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Sphingosine-kinase 1 and 2 contribute to oral sensitization and effector phase in a mouse model of food allergy

Susanne C. Diesner^{a,b,c}, Ana Olivera^c, Sandra Dillahunt^c, Cornelia Schultz^a, Thomas Watzlawek^a, Elisabeth Förster-Waldl^b, Arnold Pollak^b, Erika Jensen-Jarolim^a, Eva Untersmayr^{a,*}, and Juan Rivera^{c,*}

^aDepartment of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria.

^bDepartment of Pediatrics and Adolescent Medicine, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria.

^cLaboratory of Molecular Immunogenetics, NIAMS, National Institutes of Health, Building 10, Bethesda, Maryland 20892, USA.

Abstract

Background—Sphingosine-1-phosphate (S1P) influences activation, migration and death of immune cells. Further, S1P was proposed to play a major role in the induction and promotion of allergic diseases. However, to date only limited information is available on the role of S1P in food allergy.

Objective—We aimed to investigate the role of sphingosine-kinase (SphK) 1 and 2, the enzymes responsible for endogenous S1P production, on the induction of food allergy.

Methods and results—Human epithelial colorectal Caco2 cells stimulated *in vitro* with S1P revealed a decrease of transepithelial resistance and enhanced transport of FITC labeled OVA. We studied the effect of genetic deletion of the enzymes involved in S1P production on food allergy induction using a mouse model of food allergy based on intragastrically (ig.) administered ovalbumin (OVA) with concomitant acid-suppression. Wild-type (WT), SphK1^{-/-} and SphK2^{-/-} mice immunized with OVA alone ig. or intraperitoneally (ip.) were used as negative or positive controls, respectively. SphK1- and SphK2- deficient mice fed with OVA under acid-suppression showed reduced induction of OVA specific IgE and IgG compared to WT mice, but had normal responses when immunized by the intraperitoneal route. Flow cytometric analysis of spleen cells, revealed a significantly reduced proportion of CD4⁺ effector T-cells in both SphK deficient animals after oral sensitization. This was accompanied by a reduced accumulation of mast cells in the gastric mucosa in SphK-deficient animals compared to WT mice. Furthermore, mouse mast cell protease-1 (mMCP-1) levels, an IgE-mediated anaphylaxis marker, were reliably elevated in allergic WT animals.

Conclusion—Modulation of the S1P homeostasis by deletion of either SphK1 or SphK2 alters the sensitization and effector phase of food allergy.

Address Correspondence: Juan Rivera, Laboratory of Molecular Immunogenetics, NIAMS-NIH, Building 10, Room 13C103, Bethesda, Maryland 20892, USA. Phone: 301.496.7592; Fax: 301.480.1580; juan_rivera@nih.gov and Eva Untersmayr, Department of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology and Immunology, Medical University of Vienna, 1090, Vienna, Austria. Phone: +43 1 40400 5121; Fax: +43 1 40400 5130; eva.untersmayr@meduniwien.ac.at.

*These authors contributed equally

Conflict of interest

The authors declare that they have no conflict of interest.

Keywords

Sphingosine-1-phosphate; food allergy; sphingosine kinase; mast cells; anti-ulcer medication; ovalbumin

1. INTRODUCTION

Food allergies are an increasing and, thus, important health concern in Western societies.[1] Despite substantial research effort in this field, the mechanisms, risk factors and events leading to sensitization towards food compounds are unclear.

Sphingolipids and their metabolites, especially sphingosine-1-phosphate (S1P), were proposed to play a major role in the induction and promotion of allergic diseases.[2–3] Additionally, single nucleotide polymorphisms of ORMDL3, a gene regulating S1P homeostasis, were recently found to be associated with the occurrence of asthma in childhood.[4–5] Thus, sphingolipids, which are necessary constituent of membranes and liquid ordered domains and are components of routinely ingested food compounds, gained attention in allergy research.[6–8] The metabolites of sphingolipid catabolism, ceramide, sphingosine and S1P, have bioactive functions influencing cell motility, calcium homeostasis, cell growth, cell death and differentiation and participate in immune cell activation.[6,9] S1P is not only an intracellular mediator, but it also functions extracellularly via its five membrane-bound receptors (S1PR_{1–5}).[10] S1PR1 expression on lymphocytes is required for the egress from thymus and secondary lymphoid organs.[11–12] While S1PR1 also influences marginal zone B-cell localization, S1PR3 affects B-cell chemotaxis in marginal zones of spleen and DC chemotaxis.[13–14] These receptors are also found on important effector cells relevant for allergy, eosinophils and mast cells.[15] The chemotaxis and effector responses of mast cells are regulated by S1PR1 and S1PR2, while eosinophils also display S1PR3 on their surface.[16]

Endogenous production of S1P is regulated by two sphingosine-kinases (SphK1 and SphK2) which phosphorylate sphingosine intracellularly.[17] Upon activation of the cell, S1P can be secreted and act in an autocrine/paracrine manner. Both SphKs have diverse tissue distribution and functions. In mouse tissue SphK activity was found to be high in lung, small intestine and moderate in spleen and stomach. In the small intestine and spleen the contribution of SphK1 to total SphK activity was up to 70% while SphK2 played only a minor role.[18]

