The Journal of Immunology

REVIEW ARTICLE | FEBRUARY 01 2005

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Ana Olivera; ... et. al

J Immunol (2005) 174 (3): 1153–1158.
https://doi.org/10.4049/jimmunol.174.3.1153

THE JOURNAL OF IMMUNOLOGY

BRIEF REVIEWS

Sphingolipids and the Balancing of Immune Cell Function: Lessons from the Mast Cell

Ana Olivera[1] *and Juan Rivera*[1]

Recent studies reveal that metabolites of sphingomyelin are critically important for initiation and maintenance of diverse aspects of immune cell activation and function. The conversion of sphingomyelin to ceramide, sphingosine, or sphingosine-1-phosphate (S1P) provides interconvertible metabolites with distinct biological activities. Whereas ceramide and sphingosine function to induce apoptosis and to dampen mast cell responsiveness, S1P functions as a chemoattractant and can up-regulate some effector responses. Many of the S1P effects are mediated through S1P receptor family members ($S1P_{1–5}$). $S1P_1$, which is required for thymocyte emigration and lymphocyte recirculation, is also essential for Ag-induced mast cell chemotaxis, whereas $S1P_2$ is important for mast cell degranulation. S1P is released to the extracellular milieu by Ag-stimulated mast cells, enhancing inflammatory cell functions. Modulation of S1P receptor expression profiles, and of enzymes involved in sphingolipid metabolism, particularly sphingosine kinases, are key in balancing mast cell and immune cell responses. Current efforts are unraveling the complex underlying mechanisms regulating the sphingolipid pathway. Pharmacological intervention of these key processes may hold promise for controlling unwanted immune responses. The Journal of Immunology, **2005, 174: 1153–1158.**

Sphingolipids comprise a large family of lipids that include hundreds of distinct species, all of which contain an sphingoid base backbone (1). Sphingolipids are necessary constituents of membranes and liquid-ordered domains (referred to as lipid rafts), and receptor-mediated metabolism of sphingolipids may play prominent roles in the structure and stability of lipid microdomains affecting receptor clustering (2, 3). However, metabolites of sphingolipids, particularly those of sphingomyelin catabolism (ceramide (Cer),[1] sphingosine (Sph), and sphingosine-1-phosphate (S1P)), are also bioactive lipids mediating essential biological functions such as chemotactic motility, calcium homeostasis, cell growth, cell death, and differentiation (1, 4).

Sphingolipids as a signaling moiety

Stimulation of various cell types induces the breakdown of sphingomyelin successively and/or selectively into Cer (by activating the enzyme sphingomyelinase), Sph (by activating ceramidase), and S1P (following activation of sphingosine kinase (SphK)). Each of these lipid metabolites can directly bind proteins, activate signaling pathways, and affect cellular responses; moreover, in combination, the sphingolipid mediators can finely tune cellular function. Cer binds to a number of signaling proteins such as isozymes of protein kinase C (PKC) (α, δ, and ζ)), the kinase suppressor of Ras, the serine/threonine protein phosphatases PP2A and PP1, the protease cathepsin D, phospholipase D (PLD), cytosolic phospholipase A_2, and it can also affect other signaling pathways such as the JNK/stress-activated protein kinase or Akt pathways (reviewed in Ref. 1). The interaction of Cer with its protein partners not only localizes signaling to the sites where Cer is generated, it modifies the properties or the activity of the targeted proteins. Sph binds to and inhibits PKC and exerts other positive or negative effects on various enzymes involved in signal transduction. Of interest is Sph-induced activation of PLD and diacylglycerol kinase and inhibition of phosphatidic acid phosphohydrolase, which altogether results in the formation of phosphatidic acid over diacylglycerol, both known to be lipid messengers involved in several signaling pathways (1). However, Sph exerts an overall negative influence on cell responsiveness and has been shown to also induce apoptosis. S1P can act intracellularly, although the target of its intracellular action is not known, mediating proliferation and survival in mammalian cells as well as in plants and yeast (4–7), and it is also a specific ligand for a family of five G protein-coupled receptors ($S1PR_{1–5}$), originally named the endothelial differentiation gene receptors (4, 8). Each of these S1PRs couple to different subunits of heterotrimeric G proteins (α_i, α_q, and $\alpha_{12/13}$), and therefore they can trigger a plethora of signaling pathways (including Src family kinase activation, small GTPases, MAPK cascades, phospholipases, PKC, and calcium mobilization) (9, 10), and multiple and sometimes opposing cellular functions (8, 10, 11). Thus, modulation of the expression amount or pattern of the various S1PRs can represent a mechanism for tuning or modifying cellular responses.

