species of origin of the MCs used, the tissue from which MC progenitors are derived, and/or the conditions used for differentiation of MCs, all of which can influence the MC phenotype. Using embryonic liver-derived MCs from SphK1- and SphK2-deficient mice, we previously found that SphK2 was the major source of S1P and was essential for MC effector responses. Others have reported, using the rat cell line RBL-2H3 (30) or murine bone marrow-derived mast cells (BMMCs) (32), that silencing RNA (siRNA) used to silence SphK1, but not SphK2, resulted in impaired degranulation in MCs. In human mast cells (HuMCS), SphK1 was also reported to be required for MC degranulation (26, 31), whereas both SphK1 and SphK2 were involved in cytokine production (31).

In the current study, we thus explored the relative roles of SphK isoforms in MC function. We used a short hairpin RNA (shRNA) silencing strategy to achieve selective silencing of each SphK isoform, in contrast to the previously described (32) transient siRNA strategies, which we found to be neither sufficient nor selective to cause effective silencing of individual SphKs in murine MCs. In addition, we used MCs from different species or derived from various tissues, as well as MCs differentiated under different culture conditions, to explore the role of SphK isoforms in heterogeneous populations of MCs. Our findings indicate that a major determinant of the relative importance of each SphK isoform in MC function depends on the species of origin (SphK1 for human and SphK2 for mouse), which in part reflects the relative abundance of SphK1 and SphK2 expression. Despite the dominance of SphK2 in the murine system, redundancy was noted among the isoforms in their role in migration of mouse MCs toward Ag and in cytokine production, although the latter was restricted to a particular type of MC. Our findings also suggest that murine MC constitutes an ideal cellular system to investigate possible posttranslational mechanisms in the activation of SphK2, which have largely been elusive.

Materials and Methods

**Mice and cell culture**

SphK1- and SphK2-null mice (C57BL/6 × 129/Sv, N5) were generated as described previously (33, 34), and wild-type (WT) mice were on the same genetic background. Animals were maintained and used according to National Institutes of Health guidelines and National Institute of Arthritis and Musculoskeletal and Skin Diseases–approved animal study proposal A010-04-03. BMMCs were generated by flushing MC progenitors from the peritoneal cavity of adult WT mice and cultured in media containing 20% FBS (35). Ear skin MCs were obtained from WT, SphK1-, and SphK2-null mice. Briefly, ears were split into dorsal and ventral halves and placed in 2 ml 1% FBS in RPMI 1640 containing 0.1 mg/ml DNase I (Sigma-Aldrich) and 0.2 mg/ml Liberase TL (Roche Diagnostics). Following a 1-h incubation at 37°C, the digested tissue was placed into a 70-mm cell strainer (BD Biosciences) and teased through the mesh. The strainers were washed with 2 ml 1% FBS, 2 mM EDTA in PBS (Life Technologies) (37). All cells were cultured in RPMI media (Invitrogen) supplemented with 10% (BMMCs) or 20% (PDMCs and ear MCs) FBS (Invitrogen) and 20 ng/ml each of recombinant mouse IL-3 and stem cell factor (SCF) (Peprotech, Rocky Hill, NJ). Primary HuMCS were prepared from CD34+ peripheral blood progenitors isolated from healthy volunteers following informed consent under a protocol (NCT00001756) approved by the National Institutes of Health internal review board (38). The 293LTV cells (Cell Biolabs) were used for viral production and cultured in DMEM media supplemented with 10% FBS (Invitrogen).

**shRNA construction and gene transduction**

A lentiviral-based transduction system was used for RNA-mediated gene knockdown of SphK1 and SphK2 in murine and HuMCS. Both SphK1 and SphK2 shRNA stocks of shRNA constructs for mouse and human SphK1 and SphK2 were purchased from Sigma-Aldrich. The shRNA constructs were used as follows: mSphK1 (TRCN0000024648), mSphk2 (TRCN0000024632), huSphK1 (TRCN0000036960), and huSphK2 (TRCN0000036972). Nontarget shRNA (SHC002) was used as a negative control.

To generate virus, 293T cells were transfected with 3.9 μg shRNA vector and 39 μl lentiviral packaging mix (Sigma-Aldrich), using FuGENE HD (Roche). The virus supernatants were collected 72 h posttransfection and concentrated by centrifugation at 20,000 × g for 2 h. The viral pellet was resuspended and used to transduce 106 fully differentiated BMMCs or PDMCs or 5 × 106 HuMCS. The efficiency of lentiviral transduction was >90%, as determined by the GFP expression cassette pNUTS (data not shown). Cells were transduced with mSphK1 and following an additional 2-d recovery period, selection was started using 3 μg/ml (BMMCs), 1.5 μg/ml (PDMCs), or 0.2 μg/ml (HuMCS) puromycin (Sigma). Cells were kept in selection media for 5–7 d and allowed to recover for >2 d in puromycin-free media before being used for the experiments.