Regarding their influence on allergy effector cell functions, mast cells are potent producers of intracellular and secreted S1P.[7] Although both SphK1 and 2 have been proposed to regulate S1P production in mast cells, the circumstances in which one or both may contribute to S1P production in mast cells is not well defined. Our previous work showed that loss of SphK2 in mast cells reduced S1P production and caused a substantial inhibition of FcεRI mediated degranulation as well as diminished production of IL6, IL13 and TNFα.[7] In contrast, SphK1-deficiency was responsible for lowered circulatory S1P *in vivo*, which also altered mast cell function.[7] Recently, we found that SphK1 increases the rate of recovery from anaphylactic shock, whereas SphK2 slows the rate of recovery, events associated with the control of blood pressure and subsequent renal clearance of histamine.[19] S1P levels were not only crucial for anaphylactic outcome, but were also found to be increased in bronchial alveolar lavage of asthmatic patients after allergen challenge and were significantly correlated with eosinophil numbers in BAL fluid.[2] Collectively, these findings have led to the conclusion, that circulating S1P and S1P-mediated trafficking may represent a key event in the development of allergic diseases. However, evidence for a role

of S1P in food allergy is scarce. A recent study revealed that S1P is important for migration of CD4⁺ T-cells from the spleen to the intestine, resulting in antigen specific intestinal allergic hypersensitivity in a murine food allergy model.[3] It was further described that intraperitoneal injections of S1P caused eosinophils to infiltrate the peritoneal cavity, and mast cells carrying antigen-specific IgE migrated towards the antigen in a S1P-dependent manner leading to their accumulation at the site of disease.[3] Importantly, intestinal epithelial cells express S1PRs,[20] which regulate barrier function and, thus, could affect the transport of antigens through the intestinal mucosa.

In the current study we investigated the influence of endogenous S1P production on food allergy induction. First, the effects of S1P on epithelial cell integrity and antigen uptake were analyzed *in vitro* using an established Caco2 cell model. Based on these findings we studied *in vivo* the influence of S1P alteration on the development of food allergy in a previously established food allergy model [21] using SphK1 and SphK2 deficient mice. This model is based on recent murine as well as human studies showing that the inhibition of peptic degradation of food allergens by the use of acid-suppressive medication favors the development of IgE mediated food allergy.[22–24] Feeding OVA as a food model allergen under concomitant acid-suppression was repeatedly shown to be associated with food allergy including elevated allergen specific IgE titers, Th2 cytokines and anaphylactic symptoms after oral allergen provocation.[21] Here we report that S1P alters tight junction integrity and OVA uptake by epithelial cells *in vitro*. Furthermore, SphKs proposed as decisive for oral sensitization against food allergens, as SphK1 and SphK2 null mice failed to increase IgE production after oral immunization. While SphK1 seems to be crucial for intestinal mast cell degranulation SphK2 may be necessary for mast cell accumulation in the gastrointestinal mucosa.

2. METHODS

2.1. Mice

All experiments were performed in accordance with National Institutes of Health (NIH) guidelines and an animal study proposal A010-04-03 approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases. SphK1 and SphK2 null mice (C57BL/6 × 129Sv, N5) were generated as described previously.[7,11,25] Wild type mice (WT) were littermates obtained from heterozygous mating pairs. Genotyping was performed as described.[7,25]

2.2. Reagents

Transwell filter plates with polycarbonate membrane inserts (pore size: 3 µm; membrane diameter: 12 mm) were purchased from Sigma. S1P (Cayman Chemicals) was dissolved in methanol and dried over a nitrogen stream. Aliquots were resuspended in 4 mg/mL bovine serum albumin (BSA, fatty acid free, Sigma) in DMEM medium. The following antibodies were used for flow cytometric staining: PE rat anti-mouse CD3 (clone 17A2; 0.25µg), PerCP/Cy5.5 rat anti-mouse CD4 (GK1.5; 0.25µg), FITC rat anti-mouse CD8a (53–6.7; 0.25µg, all from BioLegend), APC rat anti-mouse Foxp3 (FJK-16s; 0.5µg, eBioscience), APC hamster anti-mouse CD11c (HL3; 0.5µg, BD Pharmingen) and AlexaFluor® 647 rat anti-mouse CD19 (6D5; 0.25µg, BioLegend), FITC rat anti-mouse CD117 (c-Kit, 2B8; 0.25µg, BioLegend) and PE Armenian-hamster anti-mouse FcεRIα (MAR-1; 0.5µg, eBioscience).

RPMI-1640 (Gibco) cell culture medium was supplemented with 10% heat-inactivated fetal calf serum (FCS), 2mM/L L-glutamine, penicillin (100U/mL) and streptomycin (100µg/mL). OVA (Grade V, >98%) was purchased from Sigma-Aldrich. For Caco2 stimulation

experiments, DMEM medium supplemented with 2mM/L L-glutamine, 1% hepes, 1% non-essential amino acids, penicillin (100 U/mL) and streptomycin (100 µg/mL) was used.

