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Sphingosine-1-phosphate in mast cell-mediated allergic responses

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SPHINGOSINE 1-PHOSPHATE IN MAST CELL-MEDIATED ALLERGIC RESPONSES

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by

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List of Abbreviations

-/-	Homozygous deletion of a gene
Ag	Antigen
AHR	Airway hyperresponsiveness
BAL	Bronchoalveolar lavage
BMMCs	Murine bone marrow-derived mast cells
C57BL/6	Inbred mouse strain
C5a	Complement component C5a, a protein fragment released from C5
CB	Cord blood
CB-MCs	Cord blood-derived mast cells
DMS	N,N-dimethylsphingosine
DNP-HSA	Dinitrophenol-linked human serum albumin, an antigen
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal related kinase
FBS	Fetal bovine serum
FcεRI	High affinity IgE receptor
Fyn	Tyrosine-protein kinase Fyn, Src family
GPCRs	G-protein coupled receptors
H&E	Hematoxylin and eosin stain
HRP	Horseradish peroxidase
IgE	Immunoglobulin E
IFN-γ	Interferon-gamma
IL	Interleukin
$InsP_3$	Phosphatidylinositol (3,4,5)-trisphosphate
i.n.	Intranasal delivery

i.p.	Intraperitoneal delivery
Lyn	Tyrosine-protein kinase Lyn, Src family
mAb	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein - 1
MC_T	$Tryptase^+$, $chymase^-$, lung-type mast cells
MC_{TC}	$Tryptase^+$, $chymase^+$, skin-type mast cells
NF-κB	Nuclear factor kappa B
OVA	Ovalbumin
PAF	Platelet activating factor
PAS	Periodic acid Schiff stain
PBS	Phosphate buffered saline
PKC	Protein kinase C
RPMI	Roswell Park Memorial Institute medium
S1P	Sphingosine-1-phosphate
$S1P_1$	Sphingosine-1-phosphate type-1 receptor
$S1P_2$	Sphingosine-1-phosphate type-2 receptor
S1PRs	Sphingosine-1-phosphate receptors
SCF	Stem cell factor
SK1-I	Sphingosine kinase 1 inhibitor
Sph	Sphingosine
SphK	Sphingosine kinase
T_H1	T helper cell type 1
T_H2	T helper cell type 2
TNF-α	Tumor necrosis factor - alpha
WT	Wild type

Abstract

SPHINGOSINE 1-PHOSPHATE IN MAST CELL-MEDIATED ALLERGIC RESPONSES

By Megan M. Price, BS

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

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Mast cells play a critical role in both acute and chronic inflammation and mature in peripheral tissues from bone marrow-derived progenitors that circulate in the blood as immature precursors. Mast cell progenitors are likely to encounter the serum-borne bioactive sphingolipid metabolite, sphingosine-1-phosphate (S1P), during migration to target tissues. Mast cells developed from human cord blood-derived progenitors cultured with stem cell factor (SCF) alone express intragranular tryptase (MC_T), the phenotype predominant in the lung. S1P accelerated the development of cord blood-derived mast cells (CB-MCs) and strikingly increased the numbers of mast cells expressing chymase. These mast cells have functional FcεRI, and similar to skin mast cells that express both tryptase and chymase (MC_{TC}), also express CD88, the receptor for C5a, and are activated by

anaphylatoxin C5a and the secretagogue compound 48/80. S1P induced release of IL-6, a cytokine known to promote development of functionally mature MC_{TC}, from cord blood cultures containing adherent macrophages, and from highly purified macrophages, but not from macrophage-depleted CB-MCs. In contrast, S1P stimulated secretion of the chemokine, monocyte chemoattractant protein 1 (MCP-1/CCL2), from these macrophage-depleted and purified CB-MCs.

S1P produced by two sphingosine kinase isozymes, SphK1 and SphK2, has been implicated in IgE-mediated mast cell responses. However, studies of allergic inflammation in isotype-specific SphK knockout mice have not clarified their respective contribution. Furthermore, the role that S1P plays *in vivo* in a mast cell- and IgE-dependent mouse model of allergic asthma has not yet been examined. We used an isoenzyme-specific SphK1 inhibitor, SK1-I, to investigate the contributions of S1P and SphK to mast cell-dependent airway hyperresponsiveness (AHR) and airway inflammation observed in this model. C57BL/6 mice received intranasal delivery of SK1-I prior to sensitization and challenge with OVA or only prior to challenge. SK1-I inhibited antigen-dependent activation of human and murine mast cells and suppressed activation of NF-κB, a master transcription factor that regulates expression of pro-inflammatory cytokines. SK1-I treatment of mice sensitized to OVA significantly reduced OVA-induced AHR to methacholine; numbers of eosinophils; levels of the cytokines IL-4, 5, 6, 13, IFN-γ, and TNF-α, and the chemokines eotaxin, and CCL2 in bronchoalveolar lavage fluid; and pulmonary inflammation as well as activation of NF-κB in the lungs of these mice. S1P and SphK1 play important roles in mast cell-dependent, OVA-induced allergic

inflammation and AHR in mice, in part by regulating the NF-κB pathway. The findings that intranasal administration of the specific SphK1 inhibitor SK1-I reduced allergic inflammation and AHR associated with asthma support the therapeutic potential of SphK1 inhibitors for the treatment of allergic airway inflammation. Overall, these results suggest crucial roles for S1P in regulating development of mast cells and their functions and reveal a complex interplay between macrophages and mast cell progenitors in the development of mature human mast cells.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Allergic disease and mast cells

Allergic disease is a hypersensitivity disorder of the immune system that occurs to innocuous agents and if severe enough may result in life-threatening anaphylactic reactions and potentially death. Over the last decades, allergic disease has become increasingly widespread in developed nations and allergic asthma is now the most common chronic disease among children in the United States and affects approximately 300 million people worldwide. Unfortunately, the etiology of allergic disease is not well understood. Allergic reactions are triggered when an allergen crosses an epithelial and/or endothelial barrier and interacts with cell-bound antibodies. The release of cellular mediators then has multi-organ consequences leading to responses in the skin, respiratory tract, cardiovascular system, and possibly the gastrointestinal tract or nervous system, all target organs heavily populated with mast cells.

Mast cells play an important role in the pathophysiology of allergy, initiating and amplifying immunoglobulin E (IgE)-mediated inflammatory responses including anaphylaxis, hay fever, eczema, and asthma. These cells are ubiquitous in vascularized tissues, but are able to relocate to sites of insult in disease. Their location at the interface between the external environment and host tissue, near blood vessels, nerves, and glands, places them in the ideal location to respond rapidly to perceived tissue insults. Mast cells express numerous receptors that allow them to respond to diverse stimuli (cytokine,

chemokine, eicosanoid, TLRs, secretagogues, etc). Nevertheless, they are best characterized based on expression of the high affinity receptor for IgE, FcεRI [1]. Cross-linking of FcεRI by IgE/Ag results in receptor aggregation and initiates intricate pathways that ultimately culminate in mast cell degranulation [2-4]. During degranulation, mast cells release and synthesize a plethora of proinflammatory mediators into the surrounding milieu. Pre-stored components are harbored in cytoplasmic granules rich in bioactive amines, such as histamine, proteoglycans, proteases, such as tryptase, chymase and carboxypeptidase A, and cytokines such as TNF-α [5, 6]. *De novo* synthesized mediators produced in rodent and human mast cells include eicosanoids (leukotrienes and prostaglandins) and a vast array of cytokines and chemokines. The pattern of mediators released from mast cells varies depending on the stimulus and mast cell phenotype, demonstrating the versatility of mast cells to initiate an appropriate inflammatory response. Importantly, sphingosine-1-phosphate (S1P) is now widely recognized as a potent lipid mediator produced and secreted by mast cells that in turn regulate mast cell responses [4, 7-14].