**Real-time PCR and Western blot**

To assess mRNA knockdown, total RNA was isolated from cells (1–4 × 106) using the RNeasy Kit (Qiagen) per the manufacturer’s instructions. RNA was converted into cDNA, using the SuperScript III First-Strand Synthesis System for quantitative RT-PCR (qRT-PCR) (Invitrogen). The qRT-PCR was performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). TaqMan Gene Expression Assays for SphK1 and SphK2 were purchased from Applied Biosystems. Relative levels of mRNA were calculated based on ∆Ct, using GAPDH as an endogenous control gene. The levels of expressions of the different SphK isoform variants were determined as described in Supplemental Table I. Western blots were performed to measure the effectiveness of protein knockdown. Cells were lysed in borate-buffered saline containing 1% Triton X-100, 60 mM octyl-β-D-glucoside, 2 mM PMSF, 10 μg/ml aprotinin, 2 μg/ml leupeptin and pepstatin, 5 mM sodium pyrophosphate, 50 mM NaF, and 1 mM sodium fluoride (22). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes, and the resolved protein was identified using Abs for SphK2 (22). Available Abs to SphK1 was used to detect the activation of Sphk2, which have largely been elusive.
activity. Following a 30-min incubation at 37˚C, the reactions stopped, radiolabeled lipids were extracted, and SIP was separated on TLC plates as described (22). Both SphK1 and SphK2 activities were linear, from 2.5 to 100 μg protein, indicating the validity of these assays for assessing SphK expression.

Measurement of MC effector responses

Murine MCs were sensitized with 1 μg/ml anti-DNP IgE (H1 DNP−e−26.82; Ref. 39) in HEPES-BSA buffer (37˚C, 10 mM HEPES [pH 7.4], 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na2HPO4, 5.6 mM glucose, 1.8 mM CaCl2, and 1.3 mM MgSO4, and 0.04% fatty acid−free BSA) for 1 h at room temperature. HuMCs were sensitized with 100 ng/ml biotinylated IgE (40) in cytokine-free media or complete media (cytokine production) overnight at 37˚C. For degranulation experiments, murine MCs and HuMCs were stimulated for 30 min with the indicated concentrations of DNP-HSA (Ag) (Sigma-Aldrich) or streptavidin (SA) (Sigma-Aldrich), respectively, and the release of β-hexosaminidase into the incubation media was determined using a colorimetric assay, as previously described (40). The extent of degranulation was calculated as the percent of β-hexosaminidase found in the supernatants following challenge versus the total content found in the cells. Cytokine production was measured from BMMCs (4 × 10^5 cells) stimulated with 25 ng/ml Ag for 3 h. Alternatively, HuMCs (1 × 10^6 cells) were stimulated with 100 ng/ml SA for 6 h. Production of IL-6 and TNF-α for murine MCs or IL-8 and TNF-α for HuMCs was determined by Bio-Plex Pro Cytokine Assays (Bio-Rad). For leukotriene production, cells were stimulated with the indicated concentrations of Ag for 30 min at 37˚C in HEPES-BSA buffer containing 10 μg/ml indomethacin (Sigma-Aldrich). The amount of leukotriene B4 (LTB4) in the supernatants was measured using a competitive ELISA (R&D Systems).

Passive cutaneous anaphylaxis

The ears of WT, SphK1−, or SphK2-null mice were sensitized s.c. with either anti-DNP IgE (75 ng in 20 μl saline) or saline in the contralateral ear. The next day, mice were challenged i.v. with Ag (250 μg) dissolved in 0.5% Evans blue/PBS. After 30 min, the mice were euthanized, and the ears were collected and minced. The minced ears were incubated in formamide for 1 h at 55˚C to extract the Evans blue, and the amount of dye was measured by absorbance at 620 nm (41).

Calcium mobilization

Calcium mobilization was measured using a Zeiss LSM-510 Meta confocal microscope. Cells were sensitized with 1 μg/ml anti-DNP IgE (75 ng in 20 μl saline) or the same volume of saline in the contralateral ear. The next day, mice were stimulated with Ag (250 μg) dissolved in 0.5% Evans blue/PBS. After 30 min, the mice were euthanized, and the ears were collected and minced. The minced ears were incubated in formamide for 1 h at 55˚C to extract the Evans blue, and the amount of dye was measured by absorbance at 620 nm (41).