2.3. *In vitro* CaCo2 cell uptake model

The colon carcinoma cell line Caco2 /Tc7 cells (a kind gift of Monique Rousset, INSERM, Paris, France), which exhibit an intestinal phenotype, was seeded in an inverted orientation on transwell filters and cultured thereafter for 21 days until the formation of a tight monolayer and a transepithelial resistance (TEER) of greater than 300 Ω·cm². Caco2 cells were either stimulated with 0.05 µM, 0.1 µM and 0.5 µM S1P or with BSA/medium as negative control for 1 and 5 hours. TEER was measured before and after the respective time points. Thereafter, FITC labeled OVA (50 µg/mL) was added to the apical side. After 60 minutes, transported FITC OVA from the basolateral side was measured at 485/530 nm. For calculation of OVA uptake, medium containing S1P or BSA but without FITC OVA was used as control and subtracted from the respective measured FITC OVA levels.

2.4. Immunization protocol

For the immunization experiments, WT, SphK1^{-/-} and SphK2^{-/-} mice were divided into 3 groups: the food allergy group (OVA ig.+ANT; n=7), a positive control group (OVA ip.; n=3–5) and a negative control group (OVA ig., n=5–6), which reveals similar immune responses as naïve animals,[21] but represents a more valid control group for our immunization regimen. According to our immunization protocol,[21] mice were treated intravenously (i.v.) with the proton-pump-inhibitor omeprazole (PPI; Losec®, AstraZeneca GmbH; 116µg diluted in 0.9% sodium chloride) on 3 consecutive days and immunized twice ig. with 0.2mg OVA mixed with 2mg sucralfate (Ulcogant®, Merck; groups OVA ig.+ANT). For control purposes, mice were injected i.p. with OVA (2µg OVA adsorbed to 1.3µg Al(OH)₃; groups OVA ip.) or were fed the allergen without acid-suppression (groups OVA ig.). As these mice do not develop food allergy receiving the allergen via the oral route and, thus, mimic the situation of non-allergic humans, who frequently ingest food allergens without developing adverse reactions the OVA ig. immunized group represents a valid negative control in our food allergy model. The immunization cycles were repeated for 4 times at 14 days intervals. Blood was drawn from the retrobulbar vein on days 0 and 56 and the serum was used for quantification of allergen specific IgE, IgG1 and IgG2a. Fifteen days after the last immunization, animals were subjected to oral OVA provocation using 5mg OVA/mouse (100µL). One hour after oral challenge mice were euthanized, sera were taken by heart puncture for measurement of mMCP-1, stomach and duodenum were removed and fixed in 4% formaldehyde (Sigma Aldrich) for further histological evaluation and spleens were taken under sterile conditions for FACS analysis.

2.5. ELISA and RBL assay

Murine sera taken on days 0 and 56 were tested for OVA specific IgE, IgG1 and IgG2a antibodies. OVA specific IgG1 was quantified with a Mouse anti-OVA-IgG1 ELISA kit (Shibayagi Co., Ltd. Gunma, Japan), according to manufacturer's instructions. Briefly, biotin-conjugated anti-mouse IgG1 was incubated either with a standard solution or with diluted sera samples (1:100) for 1 hour at room temperature. After repeated washing, horseradish-peroxidase-conjugated avidin was added to the wells, being followed by the substrate tetramethylbenzidine (TMB). The reaction was stopped with 1M H₂SO₄ and the absorbance was measured at 450–620 nm. OVA specific IgG2a was quantified as described previously.[21] Briefly, 1 µg OVA was coated on microtiter plates and blocked with 1% dried milk powder (DMP) in TBS 0.05% Tween. Mouse sera (dilution 1:100 in 0.1% DMP/TBST) were incubated over night. Antibodies were detected using a rat anti-mouse IgG2a (BD Biosciences, Franklin Lakes, NJ, 1:500) followed by a Peroxidase-labeled goat anti-rat IgG (Amersham, Buckinghamshire, UK; 1:1000). For detection, TMB

(tetramethylbenzidine, BD Biosciences) was added and the color reaction was measured at 405–630 nm. IgG1 and IgG2a antibody concentrations were calculated according to a standard curve after subtraction of pre-immune sera as background values.

OVA specific IgE was quantified with the Mouse OVA-IgE ELISA kit (MD Biosciences, St. Paul, MN, USA) following manufacturer's instructions. IgE concentrations were calculated according to a standard curve after subtraction of levels of pre-immune sera as background values. According to the manufacturer's protocol, the cross-reactivity of mouse IgG is less than 0.01%. To demonstrate that the OVA-IgE was biologically active, we used a degranulation assay in rat basophil leukemia cells (RBL-2H3 cells), a model for mast cells, which express the high-affinity IgE receptor FcεRI.[26] Briefly, an overnight culture of RBL-cells (4×10^5 cells/well) was incubated with sera (1:10) for 2 hours at 37°C. Cells were washed with Hepes/0.4% bovine serum albumin and stimulated with 1 μg/well OVA for 30 min to induce mast cell degranulation. As a measure of degranulation, we determined β-hexosaminidase activity by incubating supernatants (50 μl) with 100 μl of its substrate (3.5 mg Poly-N-acetylglucosamine (PNAG) /mL citrate buffer) for 90 min at 37°C. The reaction was stopped with 50 μL glycine buffer and the absorbance was monitored at 405 nm. Measured values were normalized and reported as a percent of the total β-hexosaminidase detected in cells lysed with triton-X, which was set 100%.