1.2 Sphingosine-1-phosphate

Over the last decade, the work of many investigators has established the importance of the bioactive lipid mediator sphingosine-1-phosphate (S1P) in regulating numerous and diverse cellular processes in various cell types, including proliferation, cell survival, motility and cytoskeletal rearrangements as wells as angiogenesis [15, 16]. S1P exerts the majority of its effects as an extracellular ligand for a family of five specific G protein

coupled receptors, denoted $S1P_{1–5}$ [15]. These receptors all bind S1P with similar affinity and couple to a variety of G proteins, initiating various downstream signaling pathways thus enabling S1P to regulate a diverse array of biological responses [16]. In addition, every cell in the body expresses at least one of the S1P receptors, which are differentially expressed from cell to cell, further complicating the understanding of the wide ranging yet distinct actions of S1P. S1P also acts independent of its receptors [15]; newly discovered intracellular targets include prohibitin 2 [17], TRAF2 [18], and histone deacetylases [19].

1.3 Sphingolipid metabolism and sphingosine kinases

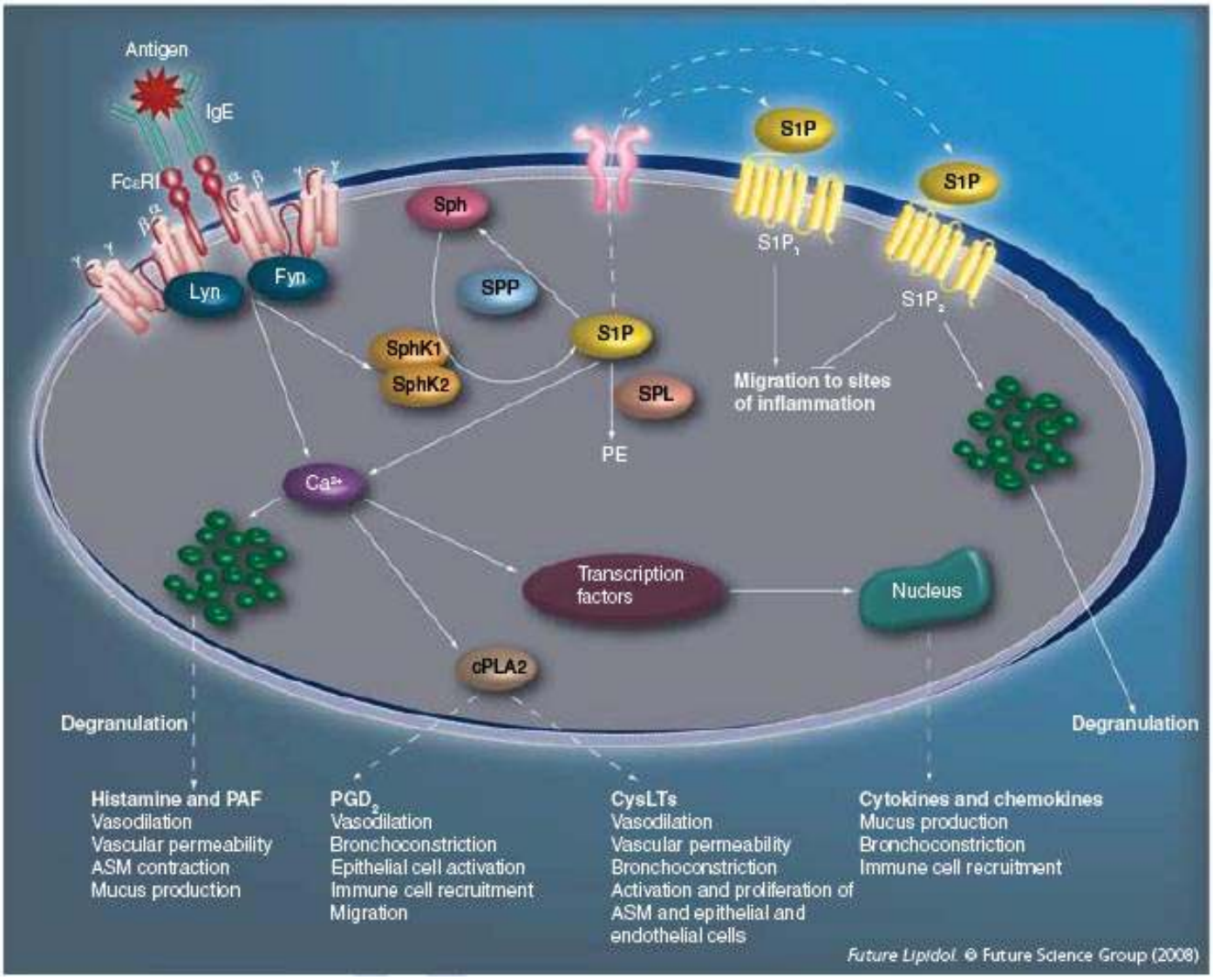
Intracellular levels of S1P are tightly regulated by the balance between its synthesis, which involves SphK1 and SphK2, and its degradation, which can occur either reversibly by two specific S1P phosphatases or irreversibly by S1P lyase (Figure 1). Therefore, this balance between S1P and its precursors – sphingosine and ceramide – and their overall regulation of opposing signaling pathways is instrumental in determining cell fate and has been termed the 'sphingolipid rheostat' [20]. SphK1 was the first isozyme discovered and characterized and is therefore, the most well studied. It is activated by numerous stimuli, including many growth factors and cytokines and crosslinking of immunoglobulin receptors [15]. Activation of SphK1, which requires its phosphorylation by ERK1/2 [21, 22], is accompanied by its translocation from the cytosol to the plasma membrane where its substrate sphingosine resides [11, 12]. Much less is known about the regulation of SphK2. Its subcellular localization is cell-type specific, appearing cytosolic in some cell types and mainly nuclear in others, and it can translocate between these

compartments in response to specific stimuli [23]. In rodent mast cells, SphK1 and SphK2 are largely cytosolic under basal conditions and translocate to the plasma membrane following IgE-receptor engagement [11, 12].

Interestingly, although SphK1 and SphK2 are highly homologous and utilize the same substrate to produce the same product, they exhibit both functional and experimental differences. In contrast to the growth and survival promoting actions of SphK1, overexpression of SphK2 in many cells induces cell death and growth arrest [24]. However, very few studies to date have examined the role of endogenous SphK2. It has recently been demonstrated that SphK2 is also activated by phosphorylation [25], as well as by antigen crosslinking of IgE receptors [12]. Mice with knockouts of either s*phk1* or *sphk2* are viable and exhibit no obvious phenotypes, however, knockout of both s*phk1* and s*phk2* results in complete loss of S1P and is embryonically fatal [26], demonstrating the necessity of S1P for life. The circulating levels of S1P in s*phk1*$^{-/-}$ mice are reduced compared to WT mice, whereas s*phk2*$^{-/-}$ mice have higher levels of circulating S1P, possibly due to increased SphK1 activity in the red blood cells of these mice [13, 27, 28 , 29]. Together, these observations also suggest that SphK1 and SphK2 may have some redundant, overlapping and/or compensatory functions.

