

Sphingosine Kinase1 Is Pivotal for FcεRI-Mediated Mast Cell Signaling and Functional Responses In Vitro and In Vivo

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Mast cell degranulation is pivotal to allergic diseases; investigating novel pathways triggering mast cell degranulation would undoubtedly have important therapeutic potential. FcεRI-mediated degranulation has contradictorily been shown to require SphK1 or SphK2, depending on the reports. We investigated the in vitro and in vivo specific role(s) of SphK1 and SphK2 in FcεRI-mediated responses, using specific small interfering RNA-gene silencing. The small interfering RNA-knockdown of SphK1 in mast cells inhibited several signaling mechanisms and effector functions, triggered by FcεRI stimulation including: Ca²⁺ signals, NFκB activation, degranulation, cytokine/chemokine, and eicosanoid production, whereas silencing SphK2 had no effect at all. Moreover, silencing *SPHK1* in vivo, in different strains of mice, strongly inhibited mast cell-mediated anaphylaxis, including inhibition of vascular permeability, tissue mast cell degranulation, changes in temperature, and serum histamine and cytokine levels, whereas silencing *SPHK2* had no effect and the mice developed anaphylaxis. Our data differ from a recent report using *SPHK1*^{-/-} and *SPHK2*^{-/-} mice, which showed that SphK2 was required for FcεRI-mediated mast cell responses. We performed experiments in mast cells derived from *SPHK1*^{-/-} and *SPHK2*^{-/-} mice and show that the calcium response and degranulation, triggered by FcεRI-cross-linking, is not different from that triggered in wild-type cells. Moreover, IgE-mediated anaphylaxis in the knockout mice showed similar levels in temperature changes and serum histamine to that from wild-type mice, indicating that there was no protection from anaphylaxis for either knockout mice. Thus, our data strongly suggest a previously unrecognized compensatory mechanism in the knockout mice, and establishes a role for SphK1 in IgE-mediated mast cell responses. *The Journal of Immunology*, 2009, 183: 221–227.

Allergic diseases are characterized by elevated IgE Abs that, in the presence of an allergen, activate the high affinity IgE receptor (FcεRI) to release inflammatory mediators from mast cells. As mast cell degranulation is pivotal to allergic diseases, investigating novel pathways that trigger mast cell degranulation could have important therapeutic potential (1–3).

The strongest known physiological stimulus for mast cell activation is cross-linking of the high-affinity receptor for IgE (FcεRI), leading to exocytosis of granules containing preformed inflammatory mediators and the de novo synthesis of pharmacologically active eicosanoids and proinflammatory cytokines and chemokines (1–3). It is well established that these mediators lead to hypersensitivity responses, as well as the propagation of inflammation.

In the last few years, it has become clear that sphingolipids are sources of important signaling molecules. In particular, the sphin-

golipid metabolite, sphingosine-1-phosphate (S1P),³ has been implicated in a variety of cellular processes, such as cell differentiation, apoptosis, and proliferation (4–7), as well as in the regulation of the immune responses (8). Interestingly, S1P levels are elevated in the bronchoalveolar lavage fluid of asthmatic patients after Ag challenge, suggesting that S1P is relevant in allergic responses (Ref. 9, reviewed in Ref. 10).

We and others have shown that in human, rat, and mouse mast cells, sphingosine kinase1 (SphK1) is required for FcεRI to trigger intracellular signals and proinflammatory responses (11–13). Moreover, we recently showed that SphK1 is one of the earliest genes to be activated during IgE-mediated mast cell priming (14), which is a prerequisite for allergic diseases. In contrast, a recent study, using mast cells-derived from SphK1 or SphK2 knockout (KO) mice, suggests that SphK2 is the isoform used by FcεRI to mediate degranulation and cytokine and eicosanoids production (15). In contrast, a more recent study using small interfering RNA (siRNA) to silence the *SPHK* genes in human mast cells, shows that SphK1, but not SphK2, plays a critical role in Ag-induced degranulation migration and cytokine and eicosanoids production, with SphK2 playing a lesser role and only for the partial secretion of TNF-α (16). Thus, given the differences between silencing *SPHK* genes in wild-type cells and those cells where the kinases were deleted in the preimplantation embryo, it is possible that the differences observed are due to a compensatory mechanism during embryonic development of the KO mice and that SphK1 may indeed also be required for mouse mast cell degranulation and for the

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³ Abbreviations used in this paper: S1P, sphingosine 1 phosphate; SphK, sphingosine kinase; siRNA, small interfering RNA; PCA, passive cutaneous anaphylaxis; HSA, human serum albumin; KO, knockout.

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release of other proinflammatory mediators from murine mast cells in vitro and in vivo.

In this study we have specifically silenced *SPHK1* or *SPHK2* in vitro and in vivo, using specific siRNAs, and demonstrate that SphK1, but not SphK2, is indeed required for the effector responses triggered by Ag/FcεRI in murine mast cells. Moreover, silencing *SPHK1* in vivo strongly inhibited mast cell IgE/Ag-mediated anaphylaxis, whereas silencing *SPHK2* had no protective effect at all. Our data indicate that SphK1 is the major isoform responsible for triggering the release of proinflammatory mediators from mast cells in vitro and in vivo, and validates SphK1 as a potential novel therapeutic target for mast cell-mediated allergic/inflammatory responses. Moreover, our results have another potential impact: the observation that siRNA methodology can generate results different to those of gene-knockout methods, which has not been well-documented.

Materials and Methods

Culture of mast cells derived from BALB/c and 129Sv × C57BL6 mice

Murine mast cells were isolated from the peritoneal cavity and cultured as previously described (17). Another source of mast cells was derived from bone marrow: total bone marrow samples from femurs of mice were cultured in RPMI 1640 medium supplemented with 10% FCS, 10 ng/ml IL-3, 20 ng/ml SCF, 2 mM L-glutamine, 100 U/ml penicillin, and streptomycin in a 5% CO₂ atmosphere. After 8 wk, bone marrow-derived mast cells were collected and tested for *c-kit* and FcεRI expression by flow cytometry using anti-*c-kit* (BD Biosciences) and anti-FcεRI (eBioscience) Abs.

For the siRNA studies, cells were incubated for 48 h with 1 μg/ml siRNAs for the mSphK1 and mSPHK2, and mixed with Lipofectamine (Invitrogen) for transfection. siRNA sequence for the mSphK1 is: (GGGCAAGGCUCUGCAGCUCdT and GAGCUGCAGAGCCUUGCCdT); siRNA sequence for the mSphK2 is: (GGCUAAGAUCUAUCAUdT and AUGAUGAUCUUAGCCdT).

In vitro assays

Mast cells were sensitized with 1 μg/ml mouse DNP-specific IgE (Sigma-Aldrich) overnight. Then cells were collected, washed, resuspended in RPMI 1640–1% FBS, and activated with 1 μg/ml Ag, DNP-human serum albumin (HSA) (Sigma-Aldrich).