Chromotaxis assay

Chromotaxis assays were performed using Transwell permeable support with a 5.0-µM pore polycarbonate membrane on 6.5-mm inserts (Costar) placed within 24-well polystyrene plates. Cells were sensitized with 1 μg/ml anti-DNP IgE overnight in full media. Following three washings with HEPES-BSA buffer, cells (3 × 10^5 cells per 100 μl) were placed in the upper chamber insert and preincubated in 600 μl HEPES buffer for 30 min. The upper chambers were then placed in contact with the lower chambers containing Ag (10 ng/ml or 30 ng/ml). After incubation for 4 h at 37˚C, the upper chambers to the lower chambers were transferred to a 96-well flat-bottom plate. Cells pellets were frozen, and the relative numbers of migrated cells were determined using the CyQUANT Cell Proliferation Assay (Invitrogen). A linear standard curve with serial dilutions of the cells, ranging from 50 to 5000 cells, was included to convert the relative fluorescence intensity to cell numbers. Fluorescence was measured using a PerkinElmer Victor 2 Microplate Reader.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical significance was determined by unpaired, two-tailed Student t test. Each culture represents cells derived from either an individual mouse or human subject. Each knockdown experiment was repeated in 3–10 separate cultures, as indicated in the legends to figures. Whenever more than one knockdown experiment was performed from a single culture, it was averaged and considered as one.

Results

MC degranulation is impaired in SphK2-null connective tissue MCs in vitro and in vivo

Our previous work (21) had established an essential function for SphK2 in embryonic cultured liver-derived mast cells (LDMCs). Although MCs derived from the bone marrow of SphK1− or SphK2-null adult mice also showed a role for SphK2, the results were less convincing, given the high degree of variability from culture to culture (21). Unlike other hematopoietic cells, MC precursors from the bone marrow migrate through the blood to the tissues, where they differentiate, responding to tissue microenvironment cues that ultimately influence their phenotype (43, 44). In vitro, the MC phenotype may also vary, depending on the tissue from which progenitors are derived, the culture conditions, or the species of origin (44). Given the MC heterogeneity in vivo and in vitro, we sought to determine whether the dominance of SphK2 over SphK1 observed in the LDMCs and BMMCs was representative of other, more mature MC populations or of in vivo MCs. BMMCs grown in the presence of IL-3 and SCF are thought to be an immature connective tissue–like mast cell (CTMC) model, with no known tissue equivalent. However, PDMCs, which differentiate and mature in vivo, are a more mature CTMC, based on their staining properties and morphology (45). In agreement with this observation, PDMCs had a denser granule content (data not shown) and expressed higher levels of murine mast cell protease (mMCP)-1, 2, 4, 5, and 9 than did BMMCs (Supplemental Fig. 1A). They also expressed mMCP5 and carboxypeptidase A3, proteases that characterize CTMCs (Supplemental Fig. 1B, 1C).

Consistent with results in LDMCs and BMMCs, SphK2 deficiency in PDMCs resulted in impaired degranulation (Fig. 1A). However, a role for SphK1 in the degranulation response was also seen in SphK1-deficient PDMCs (Fig. 1A). To determine whether the dependence of both SphK1 and SphK2 in degranulation is a characteristic of mature CTMCs or specific for PDMCs, we tested the responsiveness of skin MCs in vivo, using the MC-dependent model of passive cutaneous anaphylaxis (PCA). The skin MC is another CTMC type and shows similar content and distribution of the CTMC proteases mMCP5 and carboxypeptidase A3 as PDMCs (Supplemental Fig. 1B, 1C). Local sensitization and challenge of skin MCs resulted in similar responses in WT and SphK1-null mice, but a reduced response was observed in SphK2-null mice, as evidenced by decreased Evans blue extravasation (Fig. 1B). This reduced response was not due to differences in MC numbers in the ears, as all genotypes expressed similar cell numbers (Supplemental Fig. 1D). Skin MCs from SphK2-deficient mice showed a reduction in degranulation, as observed from MCs in the ears of mice upon PCA challenge in vivo (Fig. 1C). Furthermore, the difference in Evans blue extravasation was not due to alterations in blood vessel reactivity in SphK2-null mice because we have shown that induction of PCA by compound 48/80 (an MC secretagogue) in mice deficient for each kinase results in similar vascular permeability for all genotypes (41). The PCA responses, together with the in vitro experiments, provide evidence for an essential role for SphK2 in mediating murine MC degranulation, particularly the CTMC type, regardless of the tissue origin of the progenitor from which they are derived. However, our previous findings in a systemic challenge (passive systemic anaphylaxis) indicated that histamine release in SphK2-null mice was normal (21), suggesting that under a systemic challenge, other in vivo compensatory factors (i.e., levels of circulating S1P) may influence MC responses, as previously reported (10, 21).
pressed as percentage of WT degranulation in each separate experiment. genotypes, and each culture was from four separate ears. Data are ex-
null ear MCs and WT, **

Methods absorbance was measured at 620 nm, as described in
were euthanized and Evans Blue dye was extracted from the ears and
the contralateral ear. The next day, mice were challenged systemically with
mice ears were locally sensitized with saline or anti-DNP IgE (75 ng) in

FIGURE 1. Genetic deletion of SphK2 results in impaired degranulation in mature CTMCs in vitro and in vivo. (A) MCs were obtained by peritoneal lavage from WT and SphK1- and SphK2-null mice. After expansion of PDMCs for 11–20 d, β-hexosaminidase release was measured from supernatants of cells challenged with the indicated concentrations of Ag. Data represent the average of 10 individual cultures ± SE. Each experiment had duplicate cultures for all