Figure 1: The role of S1P in mast cell-mediated allergic responses and anaphylaxis. Antigens induce allergic responses via cross-linking of IgE-bound FcεRI receptors on the surface of mast cells, triggering elaborate signaling cascades that result in degranulation and release of a plethora of inflammatory mediators. One such pathway is the activation of SphK1 and 2 and the subsequent production of S1P. S1P can be degraded by SPP or SPL, leading to the production of PE. Intracellular S1P can then be secreted from mast cells via the ABCC1 transporter to bind its receptors extracellularly, or may act intracellularly to induce calcium release independent of phosphatidylinositol (3,4,5)-triphosphate. Extracellular signaling through $S1P_1$ is important in migration of mast cells to sites of inflammation, while $S1P_2$ inhibits motility, probably to resolve cellular movement upon arrival to target sites, and also enhances degranulation. Calcium influx is necessary for processes that are critical to the induction of allergic responses and anaphylaxis such as degranulation and activation of cPLA2, and various transcription factors. Histamine and PAF induce vasodilation, ASM contraction, and increase vascular permeability and mucus production. Activation of cPLA2 is the rate-limiting step in the production of all eicosanoids, including PGD_2 and cysLTs, which themselves induce vasodilation, bronchoconstriction, vascular permeability, epithelial and endothelial cell activation and proliferation, immune cell recruitment, migration, and ASM activation & proliferation. Release of immediate mediators of inflammation from mast cells is followed by increased transcription of various cytokine and chemokines factors that induce mucus production, bronchoconstriction, and immune cell recruitment. Collectively, mast cell mediators promote inflammation and furthermore, function to activate and recruit other immune cells, thereby exacerbating the symptoms of allergy and anaphylaxis. Price et al. *Future Lipidol.* 2008.

ASM: Airway smooth muscle; CysLT: Cysteinyl leukotriene; PAF: Platelet-activating factor; PE: Phosphatidylethanolamine; PG: Prostaglandin; SphK: Sphingosine kinases; SPL: S1P lyase; SPP: Specific phosphatases.


Antigen
IgE
FcεRI
Lyn
Fyn
Sph
SPP
S1P
SphK1
SphK2
SPL
PE
S1P
S1P1
S1P
S1P2
Migration to sites of inflammation
Ca2+
Transcription factors
Nucleus
cPLA2
Degranulation
Degranulation
Histamine and PAF
Vasodilation
Vascular permeability
ASM contraction
Mucus production
PGD2
Vasodilation
Bronchoconstriction
Epithelial cell activation
Immune cell recruitment
Migration
CysLTs
Vasodilation
Vascular permeability
Bronchoconstriction
Activation and proliferation of ASM and epithelial and endothelial cells
Cytokines and chemokines
Mucus production
Bronchoconstriction
Immune cell recruitment
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While both SphK1 and SphK2 can phosphorylate sphingosine and sphinganine (dihydrosphingosine) only SphK2 can efficiently catalyze the *in-vitro* phosphorylation of the immunosuppressive drug FTY720 (Fingolimod) [30]. The pro-drug FTY720 is a sphingosine analog that upon phosphorylation by SphK2 forms FTY720-phosphate, a S1P mimetic capable of binding with high affinity to all of the S1P receptors except $S1P_2$. Its immunosuppresive action as a potent ligand of $S1P_1$ on lymphocytes leads to prolonged downregulation of this receptor and its degradation [31-33]. Since $S1P_1$ is required for lymphocyte egress from secondary lymph nodes and lymphoid organs, these cells are sequestered in this location by FTY720 administration, resulting in lymphopenia and rendering them incapable of contributing to inflammation [28, 34].

1.4 S1P receptors

Mast cells express two of the five S1P receptors ($S1P_1$ and $S1P_2$) [11, 35]. $S1P_1$ is widely expressed, with predominant expression found in brain, kidney, spleen, lung and the cardiovascular system [36]. $S1P_1$ was first demonstrated to be important in angiogenesis when mice lacking this receptor were found to have incomplete vascular development and consequently died *in utero* [37]. This receptor is also a key player in the maintenance of vascular integrity, which is important for inflammation and asthmatic lung remodeling [38-40]. Importantly, $S1P_1$ is also critical in lymphocyte egress from the thymus and peripheral lymphoid organs. Indeed, mice lacking expression of $S1P_1$ in hematopoietic cells exhibit lymphopenia since mature T cells are unable to exit the thymus

[32]. Furthermore, $S1P_1$ signaling is strongly upregulated prior to the exit of T cells from the thymus, suggesting a role in the chemotactic responsiveness of these cells [32].

$S1P_2$ is also expressed in a variety of cell types. In contrast to $S1P_1^{-/-}$ mice, mice lacking $S1P_2$ are viable and display a defect in proper development of auditory and vestibular systems, resulting in complete deafness [41-43]. In mast cells, $S1P_2$ is important for effective degranulation [11]. In the vascular system, activation of $S1P_2$ also increases vascular permeability, contrary to $S1P_1$ [40]. Furthermore, $S1P_2$ is considered to be a 'repellant' receptor as binding of S1P to $S1P_2$ decreases motility of many cell types, including mast cells [11].

1.5 Role of SphK1 and SphK2 in mast cells

Mast cells express the high-affinity receptor for IgE – FcεRI – which is an important component of allergic diseases. Its crosslinking by monomeric IgE bound to multivalent antigens initiates an elaborate and complicated cascade of signaling events that leads to degranulation and release of histamine and other mediators of immediate responses as well as the subsequent production and secretion of cytokines and chemokines and lipid mediators, such as eicosanoids and S1P [44, 45]. These mast cell mediators promote inflammation by enhancing vascular permeability while initiating the recruitment and activation of other immune cells involved in allergic and inflammatory responses.

Crosslinking of IgE receptors on mast cells results in activation of several key regulators, including Lyn, Fyn and Syk, which are initiators of intricate pathways involving numerous downstream signaling molecules that ultimately coordinate and

control mast cell responsiveness [2]. Loss of Fyn or Lyn in mast cells has widespread effects, impairing degranulation and cytokine production. While Fyn and Lyn tyrosine kinases are associated with SphK1 and SphK2 in murine mast cells, activation of SphK1 requires Fyn but Lyn is partly dispensable [12]. Both Lyn and Fyn contribute to SphK2 translocation to the plasma membrane upon FcεRI triggering. Interestingly, SphK2 was reported to be the major contributor of S1P in murine mast cells derived from embryonic liver progenitors [13]. Mast cells derived from s*phk2*-knockout mice demonstrated impaired IgE-mediated degranulation and production of certain cytokines, primarily due to reductions in intracellular calcium levels and PKC activation. Impairment of degranulation in SphK2-deficient mast cells was partially restored by the addition of exogenous S1P. This confirmed that SphK2 is necessary, but not sufficient, for IgE-mediated responses, at least in murine mast cells [12]. By contrast, in human mast cells, SphK1 but not SphK2 is critical for antigen-induced degranulation, chemokine secretion and migration, while both isozymes are important for cytokine secretion [14]. Furthermore, downregulation of SphK1 reduced the rapid and transient increase in intracellular calcium induced by FcεRI crosslinking, which is necessary for mast cell degranulation [10]. In addition to the engagement of FcεRI, several other stimuli are capable of triggering secretion of inflammatory mediators from activated mast cells, including the anaphylatoxin C5a [46]. With regard to the actions of C5a, SphK1 expression is required for its ability to trigger calcium release, chemotaxis, degranulation, and cytokine release from human macrophages [47]. However, neutrophils isolated from s*phk1*-knockout mice showed normal responses to C5a [48].