The interconvertible sphingolipid metabolites Cer, Sph, and S1P not only differ in their physical and signaling properties, but also have counteracting effects. For example, S1P is able to counteract

Molecular Inflammation Section, Molecular Immunology and Inflammation Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892

Received for publication September 15, 2004. Accepted for publication December 2, 2004.

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[1] Address correspondence and reprint requests to Dr. Ana Olivera or Dr. Juan Rivera, Molecular Inflammation Section, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Building 10, Room 9N228, Bethesda, MD 20892-1820. E-mail addresses: oliveraa@mail.nih.gov and juan_rivera@nih.gov

[2] Abbreviations used in this paper: Cer, ceramide; Sph, sphingosine; S1P, sphingosine-1-phosphate; SphK, sphingosine kinase; PLD, phospholipase D; PKC, protein kinase C; BMMC, bone marrow-derived mast cell; IP_3, inositol 3,4,5-trisphosphate.

0022-1767/03/$02.00

Cer-mediated apoptosis, and the balance between these two metabolites influences growth and survival in eukaryotic cells (4, 5, 7, 12). The term sphingolipid "rheostat" was coined to reflect the adjustable nature of this balance. Stimulus-coupling to the sphingolipid rheostat may involve changes in the absolute levels of the bioactive sphingolipid metabolites and temporal or local differences in the relative ratios of these metabolites, providing a "built-in" inducible regulatory switch for controlling cellular responsiveness. Not surprisingly, the system offers versatility and flexibility for regulation of the enzymes involved in sphingolipid turnover. Seven distinct mammalian sphingomyelinases and three ceramidases have been characterized differing in their optimal pH (neutral, alkaline, or acid) and other biochemical properties as well as their intracellular location and mechanism of activation (13). Similarly, two mammalian SphKs differing biochemically and functionally have been cloned (5). Two S1P phosphatases that convert S1P back to Sph (12) and a S1P lyase that cleaves S1P into precursors of the biosynthesis of glycerolipids (1, 4, 12), keep the levels of S1P tightly regulated. Environmental stresses, activation of multichain immune recognition receptors, G protein-coupled receptors, and growth factor receptors, have all been reported to regulate the activity of sphingomyelinase, ceramidase, and SphK (1, 5), leading to selective enrichment of Cer, Sph, or S1P and subsequent biological effects.

The mast cell as a model

The role and the regulation of sphingolipids and S1P receptors have been a subject of intense research in immune cells. Mast cells play an important role in innate and acquired immunity and react to various stimuli by releasing a plethora of vasoactive mediators, proteases, chemokines, and cytokines that enhance vascular permeability, recruitment, and function of leukocytes, and cause local inflammation (14). The presence of IgE Abs to normally innocuous substances or Ag causes dysregulation of this normally protective response, resulting in allergic inflammatory disease. Ag-induced aggregation of IgE Ab bound to the cell surface high affinity receptor for IgE (FcϵRI) on mast cells elicits multiple biochemical events, including SphK activation, that culminate in mast cell degranulation (15). Also, engagement of FcϵRI elicits the partitioning of this receptor to lipid rafts where sphingolipids and metabolites are enriched (16). FcϵRI also regulates the secretion of S1P into the extracellular medium and the expression of S1P receptors in the mast cell. It is now clear that regulation of SphK by FcϵRI is crucial to mast cell excitability by affecting the balance between the levels of Cer or Sph (negative regulators) and S1P (positive regulator) (17, 18). Thus, these cells represent a prototype for the "sphingolipid rheostat" hypothesis and for the multiple functions of S1P both as an intracellular messenger and autocrine/paracrine mediator (Fig. 1).

Mechanism(s) of activation of SphKs in mast cells

The precise pathways involved in the activation of SphKs in mast cells are now beginning to be revealed. In human bone marrow-derived mast cells (BMMC) and rat basophilic leukemia cell line (RBL) cells, SphK translocates to the plasma membrane within minutes of FcϵRI clustering, and its activation is dependent on PLD1 activity (19). Both FcϵRI in mast cells and FcγRI in human myeloid cells activate SphK in a tyrosine kinase- and PLD-dependent manner (19–21), suggesting a possible involvement of a common subunit of the receptors, the γ-chain. In a collaborative effort with Baumruker and colleagues (22), we found that SphK1 interacts with the protein tyrosine kinase, Src kinase members Lyn and Fyn, but not Src or other tyrosine kinases such as Syk. These results were confirmed in vitro using the purified proteins.