β-hexosaminidase β-hexosaminidase released by FcεRI-triggered mast cell degranulation was measured by a colorimetric assay as previously described (11, 13). In brief, 2 × 10⁵ cells/sample were sensitized with IgE overnight and stimulated with DNP-HSA for 30 min. Samples from the supernatant and total cell lysates were analyzed. The amount of β-hexosaminidase release (in the supernatant) was determined by measuring the OD at 400 nm and represented as a percentage of total enzyme.

Cytokine/chemokine detection Supernatants from mast cells (2 × 10⁶ cells/sample) were collected 24 h after FcεRI stimulation and stored at –20°C until use. TNF-α, IL-1β, IL-3, IL-5, IL-6, IFN-γ, IL-8, MCP1, MIP1α, MIP1β, eotaxin-1, and eotaxin-2, were evaluated by ELISA (R&D Systems) or using a multiplex immunoassay (Cytokine 20-Plex, BioSource) with the Bio-Plex System (Bio-Rad) following the manufacturer's instructions.

Cytosolic Ca²⁺ measurement Cytosolic calcium was measured as described previously (11, 13). In brief, IgE-sensitized cells were loaded with fura2-AM; labeled cells were placed in a prewarmed cuvette at 37°C in a spectrofluorimeter chamber. DNP-HSA was added to the cuvette and the release of calcium from internal stores was monitored by the changes in fluorescence.

Sphingosine kinase assay SphK activity was measured by a radiometric assay as described previously (11, 13).

PGE₂ production PGE₂ production was measured by the Biotrak PGE₂ system (Amersham Biosciences, GE Healthcare).

LTB₄ production LTB₄ production was measured by the Biotrak leukotriene B₄ enzyme immunoassay system (Amersham Biosciences, GE Healthcare).

NFκB activity NFκB activity was analyzed using the Mercury TransFactor-“Profiling Kit-Inflammation” (BD Biosciences) following the manufacturer's instructions and as previously described (18).

Protein expression analysis: gel electrophoresis and Western blots Forty micrograms of protein from total cell lysates/samples were resolved in 10% polyacrylamide gels (SDS-PAGE) under denaturing conditions and then transferred to 0.45 μm nitrocellulose membranes as previously stated (11, 13). The blots were probed using the polyclonal anti-SphK1 made in-house and used as previously described (11); against SphK2 (Exalpha Biologicals); against α-tubulin (Upstate Biotechnology) for loading control. Bands were visualized using anti-IgG HRP-conjugated secondary Abs, and the ECL Western Blotting Detection System (GE Healthcare).

siRNA administration and gene-silencing in vivo

Experiments were performed in two strains (BALB/c, and 129Sv × C57BL6 mixed background) 8- to 10-wk-old mice obtained from the Laboratory Animal Holding Unit, National University of Singapore. The study was conducted in accordance with the University of Singapore Guidelines for animal experimentation. Mice were lightly anesthetized and injected i.v. with the siRNA, following a protocol we recently developed (19); for the injections, the tail was immersed in warm water (40°C) for 10 s to dilate the tail veins. Synthetic siRNA (4 μg of siRNA in 0.2 ml of PBS) was injected into one of the lateral tail veins using a 30G needle. The siRNA injection was repeated after 24 and 48 h, to achieve a significant SphK gene-silencing effect. The siRNA-sequences were the same as for the in vitro experiments.

Induction and measurement of IgE-mediated PCA

Mice were anesthetized and injected s.c. in the dorsal skin with mouse anti-DNP IgE (20 ng in 20 μl saline). Saline alone was used as negative control. Mice were injected i.v. 24 h later with 100 μg of DNP-HSA with 1% Evans blue dye. Vascular permeability was visualized 60 min later by the blue staining of the injection sites on the reverse side of the skin. Skin samples were also harvested and Evans blue extracted from the tissue, using 0.5 ml of formamide at 55°C for 48 h and quantified by OD at 610 nm.

Induction and measurement of anaphylactic shock

Mice were injected i.v. in the tail vein with mouse anti-DNP IgE (200 μg in 200 μl saline). Saline alone was used as negative control. Twenty-four hours later, mice were challenged with 100 μg of DNP-HSA. Changes in body temperature associated with systemic anaphylaxis were monitored every 10 min, by measuring changes in rectal temperature, using a rectal probe coupled to a digital thermometer (Natsume Seisakusyo). At the end of the experiment (120 min after challenge), mice were sacrificed and plasma collected and stored at –20°C until used. IL-6, IL-3, and TNF-α protein levels in the plasma were evaluated using ELISA (R&D Systems) following the manufacturer's instructions. Histamine levels in the plasma were evaluated using ELISA (IBL), following the manufacturer's instructions. Portions of lungs were excised and fixed with 10% buffered formalin (Sigma-Aldrich), dehydrated, and embedded in paraffin for histological examination. Sections (5 μm) were stained with H&E and evaluated by light microscopy and documented by photographs.

Histological analysis of mast cells

Skin samples were fixed in 10% formalin and 4-μm paraffin sections were stained with Toluidine blue and examined with an Olympus microscope (model BX 51) using ×10, ×40, and ×100 uplan Apo lenses. Mast cell numbers in the skin were calculated by counting the cells in six different sections, derived from six mice, under light microscopy. The percentage of degranulated mast cells in the skin was calculated by observing the number of cells with >10% extrusion of granules as previously described (20, 21).

Statistical analysis

Statistical differences between control and treatment groups were calculated using unpaired Student's *t* test.

Generation of SphK^{−/−} mice

Knockout mice were generated by Genoway. In brief, for *SPHK1* knockout, a targeting vector for homologous recombination was generated that contained at the 5' end a diphtheria toxin A negative selection cassette to favor the homologous recombination event, a 5' homologous region consisting of 2.8kb homologous *SPHK1* sequences including exon 1 and 2, a Neo-cassette instead of exons 3–6, and a 3' homologous region consisting of 5.9kb of the subsequent genomic sequence.

For *SPHK2* knockout, a targeting vector was generated that contained at the 5' end a diphtheria toxin A negative selection cassette, a 5' homologous region consisting of 6.1kb homologous *SPHK2* sequences including exons

1–3, a Neo-cassette flanked by two LoxP sites instead of exons 4–7, and a 3' homologous region consisting of 1.8kb targeted *SPHK2* sequences including exon 8.

Gene targeted embryonic stem cells were isolated and used to produce chimeric male mice which passed the disrupted allele to their offsprings. Heterozygous matings produced viable mice homozygous for the mutation. Mice were backcrossed to C57BL/6 in an accelerated backcrossing procedure by Charles River Laboratories.