1.6 Extracellular functions of S1P in mast cells

Inside-out signaling, whereby S1P generated intracellularly by activation of SphKs is secreted and activates $S1P_1$ and/or $S1P_2$ receptors on the same or nearby cells, plays important roles in mast cell responses [49]. For example, activation of $S1P_1$ is critical for migration of mast cells toward antigens and might be involved in the movement of mast cells up an antigen gradient to sites of inflammation [11, 50]. Furthermore, expression of the motility-inhibiting $S1P_2$ receptor in mast cells is upregulated by crosslinking of FcεRI by antigens [11], suggesting that mast cells are attracted to an inflammation site by a $S1P_1$-dependent motility process and halt upon reaching their destination owing to upregulation of $S1P_2$. Here, activation by inside-out signaling also enhances their degranulation. Thus, there appears to be an exquisite interplay of S1P controlled responses following FcεRI activation in mast cells.

1.7 Secretion of S1P from mast cells

The mechanism by which intracellularly produced S1P can exit from cells to interact with its receptors located on the extracellular leaflet of the plasma membrane has been a long standing mystery. It has been proposed that SphK1 may be secreted from cells and catalyzes the conversion of sphingosine to S1P extracellularly [51, 52], although no evidence has been found for this in mast cells [11]. A partial answer has now been provided by the discovery that the ATP-binding cassette transporter ABCC1 promotes the export of S1P across the plasma membrane of activated rodent and human mast cells

independent of their degranulation [53]. It is possible that other ABC transporters may also participate in export of S1P.

1.8 Blood levels of S1P

The concentration of S1P in blood is maintained at high levels. Plasma levels range from 0.1 to 0.6 μM, while serum levels range from 0.4 to 1.1 μM [54, 55]. S1P mainly circulates as a complex with albumin and lipoproteins. Platelets that produce, store and secrete large amounts of S1P, were long considered to be the major source of circulating S1P. However, recent studies suggest that erythrocytes may be the major source of S1P in blood [56, 57]. The vascular endothelium, in addition to the hematopoietic system, has also been suggested to be an important contributor of plasma S1P [29].

Levels of S1P in tissues are significantly lower than in blood, possibly owing to the presence of S1P phosphatase and S1P lyase, which are absent or low in platelets and erythrocytes [58]. This leads to the establishment of a concentration gradient of S1P between blood and tissues, which is important for cell trafficking. Intriguingly, deletion of either isoform of SphK in mice does not abolish this blood–tissue gradient of S1P [13], while loss of S1P lyase activity does so [58]. Similarly, secretion of S1P by mast cells may also serve to establish a gradient that aids in the recruitment of other immune cells whose chemotactic motility is stimulated by S1P. However, susceptibility to *in vivo* anaphylaxis correlated with circulating S1P generated by SphK1 that was predominantly from a non-mast cell source(s) [13].

1.9 Intracellular actions of S1P in mast cells

Although intracellular targets of S1P in mast cells have yet to be identified, S1P has intracellular second messenger actions that regulate calcium levels independently of phosphatidylinositol (3,4,5)-trisphosphate ($InsP_3$) [7]. This calcium mobilization was recently demonstrated to be dependent on clathrin [59]. It has also been suggested that both $InsP_3$ and S1P contribute to FcεRI-induced calcium release from the endoplasmic reticulum and that production of $InsP_3$ is necessary for S1P to cause calcium mobilization from the endoplasmic reticulum [60].

Intriguingly, fetal liver-derived mast cells from mice lacking SphK2 display impaired calcium mobilization upon IgE-receptor activation, even when S1P is added exogenously [13]. Additionally, exogenous S1P only partially restored degranulation to mast cells isolated from mice lacking Fyn kinase [12]. Collectively, these data suggest that S1P may be a *bona fide* second messenger in mast cells, although acting in a manner that still requires clarification.

1.10 Anaphylaxis

Anaphylaxis is a severe and potentially fatal immediate systemic allergic reaction that occurs suddenly after contact with an allergy-causing substance and is primarily triggered by rapid, IgE-mediated immune release of potent mediators from tissue mast cells and peripheral basophils [61]. Mast cells reside at mucosal, submucosal and perivascular locations in close proximity to epithelial surfaces, near blood vessels, nerves and glands, where they are able to detect invading pathogens and changes in their

environment [45]. In humans, mast cell-derived mediators contribute to the pathophysiology of allergic diseases, inducing tissue edema, bronchoconstriction, increased vascular permeability, influx of inflammatory cells and mucus secretion. In addition, mast cells express numerous receptors for cytokines, chemokines and eicosanoids, as well as Toll-like receptors, which enable them to recognize diverse allergic stimuli. The diversity in cellular location, as well as the repertoire of receptors expressed and mediators released, permits mast cells to be key regulators of innate and adaptive immunity.

Murine and human immune systems are reasonably similar and so animal models of anaphylaxis may provide information that is potentially relevant to human anaphylaxis. Systemic anaphylaxis in the mouse can be mediated via two independent mechanisms; a classical pathway mediated by IgE, FcεRI, mast cells, histamine and platelet-activating factor (PAF), and an alternative pathway mediated by IgG, FcγRIII, macrophages and PAF [62]. Most human systemic anaphylaxis is IgE-dependent, although there is some evidence for IgE-independent anaphylaxis [63]. Some potent food allergens, particularly peanuts and tree nuts, can stimulate an anaphylactic-like, non-IgE-mediated response, thereby synergizing with IgE-induced mast cell activation to exacerbate anaphylaxis.

1.11 Role of S1P in anaphylaxis

Recent studies indicate that SphKs are also determinants of anaphylaxis. SphK2 was shown to be the main isoform required for generation of S1P, calcium influx and degranulation of rodent mast cells [12]. However, susceptibility to anaphylaxis in mice

was correlated with circulating S1P generated by SphK1, predominantly from a non-mast cell source [13]. Mast cells do not contribute to basal circulating levels of S1P as mast cell-deficient mice have similar levels of plasma S1P compared with their counterparts engrafted with normal mast cells [13]. Mice deficient in SphK1 have reduced levels of circulating S1P and are resistant to anaphylaxis. They also have impaired histamine responses despite normal mast cell function. However, mice deficient in SphK2 have enhanced levels of S1P in the blood and respond normally to anaphylactic challenge with normal histamine release [13]. Moreover, IgE-triggered anaphylactic responses were significantly attenuated by the $S1P_2$ antagonist JTE-013 and in $S1P_2$-deficient mice, in contrast to anaphylaxis induced by administration of histamine or platelet-activating factor [50].

Intestinal anaphylaxis (allergic diarrhea) is almost totally IgE-dependent and mast cell-dependent, but is mediated predominantly by PAF and serotonin. In a murine intestinal anaphylaxis model, $S1P_1$ expression was preferentially associated with pathogenic $CD4^+$ T cells induced by allergen challenge in the large intestine. The immunosuppressant drug FTY720 prevented allergic diarrhea by inhibiting the migration of these cells and decreased mast cell infiltration into the large intestine, but did not affect eosinophil infiltration or serum IgE production [64].