Lentiviral shRNA silencing of SphK1 and SphK2 in marine

Because PDMCs from SphK1- and SphK2-deficient mice showed a dependence for both SphK isoforms, whereas other MC populations showed an essential role for SphK2 alone, we explored the idea that the alteration in MC function could be due to environment or epigenetic changes associated with the genetic deletion model. To address this issue, we used RNA silencing to inhibit SphK1 or SphK2 expression in fully differentiated MCs. Previously published sequences (32), along with commercially available siRNA sequences, were used; however, in our hands the previously published SphK2 siRNA sequences did not achieve selective silencing of SphK2, knocking down both SphK1 and 2 (Supple-

FIGURE 2. Specific knockdown of SphK1 and SphK2 in rodent MCs. MCs were transduced with lentiviruses containing shRNA sequences specific for SphK1, SphK2, or nontarget (NT) shRNA control. Efficient shRNA silencing of each isoform was determined by real-time PCR (A) or by enzymatic assays (B). (A) Gene expression assays specific for SphK1 (upper panel) and SphK2 (lower panel) were used to determine the relative mRNA levels after shRNA silencing. A specific probe for GAPDH was used as an internal control. Each measurement for SphK1 and SphK2 was normalized to GAPDH (ΔCT), and measurements were then compared with the NT values. (B) Cells transduced with the various shRNA constructs were assayed for SphK1 (upper panel) or SphK2 (lower panel) activities, as described in Materials and Methods. Specific assay conditions allowed for the selective detection of SphK1 activity in the presence of Triton-

Western blot analysis. Equal protein loading was confirmed using actin as a loading control and quantified by densitometry. Shown is a representative blot from PDMC lysates out of at least three separate experiments; very similar results were obtained in BMMCs. The values underneath the blots represent the average densitometry values (n = 7, including BMMC and PDMC blots) corrected by actin and normalized to NT control and are expressed as average ± SE.

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This finding was consistent with our previous one (21) in concentrations ranging from 1 ng/ml (data not shown) to 100 ng/ml. in PDMCs and BMMCs (Fig. 3A, 3C, respectively) at Ag concentrations linking to calcium responses in IgE/Ag-activated MCs (21, 24, 26), a result that contrasts with a previous report by Pushparaj et al. (32). The reasons for these discrepancies are not entirely clear. However, unlike for SphK2, incomplete knockdown of SphK1 is unlikely to explain the discrepancies with the previous study because the amount of SphK1 activity detected in the cells following siRNA knockdown was negligible (<5 pmol/mg/min) and similar to the activity reported in SphK1-knockout MCs (22, 46) (this background activity probably represents SphK2 activity; see Materials and Methods).

We therefore attempted to more effectively reduce the activity of the SphK isoforms, using lentiviral-based shRNA silencing, which allows for stable and high expression of shRNA. To assess the selectivity and efficiency of all shRNA constructs at the RNA and protein levels, we used real-time PCR and Western blot, and measured SphK1 and SphK2 activity; the latter of which can be distinguished in vitro owing to low cross-reactivity of the assay (22, 46). This multilayered strategy identified the shRNA sequences showing the highest degree of selective silencing for SphK1 and SphK2, with no apparent effect on the other isoform. As shown in Fig. 2, we achieved a reasonable degree of selective mRNA reduction (>60% on average for SphK1 and >70% for SphK2) for each of the targeted kinases, as measured by real-time PCR (Fig. 2A). This reduction was verified by the selective reduction of the enzymatic activity of each isoform (63% for SphK1 and 72% for SphK2) (Fig. 2B). Regrettably, immunoblotting for SphK1 protein expression was not possible, given that the available Abs failed to selectively detect this isoform in murine MCs. However, cells targeted with SphK2 shRNA showed a 70% reduction in SphK2 protein expression, whereas cells expressing shRNA for SphK1 or control shRNA showed no effect on SphK2 protein expression (Fig. 2C).

The shRNA-mediated silencing of SphK2 in fully differentiated MCs resulted in decreased (35–40%) IgE-dependent degranulation in PDMCs and BMMCs (Fig. 3A, 3C, respectively) at Ag concentrations ranging from 1 ng/ml (data not shown) to 100 ng/ml. This finding was consistent with our previous one (21) in SphK2-/- MCs. However, unlike PDMCs derived from SphK1-null mice (Fig. 1A), silencing of SphK1 expression in PDMCs or BMMCs did not alter β-hexosaminidase release, indicating that SphK1 activity was less critical for degranulation in this MC population. Although discrepancies between the SphK1-null PDMCs and SphK1 knockdown PDMCs could indicate that complete depletion of SphK1 is required to manifest its role in degranulation of PDMCs, this is unlikely because PDMCs obtained from mice in which SphK1 was conditionally deleted after birth (2, 3) had no defect in degranulation (Supplemental Fig. 3). In addition, conditional deletion of SphK1 in SphK2-/- mice did not result in any further reduction in the degranulation of SphK2-deficient PDMCs or BMMCs (Supplemental Fig. 3A, 3B). Thus, a more likely scenario is that general knockout of SphK1 (in contrast to SphK1 conditional knockout, mostly in the hematopoietic compartment (2, 3) causes developmental alterations that may phenotypically affect this MC population. The defective response in SphK1-null PDMCs, and perhaps other MC subtypes, may explain, at least in part, why histamine release after systemic Ag challenge (passive systemic anaphylaxis), but not PCA, in these mice was found to be lower than expected (21).