2.6. Mouse mast cell protease-1 detection

Sera taken after oral challenge were evaluated for the intestinal mast cell degranulation marker mMCP-1 by ELISA, according to manufacturer's instructions (eBioscience). Microtiter plates were coated with a capture antibody overnight at 4°C. After blocking, standards or sera (dilution 1:30) were added for 2 hours at room temperature. After incubation with a detection antibody and avidin-HRP, TMB was added and the color reaction was measured at 450–570 nm. mMCP-1 levels were calculated according to a standard curve.

Mouse MCP-1 was also measured in the sera of WT, SphK1- and SphK2-deficient mice before and after induction of passive systemic anaphylaxis. In brief, mice were intravenously injected with 3 μg DNP-specific IgE (H1-DNP-ε₂₆-IgE) and challenged 24 h later with 250 μg of antigen (DNP₃₆-HSA, Sigma-Aldrich). Injections were conducted on anesthetized mice (isoflurane (2%):oxygen (98%) mix for 2–3 min) in a closed chamber. Mice were euthanized 1 h after challenge by CO₂ inhalation and blood was immediately withdrawn by cardiac puncture. Blood for basal measurements was collected (less than 200 μl) 5 days prior to induction of anaphylaxis by lateral tail vein incision. Serum from the blood samples was obtained by centrifugation and immediately frozen at –80°C until assayed for mMCP-1 content. Mouse MCP-1 titers of sera taken 1 week before anaphylaxis experiments were subtracted from the levels measured in sera 1 hour after iv. injection of DNP-IgE and antigen to determine the levels of induced mMCP-1 release.

2.7. Spleen cell preparation, flow cytometry and cytokine evaluation

For single cell preparation, spleens were minced and passed through nylon meshes (cell strainer 40 μm, BD Biosciences). Erythrocytes were lysed with 5 mL ACK lysis buffer (Lonza) for 5 min and the reaction was stopped with medium. For flow cytometry, 2×10^6 spleen cells were washed in PBS/3% FCS. Surface antigens were detected with the respective aforementioned antibodies. Intracellular Foxp3 staining was done using the Foxp3 staining buffer kit (eBioscience) according to manufacturer's instructions. Cells were fixed with Fix/Perm buffer for 30 min at 4°C, washed and incubated with anti-Foxp3 antibody for 30 min. After repeated washing, cells were fixed in PBS/3% FCS containing 1% formaldehyde. The respective antibody isotypes were used as negative controls. Data was

collected by measuring 30,000 events on a FACS Calibur (BD Biosciences) and analyzed with CellQuest Pro (BD Bioscience) and FlowJo software (TreeStar).

For cytokine evaluation, spleen cells were stimulated as described recently.[21] In short, 4×10^5 cells per well were incubated with OVA (20 µg/mL), medium or concanavalin A (50 µg/mL) for 4 days. Supernatants were withdrawn and analyzed for cytokine production using the cytometric bead array mouse Th1/Th2 cytokine kit (BD Bioscience), following manufacturer's instruction. Mouse IFN γ , TNF α , IL4, IL5 capture bead suspension was incubated with either standard dilutions or test samples together with a PE labeled detection reagent for 2 hours. After washing, fluorescent intensity was analyzed by flow cytometry on a FACS Calibur. Cytokine concentrations were calculated according to a standard curve.

2.8. Histology

Formalin-fixed gastric and duodenal tissue samples were paraffin-embedded and sectioned at American HistoLabs, Inc. (Gaithersburg, MD). Mast cells were identified by toluidine blue staining and counted in tunica mucosa and tunica muscularis mucosae of the gastric glandular region and of the duodenum using a light microscope with 40 $\times$ magnification. Mast cell counts were calculated as mean number per field of view (FOV). Gastrointestinal tissue samples were further stained with hematoxylin and eosin for visualization of eosinophils.

2.9. Statistics

Statistical analysis was performed by a 2-tailed student's t test after logarithmic transformation with the SPSS 15.0 program and GraphPad Prism 5 software. A *p* value <0.05 was considered statistically significant.

3. RESULTS

3.1. S1P affects tight junction integrity and OVA uptake *in vitro*

To investigate the influence of e.g. autocrine and paracrine S1P secretion, we performed an *in vitro* transwell study. Caco2 cells cultured in an inverted orientation (Fig. 1A) for 21 days until the formation of a differentiated intestinal phenotype were stimulated with S1P or medium as control and subsequently evaluated for tight junction integrity and FITC OVA uptake. One hour stimulation with 0.5 µM S1P significantly decreased the transepithelial resistance (TEER) after 1 and 5 hours compared to the medium control (Fig. 1B). Being consistent with these results a significantly higher FITC OVA transport was found after S1P stimulation (Fig. 1C). To evaluate a possible dose-dependency of S1P, experiments were additionally performed with 0.05 and 0.1 µM S1P. While no significant differences of TEER levels were observed comparing the three concentrations, a trend towards an increased transepithelial OVA transport was seen in a dose-dependent manner, being especially pronounced after 1 h S1P stimulation (data not shown).

Altogether, we observed that S1P affects the epithelial permeability in *in vitro* Caco2 cell experiments underlined by an enhanced transepithelial OVA transport.