1.12 Asthma

Asthma is an obstructive lung disease that prevents exhalation from the lungs and thus reduces respiratory capacity due to allergic inflammation and narrowing of the

airways. In asthma, mast cells infiltrate the bronchial epithelium and, upon activation, release inflammatory mediators that influence bronchial epithelial function. Allergic asthma is often classified into early and late-phase reactions. Early-phase reactions are induced within seconds to minutes of allergen challenge and occur as a result of mediator secretion by mast cells at the affected site. Release of preformed mediators contributes to acute signs and symptoms of early-phase reactions. These signs and symptoms vary according to the site of reaction and the mast cell populations involved but can include vasodilation, increased vascular permeability, contraction of bronchial smooth muscle, and mucus secretion. Upon activation by IgE and allergen, mast cells also release a broad range of newly synthesized mediators that contribute to late-phase reactions and occur within several hours. Many mast cell products have the potential to recruit and/or activate other immune cells (TNF-α, IL-5, IL-6, IL-8/KC, eotaxin, CCL2, etc), and clinical features of late-phase reactions reflect the activities of both resident cells and immune cells that are recruited to the affected site, including eosinophils, neutrophils, monocytes/macrophages, and T cells.

Mast cell numbers are greater in the mucosal epithelium of patients with asthma and allergic diseases compared with disease-free controls, with no substantial change in the numbers of mast cells in the adjacent connective tissues [65]. Chronic asthma is typically associated with increased number of mucus-producing goblet cells, increased production of cytokines and chemokines, severe inflammation, and airway remodeling. Abnormal airway smooth muscle function is a key feature in the pathophysiology of asthma, with a positive correlation between mast cell numbers and bronchial hyper-responsiveness [66].

1.13 Role of S1P in asthma

Previous studies demonstrated that S1P was elevated in the airways of asthmatic individuals after antigen challenge and that S1P modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma [67] and can induce contraction of airway smooth muscle [68]. S1P can also amplify and enhance mast cell functions and may regulate their arrival to sites of inflammation [11, 69]. Rodent models in which asthma-like symptoms are introduced by sensitization and challenge with antigen are characterized by airway eosinophilia, which contributes to the observed airway hyper-responsiveness (AHR). It has recently been demonstrated that S1P induces dose-dependent contraction of bronchi and increases AHR in ovalbumin (OVA)-sensitized mice [70]. These events were associated with increased expression of SphK1 and SphK2, as well as $S1P_2$ and $S1P_3$ receptors. Local administration of S1P caused inflammation and eosinophil recruitment in a rat-paw inflammation model [71]. Furthermore, S1P and the kinases that produce it play important roles in many types of immune cells involved in allergic responses and asthma (Table 1), implicating S1P as a pleiotropic lipid mediator important in the inflammatory and allergic reactions and asthma.

FTY720 is highly effective in reducing the severity of autoimmune diseases in several animal models [72]. Neither FTY720 nor FTY720-phosphate, despite its similarity to S1P and ability to bind and activate four of the five S1P receptors, affect mast cell degranulation, yet both significantly reduce antigen-induced secretion of prostaglandin D_2 and cysteinyl leukotrienes [73]. FTY720 was suggested to be a direct inhibitor of cytosolic

phospholipase A_2, the rate-limiting enzyme in the production of all eicosanoids [73]. Indeed, oral treatment of mice with FTY720 inhibits AHR induced by adoptive transfer of T_H1 and T_H2 cells and asthma induced by active immunization and challenge with OVA [74]. In addition, inhalation administration of FTY720 prior to, or during, ongoing allergen challenge suppressed T_H2-dependent eosinophilic airway inflammation and bronchial AHR by inhibition of migration of lung dendritic cells to the mediastinal lymph nodes, thus preventing the formation of allergen-specific T_H2 cells in the lymph nodes [75].

Table 1. Involvement of SphKs in responses to stimulation of various types of immune cells.

Cell type	Source	Stimulus	SphK Involved	Effects	Ref.
Mast	Rat RBL-2H3	IgE/Ag	SphK1	Initial rise in calcium from internal stores, degranulation, cytokine production and/or migration towards antigen	[11]
	Murine BMMC	IgE/Ag	SphK2	Production of S1P, calcium influx, PKC activation, degranulation and/or cytokine production	[13]
	Human LAD2/ CB-MC	IgE/Ag	SphK1	Degranulation, CCL2 secretion and/or migration towards antigen	[14]
	Human LAD2/ CB-MC	IgE/Ag	SphK1/SphK2	TNF secretion	[14]
Macrophages	Human blood	C5a	SphK1	Intracellular calcium signaling, degranulation, cytokine generation (TNF, Il-6, IL-8) and/or chemotaxis	[45]
	Murine RAW264	LPS	SphK1	ERK1/2 and NF-κB activation	[154]
	Human U937	IFN-γ	SphK1	Vesicular trafficking	[155]
	Murine bone marrow	RANKL	SphK1	Osteoclastogenesis via regulation of p38, ERK, NFATc1 and/or cFos	[156]
	Human blood	Apoptosis Inducers	SphK2	Polarization to M2 phenotype, decreased TNF and IL-12-p70 production, increased IL-8 and IL-10 production and/or decreased NF-κB signaling	[157]
	Murine *in vivo*	C5a	SphK1	Acute peritonitis, systemic inflammation, multiorgan damage and/or release of proinflammatory mediators	[158]
B cells	Human lymphoblasts	S1P	SphK1	Resistance to Fas-mediated cell death	[159]
T cells	Murine CD4+	IL-2	SphK2	Regulation of proliferation, secretion of cytokines and/or STAT5 activation	[160]
	Murine	T-cell receptor	SphK1	Negative regulation of chemokine expression (IFN-γ, TNF, IL-2)	[161]
	Murine 2D6 clone	IL-12	SphK2	Promotes Th1 differentiation and cell-mediated immune responses	[162]
Neutrophils	Human blood	C5a	SphK1	Calcium release, degranulation, chemotaxis and/or activation of NADPH oxidase	[163]
Eosinophils	Murine *in vivo*	OVA	SphKs*	Inflammatory cell infiltration, eosinophilia, increased bronchoalveolar lavage fluid, IL-4, IL-5 and eotaxin production and/or increased serum IgI levels	[124]
	Murine *in vivo*	MeCh	SphKs*	Mucus production and/or airway hyper-responsiveness	[124]

*Isozyme not identified.
BMMC: Bone marrow-derived mast cells; CB-MC: Cord blood-derived mast cells; CCL2: CC chemokine ligand 2; ERK: Extracellular signal-regulated kinase; LPS: Lipopolysaccharide; MeCh: Methacholine; OVA: Ovalbumin; S1P: Sphingosine-1-phosphate; SphK: Sphingosine kinase.

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1.14 Dissertation Objective

It is well recognized that mast cell phenotype varies depending on the environment they populate *in vivo* or the growth factors used *in vitro* [46]. Human mast cells have two distinct phenotypes which vary in migration behavior and responses to activating stimuli, based on the content of intragranular proteases. Both phenotypes appear to be derived from a common mast-cell progenitor with the ultimate phenotype determined by micro-environmental factors in the surrounding milieu. However, the exact environmental cues governing mast cell differentiation remain to be determined. Thus, we investigated the involvement of S1P in the development of mast cells derived from human cord blood hematopoietic progenitors.