Lyn and Fyn are FcϵRI proximal kinases that initiate the signaling events following cross-linking of this receptor (23). Lyn phosphorylates tyrosine residues in the ITAMs of the β- and γ-chains of the receptor, resulting in the recruitment and further activation of both Lyn and Syk, both of which phosphorylate downstream substrates. Fyn activation results in increased PI3K activity and other unknown signals that are crucial for mast cell responsiveness (24). Using BMMC derived from Fyn- or Lyn-deficient animals, we demonstrated that both Lyn and Fyn are required for the immediate activation of SphK, whereas only Fyn is indispensable for the late activation of SphK (Ref. 22 and our manuscript in preparation). We also found that after activation of mast cells, Lyn interacts with SphK1 and recruits it to the FcϵRI and to the lipid rafts, where sphingolipids and likely its substrate Sph, reside. Interestingly, the interaction of Lyn with SphK results in the potentiation of their respective activities. The enhancement of SphK activity after interaction with Lyn occurs without any detectable phosphorylation of the lipid kinase (22), although general inhibitors of tyrosine phosphorylation (20) or selective inhibitors of Src kinase activity (our unpublished observation) abrogate stimulation of SphK by FcϵRI. These results suggest that following interaction with Lyn, SphK is recruited to particular signaling domains in the membrane where conformational changes occur in both kinases that favor their respective activities, whereas Fyn and/or Lyn provide additional signals required for the full activation of SphK. It is worth noting that, to date, the activation of SphK is the only known event where both Lyn and Fyn kinase activities cooperate to initiate subsequent signals.

Positive regulation of mast cell function by sphingolipids

S1P is one of the mediators that mast cells secrete upon activation via Ag-specific cross-linking of FcϵRI (18, 25). So far, substantial stimulation-dependent release of S1P has been demonstrated only in platelets and mast cells. Interestingly, S1P was found to be elevated in the bronchial airway lavage of ragweed-allergic, asthmatic patients 1 to 2 days after the allergen challenge (26), and a role for S1P as a mediator in allergy and asthma has been proposed (27) based on the following properties of S1P: S1P is an important chemoattractant for cells involved in inflammatory processes (25, 28–30); S1P shifts maturing dendritic cell-induced polarization of T cells into a Th2 phenotype (31); S1P is a known regulator of endothelial cell function (7) and promotes cell adhesion molecule expression in endothelial cells (32, 33); S1P induces proliferation and contractility, and stimulates IL-6 production in airway smooth muscle cells, and hence it could participate in the pathology of remodeling the bronchial walls in asthmatic patients (27) (Fig. 1).

In addition to the putative role of mast-cell released S1P in inflammatory responses, we recently reported that mast cell-released S1P could, in an autocrine fashion, enhance mast cell function by binding to its receptors ($S1P_1$ and $S1P_2$) in mast cells (25). Activation of mast cells by IgE/Ag was found to induce the transactivation of the S1P receptors. Transactivation