Results

We first stimulated murine mast cells by FcεRI cross-linking and looked at the subcellular localization of the SphK isoforms, and found that FcεRI cross-linking triggers the translocation of SphK1 from the cytosol to the plasma-membrane periphery; however, the localization of SphK2 remained unaltered (Fig. 1*a*). We then went on to specifically knockdown SphK1 or SphK2 by RNA-interference (Fig. 1*b*), and measured SphK activity and S1P generation: FcεRI-triggered SphK and S1P was substantially reduced in cells where SphK1 had been knocked down, whereas silencing SphK2 had no effect on the FcεRI-mediated SphK activity (Fig. 1, *c* and *d*). After which, we investigated the role of each of the SphK-isoforms in FcεRI-mediated activation of intracellular signaling pathways essential for mast cell degranulation, such as FcεRI cell surface expression, calcium mobilization, and the release of granular contents. Using siRNA knockdown, we show that neither SphK1 nor SphK2 is required for FcεRI cell surface expression (Fig. 2*a*). However, SphK1 was essential for the FcεRI-triggered initial rise in cytosolic calcium signals (Fig. 2*b*) and also for mast cell degranulation (Fig. 2*c*), whereas silencing SphK2 had no effect at all on any of the FcεRI-mediated responses (Fig. 2). FcεRI stimulated human mast cells to produce substantial amounts of PGD₂ and LTC₄ in a SphK1-dependent manner (Fig. 3, *a* and *b*), both of which are important lipid-derived mediators for mast cell-mediated responses. FcεRI triggered a substantial rise of cytokines, including IL-1β, IL-3, IL-5, IL-6, and TNF-α, and chemokines MIP-2, eotaxin-2, MCP-1, MIP-1α, and MIP1-β (Fig. 3, *c* and *d*). The production of several of the cytokines and chemokines was inhibited in cells pretreated with the SphK1-siRNA, whereas consistent with other results, the siRNA-SphK2 had no effect at all. These results were observed in mast cells derived from BALB/c and 129Sv × C57BL6 mice.

We investigated the effect of SphK-silencing on the activities of NFκB. FcεRI triggers the activation of NFκB; however, in cells pretreated with the siRNA specific for SphK1, but not that for SphK2, the FcεRI-stimulated NFκB activity was abrogated (Fig. 3*e*). In addition, the SphK1-siRNA prevented the generation of IL-1β, IL-3, IL-6, TNF-α, MIP-2, MCP-1, MIP-1α, and MIP1-β synthesis, whereas the SphK1-siRNA had no effect on FcεRI-triggered IL-5 or eotaxin-2 synthesis (Fig. 3, *c* and *d*). The siRNA for SphK2 had no effect at all. The results were similar in mast cells from BALB/c and 129Sv × C57BL6 mice.

We then silenced either SphK1 or SphK2, by siRNA in vivo transfection in adult mice as recently described (19), in two different backgrounds of mice, namely, in BALB/c and 129Sv × C57BL6 mice (Fig. 4*a*). We then investigated the role of each SphK-isoform in an anaphylactic response using the model of IgE-mediated passive cutaneous anaphylaxis (PCA) (22), the gold standard model of mast cell-mediated hypersensitivity. One of the key inflammatory events during an allergic reaction is an increase in vascular permeability (20–22). Thus, we sensitized mice s.c. with anti-DNP IgE Ab and then challenged i.v. 24 h later with DNP-HSA in saline-containing Evans blue to measure vascular permeability. The amount of Evans blue in control and challenged mice was quantified and showed the expected increase in vascular permeability after antigenic challenge. The PCA-triggered vascular

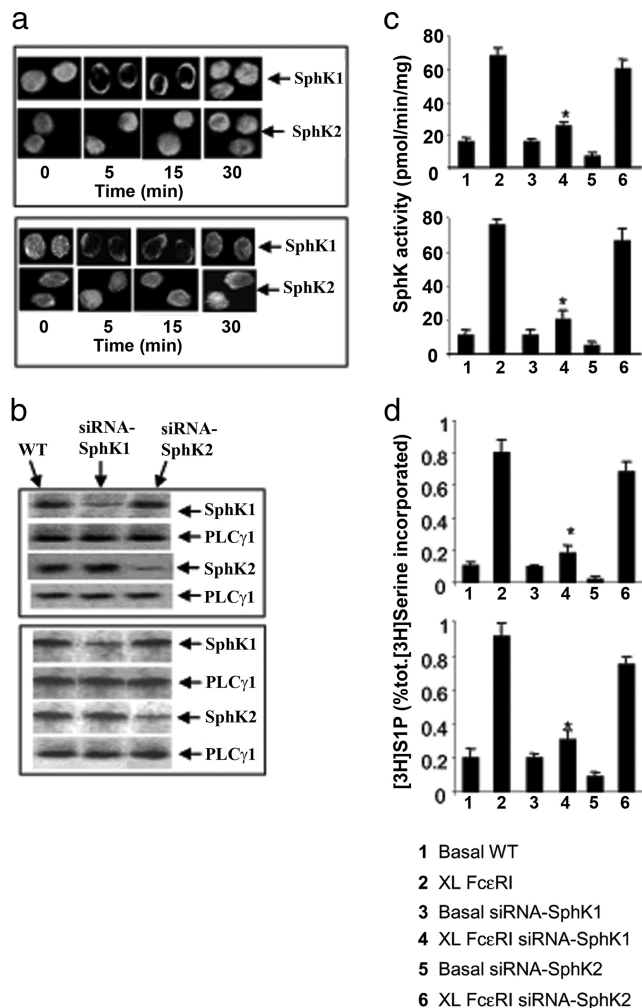


FIGURE 1. SphK1, but not SphK2, is activated by FcεRI in mouse mast cells. Mast cells derived from BALB/c (*top panels*), and 129Sv × C57BL6 (*bottom panels*) mice were stimulated by cross-linking FcεRI. *a*, Translocation of SphKs was measured by fluorescence microscopy, following FcεRI cross-linking for the times indicated in the figure. *b*, Mast cells were incubated with medium (WT) or with siRNA against SphK1 (siRNA-SphK1) or against SphK2 (siRNA-SphK2) for 48 h and the silencing effect was measured by Western blot. Results are typical from at least three independent experiments. SphK activity (*c*) and S1P generation (*d*) in mast cells was measured after 5 min of incubation from: 1, Nonstimulated untreated/control cells (Basal WT); 2, Following FcεRI cross-linking in untreated/control cells (XL FcεRI); 3, Nonstimulated cells pretreated with the siRNA against SphK1 (Basal siRNA-SphK1); 4, Following FcεRI cross-linking in cells pretreated with the siRNA against SphK1 (XL FcεRI siRNA-SphK1); 5, Nonstimulated cells pretreated with the siRNA against SphK2 (Basal siRNA-SphK2); 6, Following FcεRI cross-linking in cells pretreated with the siRNA against SphK2 (XL FcεRI siRNA-SphK2). Results are means ± SD of triplicate measurements and from at least three independent experiments. *, $p < 0.01$, compared with group 2.

permeability was considerably inhibited in mice pretreated with the SphK1-siRNA, whereas the PCA-response in mice pretreated with the SphK2-siRNA showed a similar level of vascular permeability as the positive control mice. Further analysis on the Evans blue levels in the skin confirmed the visual observation and revealed a significant decrease of vascular permeability only in mice pretreated with the SphK1-siRNA; similar results were observed in both mouse strains (Fig. 4*b*). To further test whether SphK1 was critical for mast cell degranulation in vivo, skin samples at the injection sites were removed