Calcium is needed for degranulation, and SphKs have been linked to calcium responses in IgE/Ag-activated MCs (21, 24, 26, 47). Consistent with the effect on degranulation, calcium responses were reduced in both PDMCs and BMMCs (Fig. 3B, 3D, respectively) when SphK2, but not SphK1, was silenced.

To further explore the apparent selectivity of SphK2 in murine MC responses, we also measured inflammatory mediators that are produced and secreted at later times following Ag stimulation. Silencing of SphK2 resulted in a significant impairment in production of IL-6 and TNF-α in both PDMCs (Fig. 4A, 4B) and BMMCs (Fig. 4D, 4E). LTβ4 production was also significantly impaired in PDMCs in which SphK2 was silenced (Fig. 4C); however, only a slight reduction in the secretion of LTβ4 was seen in BMMCs (Fig. 4F), suggesting that SphK2 may not be required for production of this lipid mediator in this population of MCs. Silencing of SphK1 in BMMCs also resulted in a trend toward reduced cytokine production, particularly IL-6, suggesting a possible role for SphK1 in cytokine production in this type of MC, although significance was not achieved (Fig. 4D, 4E). Collectively, the shRNA-mediated silencing of SphK1 and SphK2 in fully differentiated BMMCs and PDMCs established a predominant role for SphK2 in regulating Ag-induced MC effector functions, akin to the results seen in genetically altered mouse MCs (21). These findings indicate that the absence of SphK2 causes an intrinsic defect in MC function and resolves the muddling issue of potential environmental or developmental defects in genetically deleted SphK1 and 2 MCs [Fig. 1; (21)].

**FIGURE 3.** shRNA-mediated silencing of SphK2, but not SphK1, results in impaired degranulation and calcium responses in PDMCs and BMMCs. Lentivirus-transduced PDMCs (A, B) or BMMCs (C, D) were sensitized with anti-DNP IgE, as described in Materials and Methods. Cells were washed and then stimulated with the indicated concentrations of Ag. (A and C) Degranulation was calculated as the percentage of β-hexosaminidase released to the incubation media, compared with the total β-hexosaminidase cellular content. Data shown are the average of three to five independent cultures done in triplicate ± SE. Statistical significance compared with nontarget control, *p < 0.05, **p < 0.01. (B and D) Lentivirus-transduced cells were loaded with Fura-4 AM and FuraRed-AM. Following Ag stimulation, calcium responses were measured using confocal microscopy and are shown as a ratio of intensity of Fura-4 AM/FuraRed-AM. One representative experiment is shown averaging 15 to 20 individual cells per time point. Data shown are a mean ± SE. Similar results were obtained in three independent cultures measuring calcium as the ratio of emission at 510 nm when Fura-2AM–loaded cells were excited at 340 and 380 nm in population analysis (data not shown).

*SphK1 AND Sphk2 USAGE IN MAST CELL RESPONSES*

MCs migrate toward a variety of stimuli, such as SCF, highly cytokinergic IgE, Ag, SIP, as well as cytokines and chemokines (30, 48, 49). Jolly et al. (30) reported that MC migration to Ag was
mediated through FceRI-mediated activation of SphK1, which leads to the formation of S1P and to the subsequent transactivation of S1PR1 receptor. This model of S1P-dependent migration was also shown for HuMCs (31). Using LDMCs and BMMCs derived from SphK1- and SphK2-null mice, we found that both SphK isoforms were required for MC migration toward Ag in a Transwell migration assay (Fig. 5A, 5B), as well as migration toward SCF (30 ng/ml, data not shown). Furthermore, the absence of both SphK1 and SphK2 in LDMCs did not show additional reduction (Fig. 5A). Similarly, shRNA silencing of SphK1 and SphK2 also caused a marked impairment in MC migration in both PDMCs and BMMCs, indicating a role for both isoenzymes in MC migration (Fig. 5C, 5D) that contrasted with the dominant role for SphK2 in mouse MCs (26, 31). Alternatively, shRNA was used to silence SphK1 and SphK2 in PDMCs (C) and BMMCs (D). The ability of MCs to migrate toward increasing concentrations of Ag was measured using Transwell assays. Cells were placed in the upper inserts, and the indicated concentrations of Ag in the lower chamber. Cells were allowed to migrate for 3 h. The number of cells migrated was quantified using CyQUANT-GR dye. Data represent the mean ± SE of at least three individual cultures. Statistical significance compared with WT (A, B) or nontarget (NT) control, *p < 0.05, **p < 0.01. Despite the trends, some comparisons did not achieve statistical significance owing to variability in the total number of cells migrating between different cultures. However, in each experiment, silencing or genetic deletion of SphK1 or SphK2 showed an average of ≥50% reduction in MC migration, regardless of the MC type. Deletion or silencing of SphK1 also resulted in impairment of MC migration toward SCF (30 ng/ml), a potent MC chemoattractant. In LDMCs, migration to SCF was as follows: WT: 3402 ± 823; SphK1−/−: 1450 ± 650; SphK2−/−: 719 ± 167; SphK1−/−/SphK2−/−: 473 ± 148 (mean ± SEM).