While the first portion of this work is focused on the emerging importance of S1P in the development of mast cells, the latter will focus on the consequence of S1P in mast cell functions. Although S1P has been implicated as an important component in the regulation of immune responses, many questions remain to be answered. There has been some debate regarding the importance of each of the SphK isoenzymes in mast cell-mediated allergic responses. We examined the importance of SphK1 and S1P *in vivo* using an isotype specific SphK1 inhibitor, SK1-I, in a mast cell-dependent and IgE-dependent murine model of chronic asthma. These studies will provide the basis for enhancing existing therapeutic approaches by targeting SphKs, S1P receptors, and S1P itself, to suppress mast cell-mediated inflammation and related pathological conditions.

CHAPTER 2: SPHINGOSINE-1-PHOSPHATE INDUCES DEVELOPMENT OF FUNCTIONALLY MATURE CHYMASE EXPRESSING HUMAN MAST CELLS FROM HEMATOPOIETIC PROGENITORS

2.1 INTRODUCTION

Mast cells are key effector cells involved in orchestrating and perpetuating inflammatory responses. They are tissue dwelling cells derived from hematopoietic stem cells that circulate in the blood as committed progenitors until they enter the tissues to complete their maturation [45]. Once mature, mast cells reside in the perivascular spaces of all tissues and contain intracytoplasmic granules rich in acidic proteoglycans. There are two subpopulations of human mast cells based on the composition of their intragranular protease repertoire: those expressing tryptase only (MC_T), resemble mucosal mast cells and are predominant in lung; and those that contain chymase in addition to tryptase (MC_{TC}), are similar to connective tissue mast cells and the phenotype of skin mast cells [61, 76]. Stem cell factor (SCF), the Kit ligand, is an important growth factor required for mast cell survival and differentiation and is the only growth factor identified so far that by itself *in vitro* causes human hematopoietic progenitor cells to become tryptase producing mast cells [77, 78]. Several cytokines, including IL-3, IL-4, IL-5, IL-6, and IL-9, enhance the mitogenic effects of SCF on cord blood–derived cultured human mast cells (CB-MCs) *in vitro,* and some of them are also cytoprotective [79] [80] [81] [82]. Much less is known of how human hematopoietic progenitor cells differentiate into mature MC_{TC} and the factors that influence chymase expression. A notable exception is IL-6 which induces chymase

protein expression in SCF-dependent cord blood–derived human mast cells that normally only express tryptase [83-86].

Sphingosine-1-phosphate (S1P) is a potent lipid mediator produced and secreted by mast cells to regulate their functions (reviewed in [49, 87]). Similar to crosslinking of the high affinity IgE receptor (FcεRI), SCF also activates both isoforms of sphingosine kinase (SphK1 and SphK2), in mast cells leading to S1P formation [12]. It has been suggested that SphK2 is required in murine mast cells for production of S1P, cytokine secretion and degranulation. However, susceptibility of mice to *in vivo* anaphylaxis correlated with circulating S1P generated by SphK1 from a non-mast cell source [13]. Mast cells express two of the five known S1P receptors, $S1P_1$ and $S1P_2$, and activation of these receptors by secreted S1P is important in movement of rodent mast cells and their degranulation, respectively [11, 14].

Mast cell precursors circulate in the blood where they have the opportunity to encounter various serum-borne growth factors, including S1P. Lysophosphatidic acid (LPA), another phospholipid mediator present in serum that is structurally related to S1P was shown to increase the number of cord blood-derived mast cells (CB-MCs) [88]. Given that S1P is also present in human serum at high nanomolar concentrations [16] and can influence mast cell responses [49, 89], it was of interest to examine the involvement of S1P in development of mast cells derived from human hematopoietic progenitors. Remarkably, S1P increased the number of cord blood-derived mast cells (CB-MCs) and strikingly increased expression of chymase and CD88, the receptor for C5a. Our results also reveal

that cooperation between monocytes/macrophages and mast cell progenitors may be important for the development of mature chymase expressing mast cells.

2.2 MATERIALS AND METHODS

2.2.1 Reagents and antibodies

S1P was obtained from Biomol (Plymouth Meeting, PA). VPC23019 was from Avanti (Alabaster, AL). JTE-O13 was from Tocris (Ellsville, MO). SCF was a generous gift from Amgen (Thousand Oaks, CA). Recombinant human IL-6 was purchased from R & D Systems (Minneapolis, MN). Anti-tryptase and anti-chymase monoclonal antibodies (mAb) were obtained from Chemicon (Temecula, CA), Alexa Fluor 488-labeled goat anti-rabbit IgG was from Molecular Probes, Eugene, Ore. Dinitrophenyl-human serum albumin (DNP-HSA, Ag), heat-inactivated controlled process serum replacement medium (CPSR-3), C5a, and compound 48/80 were from Sigma-Aldrich (St. Louis, MO), anti-human IL-6 mAb was from Invitrogen (Carlsbad, CA). Anti-CD14 coated magnetic Dynabeads were from Dynal Biotech, ASA, (Oslo, Norway), and human cytokine ELISA kits were from BD Biosciences (San Diego, CA).

2.2.2 Culture of human cord blood-derived mast cells

Umbilical cord blood was obtained at the time of delivery and collected in heparin-containing tubes. The experimental protocol was approved by the Institutional Review Board at Virginia Commonwealth University. Cord blood was diluted and overlaid on Histopaque (density = 1.077 g/ml) and then centrifuged to remove erythrocytes. Mononuclear cells at the plasma-Histopaque interface were collected, washed, and subjected to a second Histopaque density gradient centrifugation. Purified mononuclear

cells were cultured in 24-well plates at 5 x 10^5 cells/ml in RPMI 1640 containing 10% heat-inactivated controlled process serum replacement medium (CPSR-3), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10 mM HEPES (pH 7.2), 50 μM 2-mercaptoethanol, 200 U/ml penicillin, 100 μg/ml streptomycin, and 100 ng/ml SCF in the absence or presence of S1P as indicated in figure legends. Culture medium with indicated supplements was replaced weekly. Slides were stained with toluidine blue to assess metachromasia and mast cell numbers. Cell numbers and viability (always >80% determined by trypan blue exclusion) were assessed immediately prior to experiments.

2.2.3 Immunomagnetic purification of CB-MCs by negative depletion of CD14-positive cells

Monocytes/macrophages were immunodepleted from cultures using anti-CD14 coated magnetic Dynabeads (4 beads per target cell), essentially as recommended by the manufacturer except for the omission of sodium citrate/EDTA. Unattached CD14-negative cells (mast cells) were collected, cultured as described above and contained 95-99% mast cells as determined by toluidine blue staining.

2.2.4 Preparation of macrophages from cord blood cultures

Macrophages were highly enriched by positive selection with biodegradable anti-CD14-MicroBeads using a SuperMACS (Miltenyi Biotec, Auburn, CA), according to the manufacturer's protocol. 90-95% of the cells were positive for CD14 expression by immunofluorescence analysis.