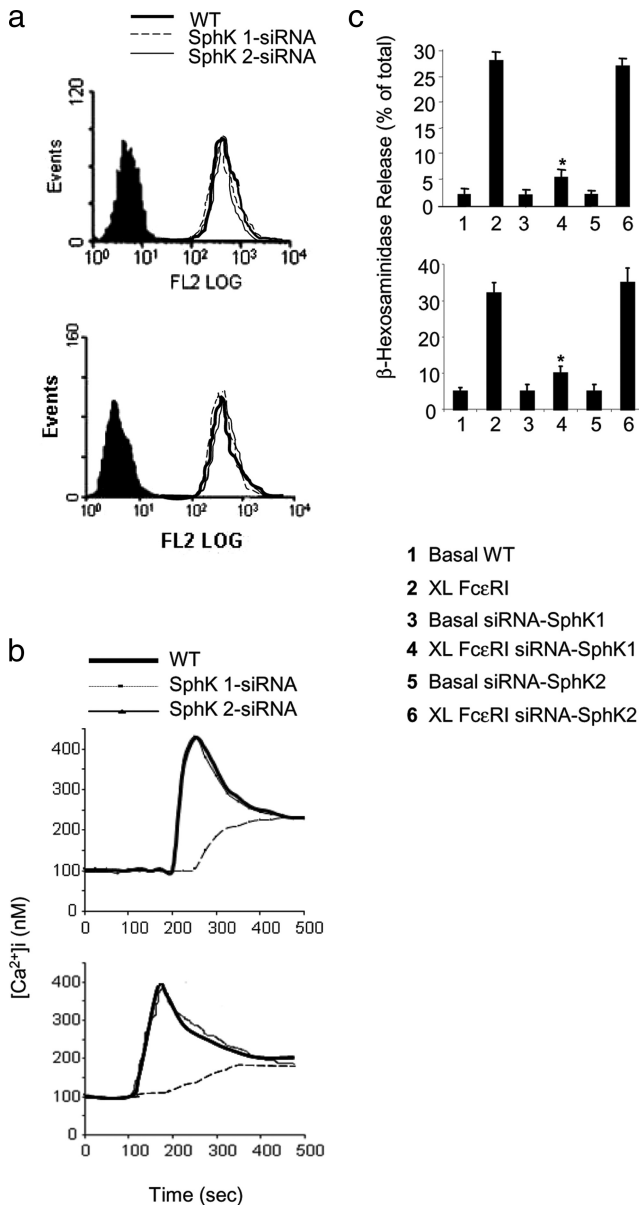


FIGURE 2. FcεRI-triggered intracellular signaling requires SphK1, but not SphK2. Mast cells derived from BALB/c (*top panels*), and 129Sv × C57BL6 (*bottom panels*) mice. *a*, Cell surface expression of FcεRI was measured in mast cells previously incubated with: medium alone (WT) or with medium supplemented with the siRNA against SphK1 (SphK1-siRNA) or with medium supplemented with the siRNA against SphK2 (SphK2-siRNA) for 48 h. Results are typical from at least three independent experiments. *b*, Cytosolic calcium was measured, following FcεRI cross-linking, in mast cells previously incubated with: medium alone (WT); or with medium supplemented with the siRNA against SphK1 (SphK1-siRNA); or with medium supplemented with the siRNA against SphK2 (SphK2-siRNA) for 48 h. Results are typical from at least three independent experiments. *c*, β-hexosaminidase release was measured after 30 min of incubation from: 1, Nonstimulated untreated/control cells (Basal WT); 2, Following FcεRI cross-linking in untreated/control cells (XL FcεRI); 3, Nonstimulated cells pretreated with the siRNA against SphK1 (Basal siRNA-SphK1); 4, Following FcεRI cross-linking in cells pretreated with the siRNA against SphK1 (XL FcεRI siRNA-SphK1); 5, Nonstimulated cells pretreated with the siRNA against SphK2 (Basal siRNA-SphK2); 6, Following FcεRI cross-linking in cells pretreated with the siRNA against SphK2 (XL FcεRI siRNA-SphK2). Results are means ± SD of triplicate measurements and from at least three independent experiments. *, *p* < 0.01, compared with group 2.

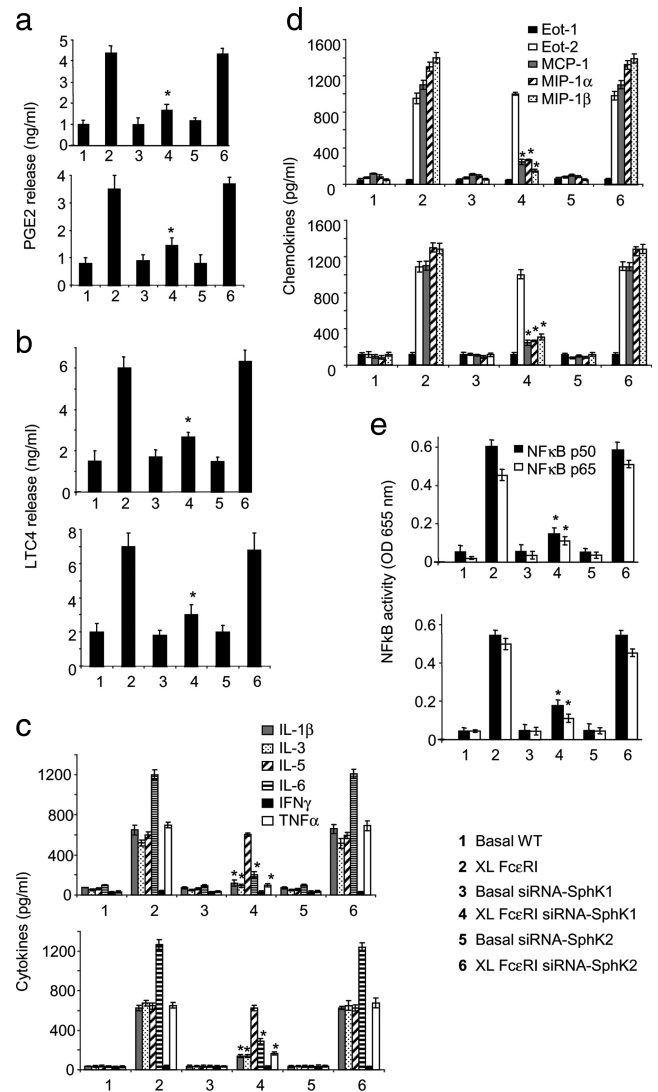


FIGURE 3. SphK1, but not SphK2, is required for FcεRI-triggered eicosanoid, proinflammatory cytokine/chemokine production, and NFκB activation. Mast cells derived from BALB/c (*top panels*), and 129Sv × C57BL6 (*bottom panels*) mice. PGE₂ (*a*); LTC₄ (*b*); cytokines (*c*); and chemokines (*d*) release was determined 24 h after FcεRI stimulation in: 1, Nonstimulated untreated/control cells (Basal WT); 2, Following FcεRI cross-linking in untreated/control cells (XL FcεRI); 3, Nonstimulated cells pretreated with the siRNA against SphK1 (Basal siRNA-SphK1); 4, Following FcεRI cross-linking in cells pretreated with the siRNA against SphK1 (XL FcεRI siRNA-SphK1); 5, Nonstimulated cells pretreated with the siRNA against SphK2 (Basal siRNA-SphK2); 6, Following FcεRI cross-linking in cells pretreated with the siRNA against SphK2 (XL FcεRI siRNA-SphK2). Results are means ± SD of triplicate measurements and from at least three independent experiments. *, *p* < 0.01, compared with group 2. *e*, NFκB was determined 30 min after FcεRI stimulation from cells treated as above 1–6. Results are means ± SD of triplicate measurements and from at least three independent experiments. *, *p* < 0.01, compared with group 2.

and examined histologically. The level of mast cell degranulation was very much reduced in the SphK1-silenced mice, whereas the siRNA-SphK2-silenced mice displayed similar responses to that of the positive control group; similar results were observed in both mouse strains (Fig. 4c).