3.2. Orally immunized SphK1 and SphK2 null mice fail to produce increasing OVA specific IgE titers

We next evaluated whether changes in endogenous S1P homeostasis by the deletion of either one of the SphKs influences sensitization and effector phase of food allergy *in vivo*. After immunizing WT, SphK1- or SphK2-deficient mice using our oral or intraperitoneal immunization protocol (Suppl. figure 1),[21] mouse sera were screened for allergen specific antibody production after the last immunization. Significantly higher titers of OVA specific

IgE were found in all ip. immunized groups, regardless of the SphK1 or SphK2 status. WT animals being fed OVA under acid-suppression (OVA ig.+ANT) revealed significantly elevated levels of IgE compared to the control group being fed the allergen alone (OVA ig.) indicating a successful oral OVA sensitization. However, both SphK1 and SphK2 null mice orally immunized with OVA under anti-acid treatment did not reveal higher IgE titers than the negative control (OVA ig.) (Fig.2A). These findings were consistent with the results of RBL-assays (Fig.2B). RBL-2H3 cells, passively sensitized with murine sera, released significantly more β -hexosaminidase after allergen triggering when sera of ip. immunized WT, SphK1 and SphK2 null mice were used. However, from the groups orally immunized with OVA under acid suppression, only incubation with sera of WT mice led to an enhanced RBL-2H3 cells degranulation. In direct comparison, significant differences between the acid-suppressed WT and SphK1 animals ($P=0.0432$, WT OVA ANT compared to SphK1 OVA ANT) were observed. To validate our recent data,[21] we compared also in this study antibody titers of negative control animals (OVA ig.) with naïve mice. Comparable antibody levels and baseline biological antibody functionality was observed (eg. β -hexosaminidase release in RBL assays WT OVA ig: mean 0.5%, WT naïve: 0.04%) (data not shown).

In line with these results OVA specific IgG1 (Fig.3A) and IgG2a (Fig.3B) levels were significantly elevated after ip. immunizations in WT, SphK1 and SphK2 null mice. SphK2 null animals being injected OVA ip, however, revealed significantly lower levels of allergen specific IgG1 than the ip. immunized WT group ($P=0.0013$). As expected, oral sensitization of WT mice with anti-acid treatment also increased IgG1 and IgG2a antibody production but the response was not as robust as in the ip. sensitized group. Also the SphK1-deficient mice being fed OVA under acid-suppression revealed an increase in IgG1 titers comparable to those found in WT animals. However, in SphK2 null mice no elevation in IgG1 or IgG2a antibodies was detected.

3.3. Orally immunized SphK1 null mice reveal impaired intestinal mast cell degranulation

The intestinal mast cell degranulation marker mast cell protease-1 (mMCP-1) was evaluated in mouse sera 1 hour after oral OVA challenge (Fig. 4). Food allergic WT mice revealed higher mMCP-1 levels compared to the negative control WT animals. Induction of food allergy in SphK1-deficient animals did not result in marked changes in the levels of mMCP-1. Interestingly, all groups without functional SphK2 appeared to have higher mMCP-1 levels (not statistically significant), which could be related to an increased number of mast cells in these mice (Suppl. fig.2 and [7]). However, under passive systemic anaphylaxis challenge, WT, SphK1- and SphK2- deficient mice efficiently released mMCP-1. While the levels of mMCP-1 released upon challenge of SphK1-deficient animals were seemingly reduced (albeit not significantly), the findings demonstrate that there was no intrinsic defect in the ability to release mMCP1 under stimulation for any of the genotypes (Suppl. fig.3).

3.4. Changes of splenocyte population patterns of transgenic mice

In flow cytometric analysis, splenocytes were gated for CD3, CD4, CD8 and Foxp3 in order to discriminate between different T-cell subpopulations (Fig. 5A). Direct comparison of splenic CD4⁺ T-cells from mice being fed OVA with anti-ulcer medication or mice immunized ip. (Fig. 5B) indicated that WT mice had significantly more CD4⁺ T-cells than SphK1^{-/-} and SphK2^{-/-} mice, while in control mice (OVA ig.) no statistical differences were observed (Fig. 5B). In agreement, Sensken et al.[27] found no differences in the numbers of CD4⁺ T-cells in the spleen of naïve SphK2 null mice. When Foxp3⁺ regulatory T-cells were gated (Fig. 5C) a significantly lower fraction was observed in SphK1^{-/-} compared with WT and SphK2^{-/-} mice fed with allergen under anti-acid medication or after ip injections. Interestingly, the SphK2^{-/-} control group (OVA ig.) showed significantly

more T-regulatory cells compared to WT and SphK1^{-/-} animals. However, it appears that the difference in the relative numbers of CD4⁺Foxp3⁺ T-cells localized in the spleen of SphK1^{-/-} and SphK2^{-/-} mice is not associated with the inability of both genotypes to respond to a food allergen. Moreover, the differentiation of T-regulatory cells and their suppressive activity are known to be inversely related to the levels of S1P (via S1PR₁). [28] Collectively, these findings argue that the observed decrease in T-regulatory cell proportion in SphK1^{-/-} mice is independent of the decreased allergic response, since a reduction in the fraction of T-regulatory cells should have increased allergic responsiveness.