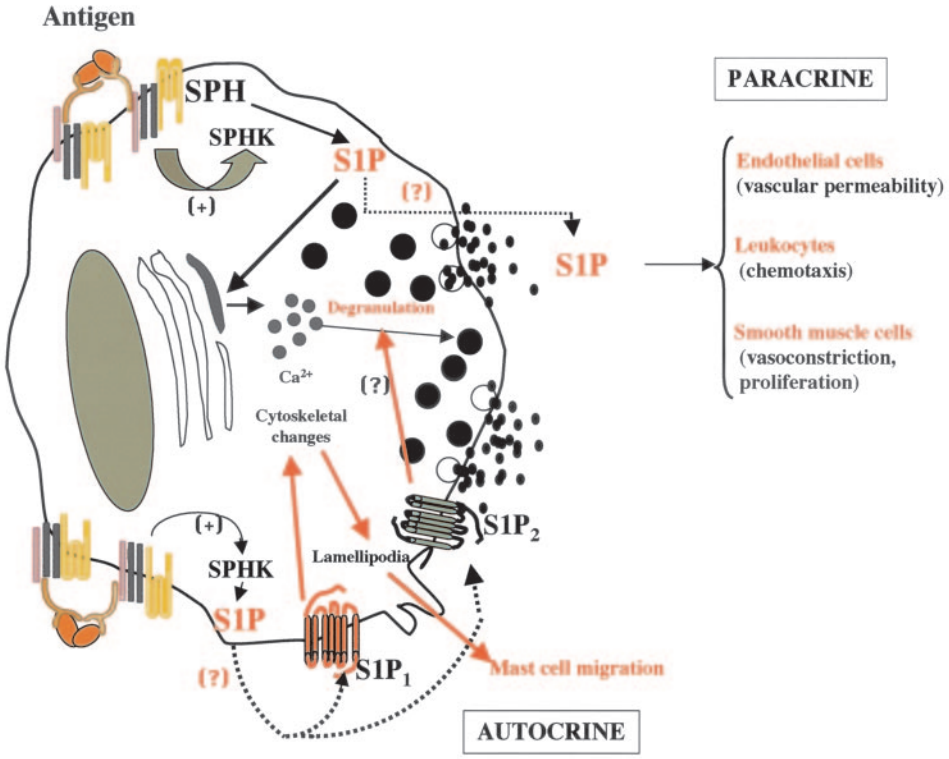

FIGURE 1. Role of S1P and its receptors in mast cell functions. Cross-linking of the FcεRI by IgE/Ag in mast cells results in the rapid activation and translocation of SphK to the plasma membrane and the generation of S1P. S1P, independently of phospholipase Cγ (PLCγ) activation and IP_3 generation, is thought to mobilize calcium from intracellular stores, which is necessary for mast cell degranulation. However, the nature of the intracellular calcium pools targeted by S1P has not been determined, although in this figure, it is illustrated as an endoplasmic reticulum calcium pool. S1P is secreted by activated mast cells to the extracellular medium by mechanisms that have not yet been elucidated. Furthermore, generated S1P is able to rapidly bind and activate its receptors $S1P_1$ and $S1P_2$ on the plasma membrane. $S1P_1$ induces cytoskeletal rearrangements, leading to the movement of mast cells toward an Ag gradient, whereas transactivation of $S1P_2$ enhances the degranulation response. Mast cell-secreted S1P can also promote inflammation by activating and recruiting other immune cells involved in allergic and inflammatory responses. Because S1P also profoundly affects endothelial cell function, and induces contraction and proliferation of airway smooth muscle cells, and its levels are elevated in the bronchial lavage of asthmatic individuals after Ag challenge, secretion of S1P by mast cells could be of relevance in this pathology. Mast cell granules are illustrated as black circles, and the process of degranulation as granules in contact with the plasma membrane emptying their content (smaller black dots). The thick, solid black arrow represents the intracellular actions of S1P, the dotted lines represent the release of S1P to the extracellular medium, and the red solid arrows, represent the signaling pathways activated via S1P receptors.

of $S1P_1$ is required for the migration of mast cells toward low concentrations of Ag, whereas $S1P_2$ transactivation is important for degranulation responses (Fig. 1). BMMC from $S1P_2$-deficient mice, or the RBL transfected with a $S1P_2$ antisense showed a 40–50% reduction in granule content release, whereas RBL transfected with a $S1P_1$ antisense did not affect granule content release but abrogated migration toward Ag. Ectopic expression of $S1P_1$ in RBL cells enhanced migration toward Ag or S1P, whereas overexpression of $S1P_2$ negatively regulated their chemotactic motility. The countermigratory effects of $S1P_2$, compared with those of $S1P_1$, have been well documented in other cell types, and are primarily related to down-regulation of Rac activity and activation of Rho (10). Interestingly, $S1P_2$ mRNA expression is enhanced as a late consequence of FcεRI cross-linking, whereas the level of $S1P_1$ expression is unchanged. Thus, one can envision a model in which an Ag gradient may play an important role in mast cell function through S1P. Low Ag concentrations can attract mast cells to the site of action via $S1P_1$, and, as mast cells approach higher concentrations of Ag, a shift in the expression of S1P receptors (enhanced $S1P_2$ expression) resolves migration while promoting degranulation, a process known to require a stronger stimulus (34). The mechanism(s) by which S1P is secreted has not been identified, and the detailed mechanism(s) involved in the transactivation of the S1P receptors also remains to be elucidated. Nevertheless, IgE-dependent SphK activation is required for S1P receptor transactivation (25). The fact that it occurs rapidly and when little (25) or no (18) S1P is detected in the medium suggests a tight spatial coordination between S1P production and $S1P_1$ receptor activation, and could potentially involve S1P transporters as described in T cells for the rapid formation of phosphorylated FTY720, an S1P mimetic drug, and its subsequent binding to S1P receptors (35).