We then investigated the role of silencing SphK1 or SphK2 in systemic anaphylactic shock. BALB/c and 129Sv × C57BL6

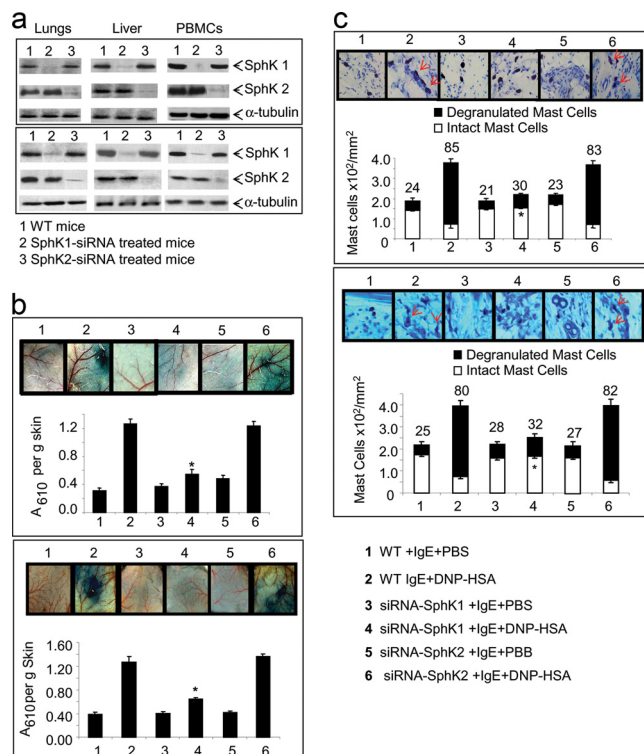


FIGURE 4. siRNA in vivo silencing. SphK1 is essential for IgE-mediated PCA. siRNA was injected into BALB/c and 129Sv × C57BL6 mice as described in *Materials and Methods*. *a*, Western blots of extracts from lungs, liver, and peripheral blood mononuclear cells (PBMCs), from: PBS/saline injected mice (WT); mice injected with siRNA against SphK1 (siRNA-SphK1); and mice injected with the siRNA against SphK2 (siRNA-SphK2). *Top panels* are from BALB/c; *bottom panels* are from 129Sv × C57BL6. Results are typical from at least three independent experiments. PCA was performed as described in *Materials and Methods*. For *b* and *c*, mice were treated as follows: 1, IgE-sensitized and saline challenged control mice (WT plus IgE plus PBS); 2, IgE-sensitized and DNP-HSA-challenged control mice (WT IgE plus DNP-HSA); 3, mice pretreated with the siRNA against SphK1 IgE-sensitized and saline challenged (siRNA-SphK1 plus IgE plus PBS); 4, mice pretreated with the siRNA against SphK1, sensitized with IgE and challenged with DNP-HSA (siRNA-SphK1 plus IgE plus DNP-HSA); 5, mice pretreated with the siRNA against SphK2 IgE-sensitized challenged (siRNA-SphK2 plus IgE plus PBS); 6, mice pretreated with the siRNA against SphK2, sensitized with IgE, and challenged with DNP-HSA (siRNA-SphK2 plus IgE plus DNP-HSA); *b*, Photographs of the reverse side of the skin, (*top panel* from BALB/c mice, *bottom panel* from 129Sv × C57BL6 mice), showing vascular permeability. The histograms shows the quantification of the vascular permeability by measuring the amount of Evans blues in the skin; *c*, microscopy photographs showing the histology of the skin (*top panel* from BALB/c mice, *bottom panel* from 129Sv × C57BL6 mice), stained with Toluidine-blue, showing in situ mast cell degranulation: arrows indicate degranulated mast cells; the histograms shows the quantification of the percentage of mast cells in the mice skin. Results are means ± SD; *n* = 10; *, *p* < 0.01, compared with group 2.

mice, which were injected i.v. with anti-DNP IgE Ab and challenged i.v. 16 h later, developed markedly reduced body temperature, reaching 30°C 30 min after antigenic challenge (Fig. 5*a*). The rapid decrease in body temperature was considerably reduced in mice pretreated with the SphK-1 siRNA. However, in mice pretreated with the SphK2-siRNA the drop in temperature was identical with the positive control mice (Fig. 5*a*). The effect of specifically silencing SphK1 in the induction of anaphylactic shock was also reflected in the plasma cytokine and chemokine concentrations (Fig. 5*b*), and histamine levels (Fig. 5*c*); *top panels*