To investigate whether the impaired IgE production in acid-suppressed SphK1^{-/-} and SphK2^{-/-} mice could be caused by reduced numbers of antigen presenting cells, spleen cells were stained for CD11c⁺ and CD19⁺ B-cells. No differences were observed between WT and SphK1 null mice under either treatment, while, surprisingly SphK2 null mice revealed a higher proportion of CD11c⁺ cells in spleen compared to WT and SphK1 animals being fed OVA either with or without acid-suppression (Suppl. fig. 4A). The CD19⁺ B-cell fraction was also unaltered between WT and mutant mice irrespective of the different immunization protocols (Suppl. fig. 4B). Thus, since ip. injection of OVA elicited antibody responses in all genotypes (Figs. 2 and 3), the lack of antibody production in the orally sensitized mice of both genotypes does not appear to be associated with changes in the antigen presenting cell fraction.

Furthermore, we evaluated whether murine spleen cells respond adequately when being stimulated with OVA. Therefore, supernatants of OVA stimulated spleen cells were analyzed for cytokine production. Interestingly, acid-suppressed SphK2-null animals revealed significantly higher levels of IFN γ compared to the respective SphK1-deleted group (data not shown). Also the OVA ip. immunized SphK2-null mice showed significantly more IFN γ than the respective WT group. No significant differences of TNF α levels were observed between the mouse strains. However, with regard to Th2 cytokines, IL5 titers were under the detection limit of the applied assay. Although IL4 levels were measurable, they were below the lowest standard dilution (WT and SphK1 null mice) or even below the detection limit (SphK2 null mice). These low Th2 cytokine titers were not surprising as it is well known from pre-experiments that C57BL/6 $\times$ 129Sv, N5 mice with a mixed Th1 and Th2 background, have very low Th2 cytokine levels.

3.5. Mast cells accumulate less efficiently in gastric mucosa of SphK1^{-/-} and SphK2^{-/-} mice

Since mast cells are key effector cells in allergy, we examined the overall numbers of mast cells (Fc ϵ RI positive/ c-kit positive) in spleen using flow cytometry analysis. We observed that SphK2 animals had significantly more mast cells in the spleen compared to WT and SphK1 mice (Suppl. fig. 3).

To investigate the migration from systemic effector cells to the organs involved in food allergies, we harvested gastrointestinal tissue to localize both mast cells and eosinophils. Mast cells were identified by staining with toluidine-blue and were counted in tunica mucosa (Fig. 6A and B) and below the lamina propria, the tunica muscularis mucosae (Fig. 6C) of the glandular segment of the stomach and in duodenum. In WT mice immunized with OVA i.p. an increase was readily apparent when compared to negative control WT mice (Fig 6A, first line and 6B). In contrast, mast cell numbers were not increased in either SphK null mice under the same conditions. SphK2^{-/-} mice fed with OVA under acid-suppression had significantly less mucosal mast cells than WT and SphK1^{-/-} mice. Interestingly, ip. immunized and naïve SphK1^{-/-} and SphK2^{-/-} mice also showed decreased mast cell numbers in the tunica mucosa compared to the WT groups. In the muscularis mucosae elevated mast cell numbers were observed only in WT mice immunized with OVA ip and

the control animals (WT OVA ig.), whereas other immunization protocols showed no differences.

Mast cells in duodenum were found only sporadically without significant differences between the mouse strains irrespective of the different immunization regimens (data not shown). Gastrointestinal samples were also stained with hematoxylin/eosin for detection of eosinophils. Only a limited number of these effector cells was found in both gastric as well as in the small intestine (data not shown). These results differ from our previous studies where eosinophils were found to migrate into the gastric mucosa after oral sensitization of BALB/c mice.[23,29] This was not necessarily unexpected as the intensity of the allergic response is known to vary depending on the genetic background of the mouse.[30]

4. DISCUSSION

Food allergy is a disease associated with elevated allergen specific IgE antibody titers, Th2 cytokine preponderance and effector cell migration towards the food allergen after allergen exposure.[31] Here we report that in a food allergy model,[21] both SphK1^{-/-} and SphK2^{-/-} mice developed significantly reduced levels of IgE when immunized via the oral route. In contrast, all 3 groups of mice immunized i.p. with OVA showed comparable, high levels of OVA specific IgE and IgG2a, while the IgG1 response was significantly reduced only in the SphK2 null group. To determine the biological functionality of the measured OVA specific IgE antibodies we performed a functional assay with RBL cells and found the functional responses were associated with OVA specific IgE levels. Thus, we confirmed significantly elevated OVA specific IgE levels in the acid-suppressed WT animals and all ip. immunized groups. These findings indicate that the route of immunization determines the impact that SphK1 or SphK2 may have on IgE and IgG production. Since the proportion of antigen presenting cells was not directly affected by the loss of SphK1 and was even elevated in SphK2 null animals it could be hypothesized that the lack of SphKs may not have a direct influence on efficient antigen presentation. A more likely explanation is that the lack of S1P may affect the uptake of OVA across the gastrointestinal epithelium thus affecting antigen availability. This is supported by studies showing that sphingolipids and S1P receptors can alter the transepithelial resistance and the uptake of dextrans and peptides.[32] In agreement, our *in vitro* data in intestinal epithelial Caco2 cells suggest that a physiologically low S1P concentration decreases tight junction integrity and enhances OVA uptake. Based on recent findings that the amount of circulating antigen influences allergic responses, especially systemic anaphylaxis,[33] our data suggest an involvement of SphK1 and SphK2 on allergen uptake and, thus, in the sensitization phase of food allergy.