**FIGURE 5.** MC migration requires both SphK1 and SphK2. Fetal liver (A) and bone marrow (B) MCs were derived from SphK1-, SphK2- mice (A, B) or SphK1/SphK2 double-deficient (A) mice. Alternatively, shRNA was used to silence SphK1 and SphK2 in PDMCs (C) and BMMCs (D). The ability of MCs to migrate toward increasing concentrations of Ag was measured using Transwell assays. Cells were placed in the upper inserts, and the indicated concentrations of Ag in the lower chamber. Cells were allowed to migrate for 3 h. The number of cells migrated was quantified using CyQUANT-GR dye. Data represent the mean ± SE of at least three individual cultures. Statistical significance compared with WT (A, B) or nontarget (NT) control (C, D), *p < 0.05, **p < 0.01, ***p < 0.001. Despite the trends, some comparisons did not achieve statistical significance owing to variability in the total number of cells migrating between different cultures. However, in each experiment, silencing or genetic deletion of SphK1 or SphK2 showed an average of ≥50% reduction in MC migration, regardless of the MC type. Deletion or silencing of SphK1 also resulted in impairment of MC migration toward SCF (30 ng/ml), a potent MC chemoattractant. In LDMCs, migration to SCF was as follows: WT: 3402 ± 823; SphK1−/−: 1450 ± 650; SphK2−/−: 719 ± 167; SphK1−/−/SphK2−/−: 473 ± 148 (mean ± SEM).

**FIGURE 4.** shRNA-mediated silencing of SphK2 results in impaired cytokine and LTB4 production, whereas partial effects are observed by silencing SphK1 in BMMCs. Lentivirus-transduced PDMCs (A–C) and BMMCs (D–F) were sensitized with anti-DNP IgE and stimulated with Ag (DNP-HSA) for 3 h for cytokine and 30 min for LTB4 production. The amount of IL-6 (A, D) and TNF-α (B, E) released into the media was determined using Bio-Plex magnetic bead–based cytokine assays. LTB4 released into the media was quantified by ELISA (C, F). Data represent the mean ± SE of at least three to five individual cultures. Statistical significance by an unpaired two-tailed Student t test compared with nontarget control, *p < 0.05, **p < 0.01.

SphK1 is necessary for effector responses in HuMCs

Studies using human BMMCs and cord blood–derived MCs have demonstrated that SphK1 is the dominant isoform in HuMC function (26, 31). Given that such studies employed transient siRNA for silencing SphK1 and 2, we wished to confirm the reported findings using shRNA to selectively target SphK1 and SphK2 in LAD2 cells, an SCF-dependent HuMC line, and in HuMCs. LAD2 cells are immortalized cells that, unlike the primary HuMC, can be sustained in long-term cultures (50), and therefore they provided an ideal system to screen the efficacy of various shRNA constructs. As in mouse MCs, the efficiency of RNA and protein silencing of SphK1 and 2 was between 60 and 75%, as determined by real-time PCR and the respective enzymatic activities (Fig. 6A, 6B). Similar knockdown efficiencies were obtained in HuMCs, using these constructs (data not shown).

The shRNA-mediated silencing of SphK1 resulted in the reduced degranulation of LAD2 cells, whereas SphK2 silencing had no effect (Fig. 6C). Similarly, shRNA silencing of SphK1 in HuMCs resulted in impaired MC degranulation, whereas silencing of SphK2 did not (Fig. 6D). This finding is consistent with previous reports of the requirement for SphK1 in cord blood–derived HuMC responses (26, 31). A drawback of the LAD2 cell line is that cytokine secretion is poor (31) and it is an immortalized cell line; thus, we used the CD34+–derived primary HuMC to study the effect of SphK1 and SphK2 on cytokine secretion as a potentially more accurate model of HuMC function. Similar to the degranulation responses, silencing of SphK1 resulted in a reduction of IL-8 and TNF-α secretion, whereas silencing of SphK2 had no effect (Fig. 6E). These results indicate that in HuMCs SphK1 is dominant and further demonstrate that the species of origin appears to be the determinant of which isoform of SphK is dominant in MC function.