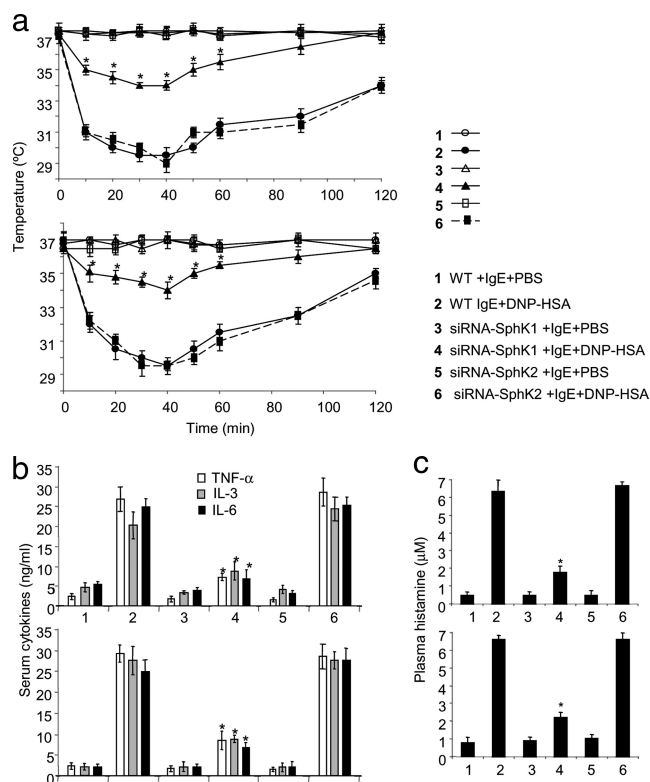


FIGURE 5. In vivo silencing SPHK1, but not SPHK2, protects mice from IgE-induced systemic shock. *Top panels* are results from BALB/c mice and the *bottom panels* are results from 129Sv × C57BL6 mice: *a*, Rectal temperature was measured at the times indicated after challenge in: 1, IgE-sensitized and saline-challenged control mice (WT plus IgE plus PBS); 2, IgE-sensitized and DNP-HSA-challenged control mice (WT IgE plus DNP-HSA); 3, Mice pretreated with the siRNA against SphK1 IgE-sensitized and saline-challenged (siRNA-SphK1 plus IgE plus PBS); 4, Mice pretreated with the siRNA against SphK1, sensitized with IgE and challenged with DNP-HSA (siRNA-SphK1 plus IgE plus DNP-HSA); 5, Mice pretreated with the siRNA against SphK2 IgE-sensitized and saline-challenged (siRNA-SphK2 plus IgE plus PBS); 6, Mice pretreated with the siRNA against SphK2, sensitized with IgE, and challenged with DNP-HSA (siRNA-SphK2 plus IgE plus DNP-HSA). *b*, Serum cytokines were examined at 120 min after challenge, for same treatment groups as above (1–6). *c*, Serum histamine was examined at 120 min after challenge, of same treatment groups as above (1–6). Data are mean ± SD; *n* = 10; *, *p* < 0.01 compared with group 2.

show data from BALB/c mice, whereas bottom panels show data from 129Sv × C57BL6 mice. Taken together, these data show that mice pretreated with the SphK1-siRNA were largely protected from IgE-mediated anaphylaxis.

As our data using the siRNA are in sharp contrast with a recently published work using *SPHK1*^{−/−} and *SPHK2*^{−/−} mice (15), we performed experiments in mast cells derived from *SPHK1*^{−/−} and *SPHK2*^{−/−} mice. Firstly, we show that mast cells derived from either KO mice do not express the relevant SphK-isoforms (Fig. 6*a*), we then show that the calcium response and degranulation, triggered by FcεRI-cross-linking, is similar in wild-type and mast cells derived from either knockout mice (Fig. 6, *b* and *c*). We then investigated the IgE-mediated anaphylaxis in the knockout mice, we show that either mice is negative for the corresponding KO-SphK (Fig. 6*d*), and found that following the induction of systemic anaphylaxis, similar temperature changes and serum histamine levels, were observed for both knockout mice as well as for the wild-type mice (Fig. 6, *e* and *f*), indicating that no protection was observed for either knockout mice.

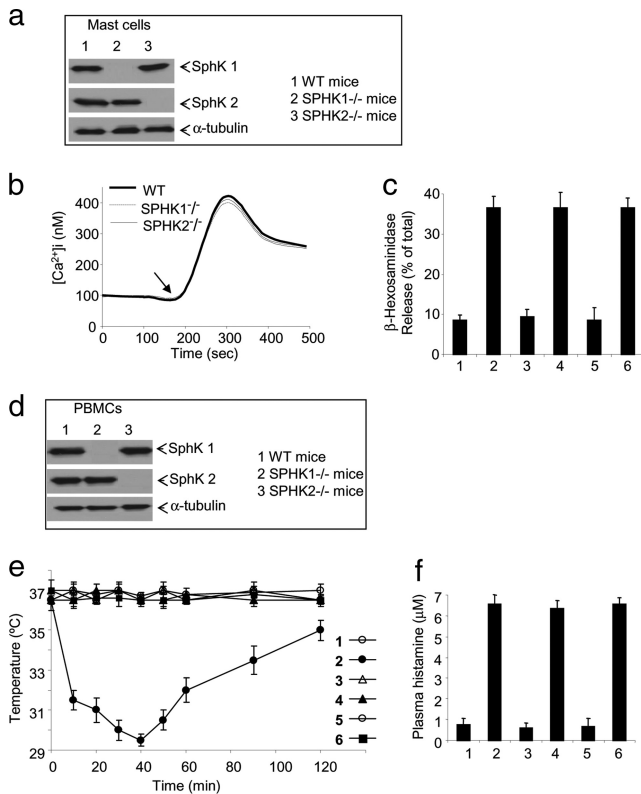


FIGURE 6. FcεRI triggered calcium signals and degranulation and systemic anaphylaxis *SPHK1*-null and *SPHK2*-null mice. *a*, Western blot analysis of SphK1 and SphK2 in mast cells. *b*, Cytosolic calcium was measured, following FcεRI cross-linking, in mast cells from *SPHK1*-null and *SPHK2*-null mice. Results are typical from at least three independent experiments. *c*, β-hexosaminidase release was measured after 30 min of incubation from: 1, Nonstimulated untreated/control cells (Basal WT); 2, Following FcεRI cross-linking in untreated/control cells (XL FcεRI); 3, Nonstimulated *SPHK1*-null cells (Basal *SPHK1*^{-/-}); 4, Following FcεRI cross-linking in *SPHK1*-null cells (XL FcεRI *SPHK1*^{-/-}); 5, Nonstimulated *SPHK2*-null cells (Basal *SPHK2*^{-/-}); 6, Following FcεRI cross-linking in *SPHK2*-null cells (XL FcεRI *SPHK2*^{-/-}). Results are means ± SD of triplicate measurements and from at least three independent experiments. *, *p* < 0.01 compared with group 2. IgE/Ag-mediated systemic anaphylaxis *SPHK1*-null and *SPHK2*-null mice: *d*, Western blot analysis of SphK1 and SphK2 in PBMCs. *e*, Rectal temperature was measured at the times indicated after challenge in: 1, IgE-sensitized and saline-challenged control mice (WT plus IgE plus PBS); 2, IgE-sensitized and DNP-HSA-challenged control mice (WT IgE plus DNP-HSA); 3, *SPHK1*-null mice IgE-sensitized and saline-challenged (*SPHK1*^{-/-} plus IgE plus PBS); 4, *SPHK1*-null mice sensitized with IgE and challenged with DNP-HSA (*SPHK1*^{-/-} plus IgE plus DNP-HSA); 5, *SPHK2*-null mice sensitized with IgE and saline-challenged (*SPHK2*^{-/-} plus IgE plus PBS); 6, *SPHK2*-null mice sensitized with IgE, and challenged with DNP-HSA (*SPHK2*^{-/-} plus IgE plus DNP-HSA). *f*, Serum histamine was examined at 120 min after challenge, of same treatment groups as above (1–6). Data are mean ± SD; *n* = 10; *, *p* < 0.01 compared with group 2.

Taken together, these data strongly suggest a previously unrecognized compensatory mechanism in the knockout mice, and that silencing SphK1 in adult mice using siRNA, clearly establishes a role for SphK1, but not SphK2, in the IgE-mediated mast cell degranulation and anaphylaxis.