Recently, it was described that the spleen plays an important role during the sensitization phase and early period of food induced allergic diarrhea and that S1P dependent migration of CD4⁺ T-cells from the spleen to the intestine is crucial for the initiation of intestinal allergies.[3] We found that WT animals immunized with OVA ip. or ig. under acid suppression had a significantly higher fraction of CD4⁺ T-cells but not of CD4⁺Foxp3⁺ regulatory T-cells, suggesting an expansion of activated or effector CD4⁺ cells. In contrast, the number of CD3⁺CD4⁺ splenic T-cells in SphK1 and SphK2 null mice did not increase suggesting defective expansion or increased migration of CD4⁺ T-cells to the intestine. However, we did not find higher numbers of infiltrating immune cells in the gastrointestinal tract of SphK null mice, when compared to control or WT mice, making the latter possibility unlikely. It is known that infiltrating CD4⁺ T-cells produce cytokines and chemokines that contribute to the recruitment of mast cells to the intestine,[3] but mast cells were not increased at this site in SphK1 and SphK2 mice. This argues that the reduced numbers of splenic CD4⁺ T-cells in the mutant mice during the disease is likely a consequence of impaired expansion. Nonetheless, the lower proportion of CD4⁺ T-cells in SphK null mice

did not correlate with antibody production, since mice sensitized ip. with OVA showed similar high titers of IgE and IgG as WT mice in the absence of increased numbers of CD4⁺ T-cells in the spleen. This finding is of considerable interest and suggests that further evaluation of the numbers and function of circulating and/or gastrointestinal resident DCs, B-cells and T-cells may improve our understanding of the requirements for antigen presentation and how SphKs and S1P may influence oral versus systemic sensitization. Additionally, the significantly higher numbers of splenic mast cells in SphK2-deficient animals in concert with lower titers of IgG1 and IgG2a might be seen in the current concept of the mast cell functional plasticity in ongoing immune responses.[34] Thus, also in our model these cells could contribute to immunomodulatory effects during establishing an immune response via the oral route.

We also investigated the effector phase of food allergy and measured mMCP-1 after oral provocation as a bio-marker of severe type I hypersensitivity reaction.[35] Mast cell tryptase is a widely used marker for anaphylaxis also in human patients and strongly correlates with severity of symptoms.[36] Mouse MCP-1 being predominantly produced by intestinal mucosal mast cells was found to be consistently elevated in WT acid-suppressed animals compared to the negative control WT group. Even though the measured mMCP-1 concentrations were lower compared with other reports in literature, we measured a substantial increase in mMCP-1 levels 1 hour after provocation with a 10-fold lower OVA dose than used in other studies.[35] However, SphK1- or SphK2-deficient mice did not show increased levels of mMCP-1 in the food allergy group treated with antacids compared to the respective negative control group. These results were not a consequence of an overall defect in mMCP-1 release from SphK1- and SphK2-deficient mast cells, as similar increases of mMCP-1 were observed when anaphylaxis was induced by a strong systemic stimulus in a passive IgE-mediated anaphylaxis model. While there was a trend towards reduced mMCP-1 release in the SphK1-deficient mice in the passive anaphylaxis model, the levels observed were markedly higher than that seen in the OVA sensitized, acid-suppressed SphK1-deficient mice. Thus, our data suggest that the poor release of mMCP-1 in SphK1- and SphK2-deficient mice does not result from reduced content of this protease. Instead, mast cell activation (and thus mMCP-1 release) might be affected by the route of the allergic challenge. Furthermore, when we evaluated mast cell numbers in gastrointestinal mucosa we observed an inability of SphK1 and SphK2 null mice to accumulate mast cells in the gastric mucosa after oral allergen provocation. As discussed above, this may in part be due to the impaired migration of CD4⁺ T-cells to the intestine. On the other hand, deficiency of SphK1 in mast cells was shown to impair their chemotactic responses, particularly when IgE/antigen triggered.[37] The failure to produce antigen-specific IgE may well have contributed to the lack of intestinal recruitment of mast cells in OVA fed, acid-suppressed SphK1 and SphK2 null mice. However, ip. immunized SphK null mice, which produced OVA specific IgE at levels similar to WT mice, also had significantly less mast cells in the intestinal mucosa, suggesting that other chemotactic signals might be impaired. The expression of S1PR₁ on mast cells as well as on other immune cells is crucial for migration.[16] Jolly et al. reported that chemotaxis of mast cells to the antigen requires FcεRI-mediated S1P production and transactivation of S1PR₁. [38] Similarly in human mast cells, down-regulation of SphK1 but not SphK2 was reported to inhibit their migration towards the antigen.[37] Our *in vivo* food allergy model indicates that both SphK1 and SphK2 may influence mast cell migration into the gastric but not the duodenal mucosa during intestinal allergic hypersensitivity, although the specific chemoattractant or whether this is mediated by S1PR₁ remains to be determined. Importantly, the presence of fewer mast cells in the gastric mucosa of control SphK1 and SphK2 null mice (Fig. 6) may also have implications for sensitization. Mast cell proteases produced by distinct populations of intestinal mast cells were shown to regulate intestinal barrier permeability during homeostasis and challenge.[39] Thus, the underrepresentation of mast cells in the gastrointestinal tract of non-allergic mice

might cause decreased permeability of the intestinal mucosa to OVA. Since increased intestinal mast cell numbers have been associated with increased intestinal permeability and the development of new-onset food allergies,[39–41] this suggests a previously unrecognized role for SphKs in regulating intestinal permeability.