It is well known that S1P is also found inside mast cells immediately after FcεRI activation, and that the early generation of this lipid mediator plays a role in the induction of cellular signals. Inhibition of SphK activation, and thus S1P generation, by either competitive analogs of Sph in RBL cells (36) or by antisense SphK mRNA in human mast cells (19) prevented IgE-triggered calcium responses and inhibited degranulation, without affecting Syk phosphorylation or inositol 3,4,5-trisphosphate (IP_3) production. The involvement of S1P in calcium release by a phospholipase Cγ-independent route implies its direct or indirect action on an unidentified IP_3-insensitive

calcium store or channel, as has been suggested for other cell types (4). Addition of S1P to mast cells activates the MAPK cascade and results in leukotriene synthesis, TNF-α production when added in combination with ionomycin (18), chemokine production (25), and a modest induction of β-hexosaminidase release (18, 25), suggesting its involvement in additional pathways. However, evidence involving intracellular S1P targets, or the mechanistic role of $S1P_1$ and/or $S1P_2$ receptors in modulating FcϵRI responses is currently unavailable.

Negative regulation of mast cell signals and function

Contrasting with the positive effects of S1P in mast cells, Cer and Sph function as negative regulators of mast cell activation. Addition of exogenous Sph to CPII mast cells and BMMCs abrogates MAPK activation and IL-5 and TNF-α induction by IgE/Ag (18). The inhibitory effects of high intracellular concentrations of Sph can be effectively reversed by addition of equivalent amounts of S1P (18). Indeed, after FcϵRI stimulation, concomitantly with the elevations of the positive mediator S1P, the levels of the inhibitory mediator Sph are significantly reduced favoring the activated (responsive) state of mast cells, whereas in the resting state, Sph levels prevail over those of S1P maintaining cells in a resting (unresponsive) state.

Consistent with this notion, Mathes et al. (37) found that Sph completely inhibits store-operated calcium currents (calcium release-activated calcium current (I_{CRAC})) in RBL-2H3 cells and proposed that steady-state levels of Sph would maintain I_{CRAC} in a blocked, resting state. Decreases in Sph levels upon stimulation would lead to the disinhibition of these channels, allowing for the full calcium responses induced by FcϵRI. The significance of Sph may not be only related to maintaining mast cells unresponsive, but under particular circumstances, the regulation of its levels can facilitate important functions in these cells. For example, Sph present in lipid rafts may participate in enhancing Lyn activity in these domains after FcϵRI engagement (22). Sph directly binds Lyn, enhancing its activity or further potentiating Lyn activity when bound to SphK, whereas S1P terminates Lyn activation (22). A plausible hypothesis in line with the "Sph/S1P rheostat" concept in mast cells, is that after IgE/Ag triggering, Lyn interacts with SphK and moves to lipid rafts where Sph binds both Lyn and SphK. As a result, enhanced Lyn activity increases phosphorylation of the γ- and β-chain of the FcϵRI, initiating and/or sustaining signaling cascades. Signals derived from Fyn and complemented by Lyn result in the maximal activation of SphK, which converts Sph into S1P, and S1P in turn actively downregulates Lyn, whose excessive activity would negatively affect mast cell responsiveness (38).

As in other cell types, treatment with Sph, soluble Cer analogs, or bacterial sphingomyelinase induced apoptotic cell death in murine BMMC even in the presence of the growth and survival factors IL-3 and/or stem cell factor (39). Short chain Cer analogs added exogenously to mast cells inhibit Ag-stimulated MAPK phosphorylation and prostaglandin D_2 production, calcium influx, plasma-membrane lipid order, and PLD1 activity (37, 40, 41). However, in most of these studies, the effects of C2-Cer closely resembled those of Sph, a metabolite into which C2-Cer can be converted. Moreover, long-chain ceramides that are structurally closer to natural ceramides or sphingomyelinase treatment failed to show these effects (37, 41). Although the physiological relevance and molecular targets of the Cer and Sph production in mast cells remains to be further defined, the inhibitory potential of short Cer analogs and nonmodifiable Sph analogs in the allergic response represents an interesting avenue for pharmacological research.