2.2.5 Immunofluorescence and immunocytochemistry

CB-MCs (5 x 10^4) were smeared onto glass slides, fixed in methanol containing 0.6% H_2O_2 for 30 min at room temperature, and stored at –80 ˚C. Slides were incubated with 10 μg/ml tetramethylrhodamine isothiocyanate conjugated anti-tryptase G3 mouse mAb (G3-TRITC) or isotype-matched negative control (MOPC-TRITC) for 1 h at 37 ˚C, washed three times in 0.01 M Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBST). Cells were visualized by fluorescence microscopy with a Nikon TE300 and percentage of positively stained cells was calculated. At least 200 cells were scored in a double-blinded manner. Images were also collected with a Zeiss LSM 510 Meta confocal microscope with the optical slice set to 1 μm for all channels. All images were exported directly using Zeiss LSM Image Examiner (v. 3.2.0.70) to 8 bit TIFF files without compression, contrast, or gamma adjustments.

Slides were stained for chymase with biotin-conjugated anti-chymase B7 mAb (B7-B) or isotype-matched negative control (MOPC-B) overnight at 4 ˚C, washed in TBST and incubated with streptavidin-peroxidase conjugate (20 μg/ml) for 1 h at room temperature. After washing, slides were incubated with 3-amino 9-ethylcarbazole in 0.01% H_2O_2 for 7 min at room temperature, and chymase positive mast cells identified by brown staining. Slides were examined with a Nikon Eclipse E800 microscope equipped with a 100X objective and percentage of positively stained cells was calculated. At least 200 cells were scored in a double-blinded manner. In some experiments, after washing, slides were incubated with alkaline phosphatase-conjugated anti-tryptase G3 mAb (10 μg/ml) at 4 °C.

Slides were then washed and incubated with SIGMAFAST Fast Red TR/Naphthol AS-MX phosphate (4-Chloro-2-methylbenzenediazonium/3-hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate) and tryptase positive mast cells identified by pink-red staining.

2.2.6 Degranulation

CB-MCs were sensitized with 1 μg/ml anti-dinitrophenyl (DNP)-IgE overnight, washed once to remove unbound IgE, and then stimulated without or with DNP-HSA (Ag, 30 ng/ml) at 37˚C. Degranulation was determined by measuring the release of the granule marker, β-hexosaminidase, with a colorimetric enzyme assay. Values are expressed as percentage of total cellular β-hexosaminidase released into the medium. Spontaneous degranulation of unstimulated cells was <10%.

2.2.7 Flow cytometry

To determine expression of surface CD88, CB-MCs were incubated with rabbit anti-human CD88 mAb (10 μg/mL) or a non-immune rabbit IgG (10 μg/mL) as a negative control, followed by staining with Alexa Fluor 488-labeled goat anti-rabbit IgG (5 μg/mL). After staining cells were washed once with PBS and re-suspended in FACS buffer. Flow cytometric analysis was performed using the FC500 combined with CXP software (Beckman Coulter, Fullerton, CA).

2.2.8 ELISA

Human IL-6 and CCL2/MCP-1 were measured by ELISAs with purified biotinylated mouse or rat mAbs specific for each cytokine. Standard curves were prepared with recombinant cytokines (BD Biosciences, San Diego, CA). Assays were performed in Maxisorb 96-well plates (Nunc) according to the manufacturer's protocols. Briefly, wells were coated overnight at 4°C with capture mAbs, blocked with PBS containing 10% FBS, washed in PBS containing 0.05% Tween 20, and incubated for 2 h at room temperature with standards or samples diluted in PBS with 10% FBS. Wells were washed, incubated with biotin detection mAbs and streptavidin-HRP conjugate for 1 h at room temperature, washed, and incubated with peroxidase substrate. Absorbance was measured at 450 nm with an EL800 microplate reader (Biotek, Winooski, VT). The lower limits of detection for IL-6 and MCP-1/CCL2 were 4.7 and 7.8 pg/ml, respectively.

2.2.9 Quantification of lipids

Lipids were extracted from media and cells by the Lipidomics Core at VCU. Internal standards were added (0.5 nmol each, Sphingolipid Mixture II/LM-6005, Avanti Polar Lipids), lipids extracted, and sphingolipids quantified by liquid chromatography, electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS; 4000 QTRAP; ABI), as described [90].

2.2.10 Statistical analysis

Experiments were repeated at least 3 times with consistent results. For each experiment, data from triplicate samples were calculated and expressed as means ± SD. Differences between groups were determined with the paired Student's test, $p \leq 0.05$ was considered significant.

2.3 RESULTS

2.3.1 S1P induces development of cord blood progenitor cells to chymase expressing mast cells

Mast cell precursors circulate in the blood where they have the opportunity to encounter various serum-borne growth factors, including S1P [16, 91]. Therefore, we sought to examine whether S1P might influence the development of mast cells derived from human hematopoietic progenitors. To this end, S1P was added to cord blood mononuclear progenitors cultured in chemically defined medium containing human SCF, a growth factor that is able to induce hematopoietic progenitor cells cultured *in vitro* to become mast cells [78]. The addition of 1 μM S1P, the concentration found in normal human plasma [55], had no significant effects on viability of cord blood mononuclear cultures compared to cells cultured in the presence of SCF alone. To examine the effects on development of mast cells, cultures were immunostained for tryptase, an intragranular marker of mast cells. After prolonged culture in the presence of S1P, a higher proportion of cells stained positively for tryptase compared to cultures with SCF alone (Figure 2A). Although co-culture of progenitors with SCF and S1P for 3 weeks had no significant effects on the proportion of tryptase-positive cells or on metachromasia (Figure 2B), by 6 to 7 weeks, 1 μM S1P increased the proportion of tryptase positive cells compared to cells cultured with SCF alone. Similarly, after 8 weeks of culture with concentrations of 0.1 and 1 μM S1P, there was an enhancement of numbers of tryptase-positive mast cells of 1.6-

and 3-fold, respectively (Figure 2A, 3). Lower concentrations of S1P did not show consistent effects.

Figure 2: S1P accelerates tryptase expression in developing human mast cells. A,B, Cord blood mononuclear cells were cultured for the indicated weeks with SCF (100 ng/ml) alone (none) or in the absence (vehicle) or presence of S1P (0.1 and 1 μM). Cultures were stained with anti-tryptase mAb (G3-TRITC) to assess tryptase expression and tryptase positive cells quantified as described in Materials and Methods. A minimum of 200 cells was scored in a double-blind manner. **A,** Data are expressed as total number of tryptase positive cells. **B,** Results from a cord blood culture from another donor are expressed as percent tryptase positive cells. Data are means ± SD. *, $p < 0.05$, **, $p < 0.01$, compared to untreated controls. Similar results were obtained with two additional cord blood cultures from other donors.