Discussion

Recently, it has been suggested that FcεRI, activates both SphK1 and SphK2 to drive different responses in mouse and human mast cells (15, 16). However, this is in contradiction with previous re-

ports showing that SphK1 is activated by FcεRI, and mediates proinflammatory responses, in rat, mouse, and human mast cells (11, 13). In this study, we show that only SphK1 is activated by FcεRI in mast cells derived from two different strains of mice, BALB/c, and 129Sv × C57BL6 mice, and provide solid evidence for the specific role of SphK1 in FcεRI-mediated calcium mobilization and degranulation in vitro. During an anaphylaxis FcεRI-mediated mast cell activation results in degranulation, and sustained de novo production of a number of cytokines and eicosanoids, which contribute to the pathology underlying allergic disease (20–24). As SphK1 is a key enzyme in degranulation and elevates the cytoplasmic Ca²⁺ levels necessary for cPLA₂ and for NFκB activation, we investigated the ability of SphKs to mediate the production of eicosanoids and cytokines. FcεRI triggered a substantial rise of the eicosanoids PGE₂ and LTC₄, as well as the cytokines IL-1β, IL-3, IL-5, IL-6, and TNF-α, and chemokines MIP-2, eotaxin-2, MCP-1, MIP-1α, and MIP-1β. The production of PGE₂, LTC₄, and of several of the cytokines and chemokines was inhibited in cells pretreated with the SphK1-siRNA, whereas consistent with other results, the siRNA-SphK2 had no effect at all.

Taken together, these results therefore demonstrate that SphK1, but not SphK2, is able to mediate a wide range of mast cell-mediated responses. It has been suggested that while the production of proinflammatory cytokines in mast cells is NFκB (p50 and p65) dependent (25, 26), the induction of Th2-like cytokines is NFκB independent (27). We therefore investigated the effect of SphK silencing on the activities of NFκB. FcεRI triggers the activation of NFκB; however, in cells pretreated with the siRNA specific for SphK1, but not that for SphK2, the FcεRI-stimulated NFκB activity was abrogated. This is in agreement with an earlier observation that Ca²⁺ signal amplitude and frequency play a role in NFκB activation (28). Consistent with this, the SphK1-siRNA prevented the generation of IL-1β, IL-3, IL-6, TNF-α, MIP-2, MCP-1, MIP-1α, and MIP-1β synthesis, which are dependent on NFκB activity (25), whereas the SphK1-siRNA had no effect on FcεRI-triggered IL-5 or eotaxin-2 synthesis, which is independent of the NFκB pathway (27).

Taken together, our in vitro data is in agreement with previous studies in rat, mouse, and human mast cells, which showed that SphK1 is critical for mast cell-mediated responses (11, 13). However, the relevance of these in vitro studies to the physiological role of SphK1 is unclear as a recent study, using murine mast cells from *SPHK1*^{-/-} and *SPHK2*^{-/-} mice, showed that SphK2 is the important isotype for murine mast cell-mediated responses (15).

Genetic robustness enables any organism to tolerate changes in its environment or genetic composition (29). The mechanisms may be explained by genetic buffering, where alternative mechanisms are present for a particular biological process in an organism, or by functional complementation, where genes are at some point redundant in their particular biological task due to the presence of two or more isoforms (30, 31). Two genes are said to be redundant if they can partially, or fully, compensate with each other functions and it may be considered that the genetic redundancy gives an organisms an advantage, as having two genes performing similar biological tasks, being able to compensate for each other should one of the genes be mutated (32). In our case, the SphK isoforms execute a similar task, but under normal circumstances their regulation may be different and their activation may be differentially modulated in different signal-transduction pathways. However, as Nowak et al. (33) mentioned on the evolution of genetic redundancy, we may conclude that the presence of SphK isoforms would certainly increase the robustness of the knock out mice to function normally in more than one state or situation (34), and this could well be the reason why our data are in sharp contrast with previous

reports that demonstrate a clear dependence on SphK1 expression for mast cell-mediated responses. Thus, suggesting that possible adaptation and/or the existence of compensatory mechanisms during embryonic development of the knockout mice, because complete deficiency of SphK activity in double knockout is embryonic lethal, but mice lacking either isoform appear normal (34, 35).

To overcome this genetic robustness in the *SPHK1* and *SPHK2* KO mice, we have adopted RNA-interference technology to assess the individual role of each SphK isoform in wild-type mice. We selectively and specifically silenced the *SPHK1* or *SPHK2* expression using siRNA (19), and investigated the specific roles of SphK1 or SphK2 in passive cutaneous and systemic anaphylactic shock in both BALB/c and 129Sv $\times$ C57BL6 mice. The rapid cutaneous and systemic inflammatory responses were considerably inhibited in SphK1-silenced mice. However, in *SPHK2*-silenced mice all the inflammatory parameters were identical with the positive control mice. Interestingly, we show in this study that in the *SPHK1*^{-/-} and *SPHK2*^{-/-} mast cells and mice the FcεRI-mediated degranulation and the IgE-mediated anaphylaxis are unchanged, indicating marked differences between the siRNA-gene silencing and the knockout mice.

In conclusion, the results presented in this study demonstrated that SphK1 is a key mediator of mast cell degranulation, leading to anaphylaxis. Moreover, our results show another important finding, that is, the observation that siRNA methodology can generate results different to those of gene-knockout methods, a phenomenon which has not been well-documented. The expanding view on the roles of mast cells in innate and adaptive immune responses, suggests a potential role for mast cells in various diseases including autoimmune diseases (36). Thus, suppression of mast cell function by SphK1 blockage offers a new potential therapeutic approach for diseases, such as allergic anaphylaxis and other diseases where mast cell activation may play a pathological role.

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Disclosures

The authors have no financial conflict of interest.