Concluding remarks and future prospects

Recent progress has increased our understanding on the functional roles of Cer, Sph, and S1P in mast cell responsiveness (17), in the priming and inactivation of neutrophils and macrophages (42–47), and in the chemotactic motility of various immune cells (25, 28–30, 48). Other studies have underscored the importance of sphingolipids in additional immune cell processes such as differentiation of monocytes into macrophage or granulocyte lineages (49, 50), regulation of apoptotic cell death and survival of lymphocytes (51) and macrophages (52), overall suggesting a role of these lipids in the regulation of various physiological aspects of immune cell function. An underlying theme is that sphingolipids function to regulate cell responsiveness. Thus, therapeutic strategies based on local or systemic delivery of sphingolipids, sphingomimetic drugs, or the development of pharmacological agents that interfere with the balance or the function of sphingolipid metabolites, although in early stages, may have promise in ablating unwanted immune responses.

To date, the most compelling example of the use of sphingolipid metabolites in therapeutics has surfaced from studies with the synthetic Sph analog FTY720. FTY720 is an immunosuppressive drug that induces lymphopenia by preventing the egress of lymphocytes from secondary lymph nodes and thymus (53, 54). FTY720 is phosphorylated in vivo by SphK, thus creating a S1P mimetic with the ability to bind to and stimulate at least four of the five S1PRs (53). This stimulation causes downregulation of cell surface receptor by internalization, rendering the cells incapable of responding to endogenous S1P (48, 55). Elegant studies using transplants of fetal liver cells from $S1P_1$ knockout donors into lethally irradiated wild-type mice, have demonstrated that $S1P_1$ is the key receptor involved in the egress of lymphocytes (48). FTY720 administration extended allograft transplant survival in animal models of allotransplantation (56) and in human renal transplantation (57, 58) and has also been used in experimental autoimmune disease models such as multiple sclerosis and systemic lupus erythematosus (59). Furthermore, FTY720 treatment suppressed Th1- and Th2-mediated airway inflammation in mouse models demonstrating its potential for treatment of asthma (60). One of the serious drawbacks of this drug is that phosphorylated FTY720 specifically binds S1P receptors, which are widely expressed in most cells, and thus unwanted secondary effects might be expected. In fact, in humans, it has been reported to cause a transient, dose-dependent bradycardia (57). Importantly, Sanna et al. (61) reported a new compound, 5-(4-phenyl-5-trifluoromethyl-thiophen-2-yl)-3-(-3-trifluoromethyl-phenyl)-[1,2,4] (SEW2871), that binds and signals selectively through $S1P_1$ (but not $S1P_{2-5}$), and maintains lymphopenia when injected in mice without inducing bradycardia.

The allergic inflammatory skin disease, atopic dermatitis, is accompanied by an abnormal barrier function of the stratum corneum that is associated with a loss of Cer in the extracellular lamellar membranes. Topical application of Cer, as a lipid

emolument, on the skin of children with this disease significantly improved the severity score, and stratum corneum cohesion and hydration (62). Alterations in the levels of the lipid mediator S1P have also been reported in some inflammatory conditions such as asthma (27), although its contributions to the pathology of disease need to be further assessed. The involvement of SphK in the production of S1P by mast cells and in the priming and the activation of neutrophils, make local targeting of SphK or of specific S1P receptors interesting avenues for future development of drugs against asthma and other inflammatory diseases.

Generally, it can be concluded that sphingolipids and metabolites can intervene in the regulation of multiple aspects of immune cell function. A unique feature is the interconvertible nature of the metabolites and their opposing and redundant roles in immune cell activation. As a cell whose excitability is determined by the balance of these metabolites, and that secretes copious amounts of S1P, the mast cell is positioned as key modulator of the immune response consistent with its role in innate and acquired immunity (14). The increased understanding of how sphingolipids balance the immune response is opening new avenues with therapeutic promise in controlling undesired immune responses.

Acknowledgments

We thank the laboratories of Drs. T. Baumruker (Novartis, Vienna, Austria), Richard L. Proia (National Institutes of Health, Bethesda, MD), and S. Spiegel (Virginia Commonwealth University, Richmond, VA) for their collaboration and key contributions in the study of SphK in mast cells.

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