SphK1 splice variants have been described in both mouse and human cells, whereas SphK2 variants have been described in human cells (14–18). Because these variants may differ in their cellular localization and thus potential function (12, 14), we sought to explore whether human and mouse MCs showed dif-

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A model of SphK1 and SphK2 involvement in human and murine MC responses is presented in Fig. 7.

FIGURE 6. SphK1 is required for IgE/Ags-mediated responses in HuMCs. LAD2 and CD34+-derived HuMCs were transduced with lentivirus containing shRNA sequences specific for SphK1, SphK2, or nontarget shRNA control. Specific knockdown of SphK1 (upper panels) or SphK2 (lower panels) in LAD2 cells was confirmed at the mRNA level using qRT-PCR (A) and by measuring enzymatic activity (B), as described above. Degranulation was determined in LAD2 MCs (C) and CD34+-derived HuMCs (D) transduced with the indicated SphK isoform shRNA. MCs were sensitized with 100 ng/ml biotinylated IgE overnight and stimulated for 30 min with the indicated concentrations of SA; the release of β-hexosaminidase into the media was determined as described in Materials and Methods. (E) The effect of silencing SphK1 or SphK2 on IL-8 (left) and TNF-α (right) production was also determined in CD34+-derived HuMCs. HuMCs sensitized as in (C) and (D) were stimulated with 100 ng/ml SA for 6 h, and the amount of cytokines released was determined by Bio-Plex Pro Cytokine Assays. Data represent the mean ± S.E. of two or three individual cultures. Statistical significance compared with nontarget control, *p < 0.05, **p < 0.01.

Discussion

SphKs and their product, S1P, have been implicated in regulating MC responses. SphK1 and SphK2 are activated upon FcεRI activation of MCs (22–26). In mouse MCs, this activation is dependent on the Src family protein tyrosine kinase Lyn and Fyn, which function to promote translocation of both SphK1 and SphK2 as well as enhance their enzymatic activity (22, 51). Translocation of these kinases to the plasma membrane enables the formation of S1P, which is vital for both immediate and delayed MC responses, including degranulation, cytokine production, arachidonic acid release, and leukotriene production. However, the specific contribution of each SphK isoform to the various MC functions is controversial (10). In this study, we found that in a particular MC type, one SphK isoform appeared to be dominant for most MC responses, whereas the other isoform did not contribute noticeably to each measured response. Furthermore, the dominance of a particular SphK isoform depended on the species of MC origin. Thus, SphK1 is key for regulating most HuMC responses, whereas SphK2 is generally predominant in most mouse MC responses. In addition, we found that, in the same species, the contributions of a given isoform may differ, depending on its tissue of origin. Whereas in most murine MCs studied, SphK2 is functionally required, in BMMCs, a less differentiated type of MC, SphK1 also seemed to contribute to cytokine production. Of interest, SphK1 and SphK2 also showed redundancy in mouse MC migration regardless of the tissue origin of a given MC (Fig. 7).

MCs are heterogeneous cells. Their phenotype varies, depending on the in vivo microenvironment where they differentiate or on the in vitro experimental culture conditions used (19). The expression of SphK1 and 2 varies among tissues and cells and during de-

![Human Mast Cells](http://www.jimmunol.org/)

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differ. Because of the predominance of Sphk2 activity in mouse the mechanism of phosphorylation/activation of SphK2 is likely to has been linked to plasma membrane association and biological the species, the cell type, or the stimulus, thus favoring one isoform translocation/activation of each isoform may differ depending on for SphK1 or 2 could be that the signaling mechanisms facilitating variant localization in mouse MCs and HuMCs warrants future investigation. Another possible factor determining the preference which expression of SphK isoforms depends on the tissue origin and possibly the differentiation state of the HuMC. In contrast to HuMCs, murine MCS showed markedly different levels of SphK1 and SphK2 expression and activity, with the latter being an order of magnitude higher [(21); Fig. 2B, Supplemental Table I]. This difference was independent of the tissue from which progenitors were derived (bone marrow versus embryonic liver) or the conditions of differentiation (SCF and IL-3 or differentiated in the peritoneum in vivo and expanded in vitro). This finding suggests that, unlike in HuMCs, expression of SphKs in the mouse is less dependent on environmental signals.