References

- Galli, S. J., and B. K. Wershil. 1996. The two faces of the mast cell. *Nature* 381: 21–22.
- Galli, S. J., J. Kalesnikoff, M. A. Grimaldeston, A. M. Piliponsky, C. M. Williams, and M. Tsai. 2005. Mast cells as “tunable” effector and immunoregulatory cells: recent advances. *Annu. Rev. Immunol.* 23: 749–786.
- Bischoff, S. C. 2007. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nat. Rev. Immunol.* 7: 93–104.
- Choi, O. H., J. H. Kim, and J. P. Kinet. 1996. Calcium mobilization via sphingosine kinase in signalling by the FcεRI antigen receptor. *Nature* 380: 634–636.
- Meyer zu Heringdorf, D., C. J. van Koppen, and K. H. Jakobs. 1997. Molecular diversity of sphingolipid signalling. *FEBS Lett.* 410: 34–38.
- Prieschl, E. E., R. Csonga, V. Novotny, G. E. Kikuchi, and T. Baumruker. 1999. The balance between sphingosine and sphingosine-1-phosphate is decisive for mast cell activation after Fcε receptor I triggering. *J. Exp. Med.* 190: 1–8.
- Bornfeldt, K. E., L. M. Graves, E. W. Raines, Y. Igarashi, G. Wayman, S. Yamamura, Y. Yatomi, J. S. Sidhu, E. G. Krebs, S. Hakomori, et al. 1995. Sphingosine-1-phosphate inhibits PDGF-induced chemotaxis of human arterial smooth muscle cells: spatial and temporal modulation of PDGF chemotactic signal transduction. *J. Cell Biol.* 130: 193–206.
- Baumruker, T., and E. E. Prieschl. 2002. Sphingolipids and the regulation of the immune response. *Semin. Immunol.* 14: 57–63.
- A. J. Ammit, A. T. Hastie, L. C. Edsall, R. K. Hoffman, Y. Amrani, V. P. Krymskaya, S. A. Kane, S. P. Peters, R. B. Penn, S. Spiegel, and R. A. Panettieri. 2001. Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J.* 15: 1212–1214.
- Oskeritzian, C. A., S. Milstien, and S. Spiegel. 2007. Sphingosine-1-phosphate in allergic responses, asthma and anaphylaxis. *Pharmacol. Ther.* 115: 390–399.
- Melendez, A. J., and A. K. Khaw. 2002. Dichotomy of Ca²⁺ signals triggered by different phospholipid pathways in antigen stimulation of human mast cells. *J. Biol. Chem.* 277: 17255–17262.
- Jolly, P. S., M. Bektas, A. Olivera, C. Gonzalez-Espinosa, R. L. Proia, J. Rivera, S. Milstien, and S. Spiegel. 2004. Transactivation of sphingosine-1-phosphate receptors by FcεRI triggering is required for normal mast cell degranulation and chemotaxis. *J. Exp. Med.* 199: 959–970.
- Melendez, A. J., M. M. Harnett, P. N. Pushparaj, W. S. Wong, H. K. Tay, C. P. McSharry, and W. Harnett. 2007. Inhibition of FcεRI-mediated mast cell responses by ES-62, a product of parasitic filarial nematodes. *Nat. Med.* 13: 1375–1381.
- Jayapal, M., H. K. Tay, R. Reghunathan, L. Zhi, K. K. Chow, M. Rauff, and A. J. Melendez. 2006. Genome-wide gene expression profiling of human mast cells stimulated by IgE or FcεRI-aggregation reveals a complex network of genes involved in inflammatory responses. *BMC Genomics* 7: 210.
- Olivera, A., K. Mizugishi, A. Tikhonova, L. Ciaccia, S. Odom, R. L. Proia, and J. Rivera. 2007. The sphingosine kinase-sphingosine-1-phosphate axis is a determinant of mast cell function and anaphylaxis. *Immunity* 26: 287–297.
- Oskeritzian, C. A., S. E. Alvarez, N. C. Hait, M. M. Price, S. Milstien, and S. Spiegel. 2008. Distinct roles of sphingosine kinases 1 and 2 in human mast-cell functions. *Blood* 111: 4193–4200.
- Malbec, O., K. Roget, C. Schiffer, B. Iannascoli, A. R. Dumas, M. Arock, and M. Daeron. 2007. Peritoneal cell-derived mast cells: an in vitro model of mature serosal-type mouse mast cells. *J. Immunol.* 178: 6465–6475.
- Zhi, L., B. P. Leung, and A. J. Melendez. 2006. Sphingosine kinase 1 regulates pro-inflammatory responses triggered by TNFα in primary human monocytes. *J. Cell Physiol.* 208: 109–115.
- Pushparaj, P. N., C. S. H'Ng, and A. J. Melendez. 2008. Refining siRNA in vivo transfection: Silencing SPHK1 reveals its key role in C5a-induced inflammation in vivo. *Int. J. Biochem. Cell Biol.* 40: 1817–1825.
- Martin, T. R., S. J. Galli, I. M. Katona, and J. M. Drazen. 1989. Role of mast cells in anaphylaxis: evidence for the importance of mast cells in the cardiopulmonary alterations and death induced by anti-IgE in mice. *J. Clin. Invest.* 83: 1375–1383.
- Brightling, C. E., P. Bradding, F. A. Symon, S. T. Holgate, A. J. Wardlaw, and I. D. Pavord. 2002. Mast-cell infiltration of airway smooth muscle in asthma. *N. Engl. J. Med.* 346: 1699–1705.
- Dombrowicz, D., V. Flamand, K. K. Brigman, B. H. Koller, and J. P. Kinet. 1993. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor α chain gene. *Cell* 75: 969–976.
- Blank, U., and J. Rivera. 2004. The ins and outs of IgE-dependent mast-cell exocytosis. *Trends Immunol.* 25: 266–273.
- Kinet, J. P. 1999. The high-affinity IgE receptor (FcεRI): from physiology to pathology. *Annu. Rev. Immunol.* 17: 931–972.
- Marquardt, D. L., and L. L. Walker. 2000. Dependence of mast cell IgE-mediated cytokine production on nuclear factor-κB activity. *J. Allergy Clin. Immunol.* 105: 500–505.
- Stassen, M., C. Muller, M. Arnold, L. Hultner, S. Klein-Hessling, C. Neudorff, T. Reineke, E. Serfling, and E. Schmitt. 2001. IL-9 and IL-13 production by activated mast cells is strongly enhanced in the presence of lipopolysaccharide: NF-κB is decisively involved in the expression of IL-9. *J. Immunol.* 166: 4391–4398.
- Masuda, A., Y. Yoshikai, K. Aiba, and T. Matsuguchi. 2002. Th2 cytokine production from mast cells is directly induced by lipopolysaccharide and distinctly regulated by c-Jun N-terminal kinase and p38 pathways. *J. Immunol.* 169: 3801–3810.
- Dolmetsch, R. E., K. Xu, and R. S. Lewis. 1998. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 392: 933–936.
- Barbaric, I., G. Miller, and T. N. Dear. 2007. Appearances can be deceiving: phenotypes of knockout mice. *Brief Funct. Genomic Proteomic.* 6: 91–103.
- Gu, J., and X. Gu. 2003. Induced gene expression in human brain after the split from chimpanzee. *Trends Genet.* 19: 63–65.
- Gu, X. 2003. Evolution of duplicate genes versus genetic robustness against null mutations. *Trends Genet.* 19: 354–356.
- Kafri, R., A. Bar-Even, and Y. Pilpel. 2005. Transcription control reprogramming in genetic backup circuits. *Nat. Genet.* 37: 295–299.
- Nowak, M. A., M. C. Boerlijst, J. Cooke, and J. M. Smith. 1997. Evolution of genetic redundancy. *Nature* 388: 167–171.
- Zemann, B., N. Urtz, R. Reuschel, D. Mechtcheriakova, F. Bornancin, R. Badegruber, T. Baumruker, and A. Billich. 2007. Normal neutrophil functions in sphingosine kinase type 1 and 2 knockout mice. *Immunol. Lett.* 109: 56–63.
- Mizugishi, K., T. Yamashita, A. Olivera, G. F. Miller, S. Spiegel, and R. L. Proia. 2005. Essential role for sphingosine kinases in neural and vascular development. *Mol. Cell Biol.* 25: 11113–11121.
- Wedemeyer, J., M. Tsai, and S. J. Galli. 2000. Roles of mast cells and basophils in innate and acquired immunity. *Curr. Opin. Immunol.* 12: 624–631.