In conclusion, we propose that both, SphK1 and SphK2, contribute to the development of food allergy by facilitating sensitization and formation of allergen specific IgE. Our results suggest that a possible mechanism could be the regulation of OVA uptake through the gastrointestinal tract. Furthermore, SphKs influence the effector phase of food allergy as they are required for normal accumulation of mast cells in mucosa. Nevertheless, SphK1 and SphK2 deficient mice also show distinct immune responses, e.g. the production of OVA specific IgG1. Despite important differences in the way these kinases may regulate the immune system, they also might share some functions that affect similarly the response to food allergens.

Although our oral immunization protocol did not induce substantial food allergic inflammation (as indicated by eosinophil migration) and anaphylactic symptoms in the strains of mice used herein, our findings provide evidence on the contribution of S1P in IgE induction as well as mast cell activation when an antigen is administered via the oral route, a mimic of food allergy induction in humans.[24] Further investigation on the underlying mechanism(s) is required. However, our observations point to the possible development of novel therapeutic strategies for food allergies, whereby alterations of SphK activity or S1P levels may be beneficial.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ANT	Anti-acid medication
anti-DNP-IgE	anti-Dinitrophenol-IgE
DC	Dendritic Cells
FCS	Fetal calf serum
i.g.	Intragastric
IL	Interleukin
i.p.	Intraperitoneal
MC	Mast cells
mMCP-1	Mouse mast cell protease-1
OVA	Ovalbumin
PNAG	Poly-N-acetylglucosamine
S1P	Sphingosine-1-phosphate
S1PR₁₋₅	Sphingosine-1-phosphate receptors 1–5
SphK	Sphingosine-kinase
TNFα	Tumor necrosis factor alpha
WT	Wild type

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Figure 1. S1P affects tight junction integrity and enhances OVA uptake *in vitro*

In a transwell model (A), transepithelial resistance (TEER) was significantly decreased after 1 and 5 hours of 0.5 μ M S1P stimulation (B). FITC OVA uptake after 60 min (C) was assessed after S1P stimulation and was significantly increased compared to medium as control (ctrl). Data are single measure points. ** $P < 0.01$, * $P < 0.05$; determined by student's t-test after logarithmic transformation.

Figure 2. OVA specific IgE is suppressed in orally sensitized acid suppressed SphK1^{-/-} and SphK2^{-/-} mice but not in ip. injected groups

IgE titers in sera were evaluated by specific ELISA (A) or by a functional degranulation assay (B) using RBL2H3 cells. WT, SphK1 and SphK2 knock-out mice were fed OVA with anti-ulcer medication (1; OVA ig.+ANT). Control animals were fed OVA without acid-suppression (2; OVA ig.) or injected OVA i.p. (3; OVA ip.). Data are mean +SD. ** $P<0.01$, * $P<0.05$; determined by student's t-test after logarithmic transformation.

Figure 3. Increased titers of IgG1 and IgG2a are observed in i.p. immunized mice of all genotypes

Serum levels of OVA specific IgG1 and IgG2a were measured by ELISA. Groups represent the respective immunization protocols (1: OVA ig.+ANT; 2: OVA ig., 3: OVA ip). Data is mean +SD. ** $P < 0.01$, * $P < 0.05$; determined by student's t test after logarithmic transformation.

Figure 4. High levels of mast cell protease-1 in acid-suppressed WT animals

mMCP-1 was measured in serum samples obtained 1 hour after oral OVA provocation. Groups represent different immunization regimen (1: OVA ig.+ANT, 2: OVA ig, 3: OVA ip) in WT, SphK1 or SphK2-null mice. The mMCP-1 levels in the naïve mice did not significantly differ from those in the negative control mice (OVA ig.) (data not shown). The boxes represent the inner quartiles value range with the median indicated as black line. Signals with more than 1.5-fold interquartile range deviation from the end of the box were defined as outliers and marked as circles. Brackets indicate the statistically significant difference of mMCP-1 (* $P < 0.05$, ** $P < 0.01$; determined by student's t test after logarithmic transformation).

Figure 5. Altered proportions of CD3⁺CD4⁺ T-cells and CD4⁺Foxp3⁺ T-regulatory cells in spleens of mutant mice

Spleen cells were stained and gated for CD3, CD4, CD8 and Foxp3 in flow cytometric analysis (A). The fraction of CD4⁺ T-cells are shown in relation to absolute CD3⁺ cell numbers (B). T-regulatory cells were gated as CD4⁺Foxp3⁺ (the percent is in relation to CD4⁺ cells) (C). Means +SD are indicated. **P<0.01, *P<0.05; determined by student's t test after logarithmic transformation.

Figure 6. Migration of mast cells into gastric mucosa is impaired in SphK1^{-/-} and SphK2^{-/-} mice

Gastric sections of the tunica mucosa (A) were histologically evaluated for mast cell numbers (MC) by toluidine-blue staining. MCs in the tunica mucosa (B) of all fields of view of the glandular part of the stomach and in the tunica muscularis mucosae (C) were counted and the mean MCs per field of view (FOV) were calculated. The results of each group are presented as mean +SD. **P<0.01 and *P<0.05 were determined by student's t test after logarithmic transformation.