The higher proportion of SphK2 activity in murine MCS appears to correlate with its functional dominance, yet the division of function of these isoforms may not be related solely to their relative expression. In fact, we show that Sphk1 has a role in chemotaxis in murine MCs, despite its lower expression, suggesting that this isoform is more signaling efficient in regulating chemotaxis. Additional evidence for this view is provided by our findings with CD34+ derived HuMCs, which have equivalent levels of SphK1 and SphK2 activity (Fig. 6B) and, yet, in response to IgE/Ag, showed dependence on SphK1, but not on SphK2. One hypothesis for the differential use of SphK isoforms in HuMCs may be the intracellular location of these enzymes, which may differ between species. Translocation of SphKs from cytosol to the plasma membrane (12), and, in some instances, their relocation to lipid microdomains (12, 52), seems to be crucial for their signaling roles. We previously reported that in IgE/Ag-stimulated mouse BMCCs, both SphK1 and SphK2 translocated to the plasma membrane in proximity to the FceRI in lipid rafts (22, 51). Translocation of these isoforms in HuMCs remains to be explored; however, it may be possible that SphK1 (26, 31), but not SphK2, translocates in these cells following FceRI engagement. Interestingly, HuMCs express a variant of SphK1 not found in mouse MCS, SphK1c, which showed enhanced plasma membrane localization in human endothelial cells (18), raising the possibility of a preferential membrane association of SphK1 in HuMCs when compared with mouse MC. Among these lines, the localization of SphK2 in human cells has often been described as vesicular or nuclear (17, 18, 53, 54), particularly the long isoform (2b), and the only example of receptor-mediated activation of SphK2 in human cells was in cell types in which SphK2 was mostly localized at the plasma membrane (55). Thus, the differences in SphK isoform or variant localization in mouse MCs and HuMCs warrants future investigation.

Another possible factor determining the preference for SphK1 or 2 could be that the signaling mechanisms facilitating translocation/activation of each isoform may differ depending on the species, the cell type, or the stimulus, thus favoring one isoform over the other. Although phosphorylation of Ser225 in SphK1 has been linked to plasma membrane association and biological functions, this site is not conserved in SphK2 (12, 56), and thus the mechanism of phosphorylation/activation of SphK2 is likely to differ. Because of the predominance of Sphk2 activity in mouse MCs (Fig. 2B), this may prove a valuable cellular system for an in-depth evaluation of the molecular mechanisms regulating SphK2 activity.

Despite the overall dominance of function for one or the other isoenzyme in a particular MC type, we also found evidence for the redundant function of SphK1 and SphK2 in cytokine production and chemotaxis. This finding was not unexpected, because there are multiple examples of SphK1 and 2 exhibiting overlapping functions. It has been shown that both SphK genes are needed for proper neurotube and vascular formation during embryogenesis (34); both isoforms are also required for migration in MDA-MB-453 breast cancer cells (55) and for survival of cardiomyocytes and cardioprotection by ischemic preconditioning (57, 58). In addition, both isoforms seem to contribute to glioblastoma cell growth (59) and to resistance to drug treatment in human colon cancer cells (60). In our study, the joint involvement of SphK1 and SphK2 in the regulation of cytokines was restricted to only BMNC cultures (Fig. 4D), whereas no involvement for SphK1 was observed in PDCMs or MCs derived from embryonic liver progenitors. This finding supports the concept that differences in phenotype between MC populations can contribute to determining which SphK isoforms are involved in certain MC responses. Both isoforms of SphK were also required for migration toward Ag in murine MCs, regardless of their progenitor or phenotype (Fig. 5). Nevertheless, their contribution to cell migration was neither synergistic nor redundant because deletion of both kinases did not have additional effects on migration responses of MCs and the lack of one isoform could not compensate for the lack of the other one. In agreement, neither SphK1- nor SphK2-deficient mice failed to accumulate MCs in the gastric mucosa after induction of food allergy (61). It should be noted that MCs derived from closely related species may differ in SphK usage because only SphK1 has been involved in cell migration toward Ag in the rat MC line RBL-2H3 (30). In this regard, RBL-2H3 cells are more akin to the HuMC line LAD2 as well as cord blood-derived HuMCs (31), which showed dependency on SphK1 in their migration to Ag. However, migration of HuMCs to Ag, unlike rodent MC migration, was slow (within 24 h) (31) and was not observed within 4 h, a time period long enough for their robust migration to SCF (data not shown). This finding suggests that the dependency on SphK1 in HuMCs, unlike RBL-2H3 cells, may not be a direct consequence of FceRI engagement, but of secreted chemokines or cytokines. Thus the mechanisms and regulation of migration toward Ag vary between species, and the involvement of SphK isoform usage is also highly complex within and across species.

In summary, our findings conclusively demonstrate a more general role for SphK2 in murine MC function in vitro and in vivo, whereas HuMCs revealed a dominant role for SphK1. However, considering that the expression and/or function of SphK1 and SphK2 can be influenced by environmental cues, one cannot conclude that the dominance of SphK1 or SphK2 in HuMCs or mouse MCs, respectively, can be generalized to all MCs under all circumstances and for all MC responses. It has been well documented that mature in vivo–differentiated MCs differ functionally from in vitro–differentiated MCs, presumably owing to environmentally driven phenotypic differences and maturation (62, 63).

Studies to define SphK expression in in vivo–differentiated HuMCs would be of considerable value in determining SphK isoform usage in specific tissues. On the basis of such studies, specific inhibitors for SphK1 or inhibitors for both SphK1 and SphK2 could deserve some consideration for blocking allergic responses.

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References