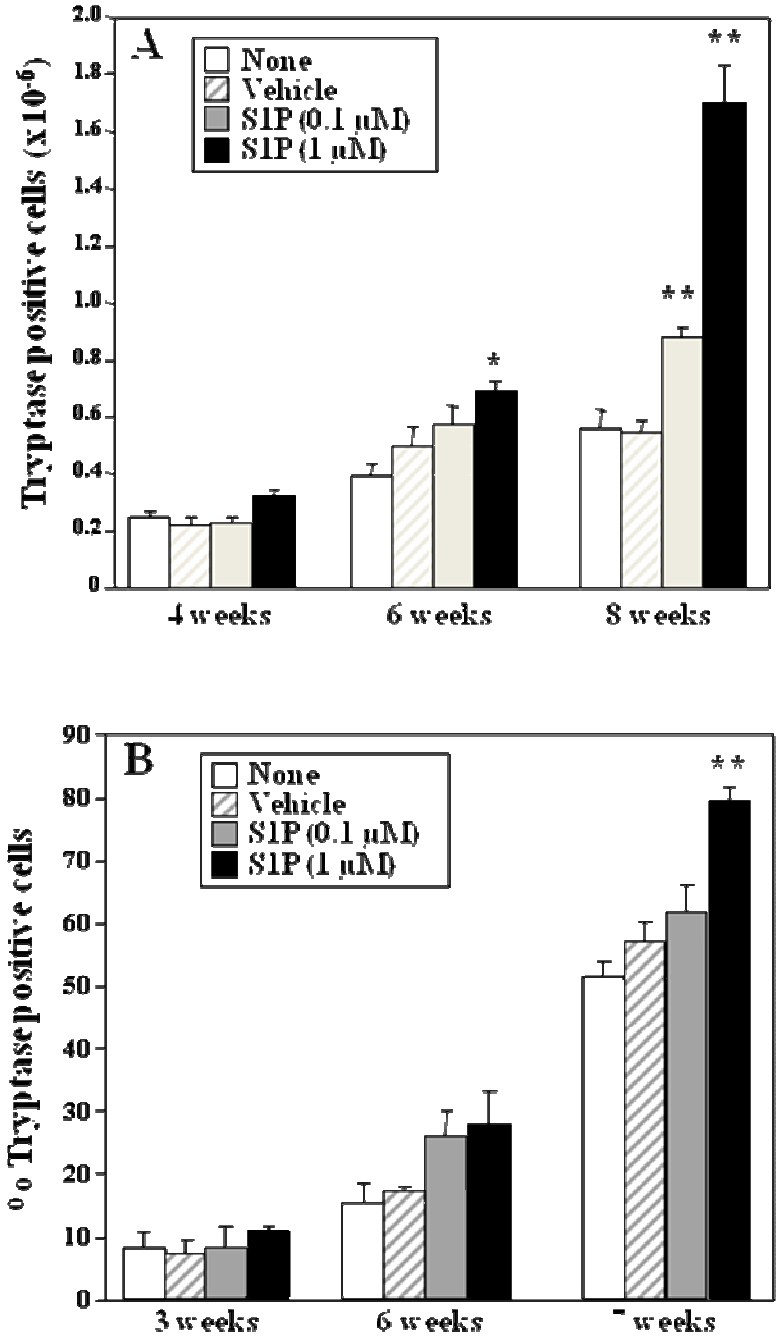
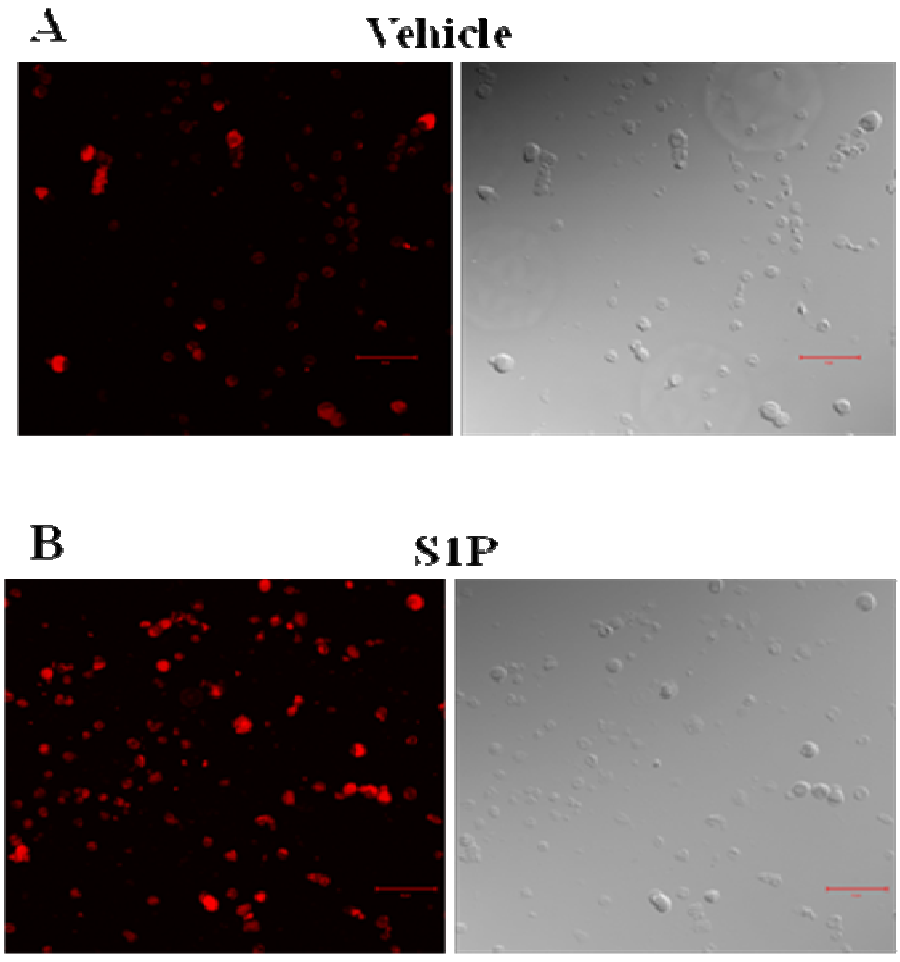

A
None
Vehicle
S1P (0.1 μM)
S1P (1 μM)
Tryptase positive cells (x10-6)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
**
**
4 weeks
6 weeks
8 weeks
B
None
Vehicle
S1P (0.1 μM)
S1P (1 μM)
% Tryptase positive cells
90
80
70
60
50
40
30
20
10
0
**
3 weeks
6 weeks
8 weeks

Figure 3: Effect of S1P on tryptase expression in developing human mast cells. Panels show representative fields of cells from a different donor stained with G3-TRITC and corresponding DIC images after treatment without (vehicle) (**A**) or with S1P (0.1 μM) (**B**) for 8 weeks. Size bars, 50 μm. Similar results were obtained with two additional cord blood cultures from other donors.


A
Vehicle
B
S1P

In agreement with previous studies [83, 86, 88], human progenitor cells cultured in the presence of SCF alone only expressed tryptase and had no detectable chymase in their granules, as determined by immunocytochemistry (Figure 4A, 5A). Remarkably, culturing in the presence of S1P not only increased the number of mast cells but strikingly increased chymase expression (Figure 5, 6). A small increase was detected within 4 weeks of culture in the presence of 1 μM S1P (Figure 4A). However, after 6 weeks of culture, even a concentration of S1P as low as 0.1 μM induced a significant increase in chymase-positive mast cells (Figure 4A, B). Tryptase staining in the granules of cells cultured for 8 weeks in the presence of S1P was observed by confocal microscopy (Figure 6B). These cells also contained granules that stained strongly with toluidine blue (Figure 6C). Immunocytochemistry of these S1P treated mast cells revealed strong chymase staining compared to cells cultured with SCF alone (Figure 5A, 6D). Moreover, as expected, the chymase expressing mast cells also expressed tryptase (Figure 6E). These results are reminiscent of many previous studies showing that after culturing cord blood mononuclear cells in the presence of SCF and IL-6 for 8 weeks, all mast cells had tryptase-positive granules, while ~ 20% also expressed chymase [79, 82, 92].

Figure 4: S1P induces chymase expression in developing human mast cells. A, B, Cord blood mononuclear cells were cultured for the indicated weeks with SCF (100 ng/ml) alone (none) or in the absence (vehicle) or presence of the indicated concentrations of S1P. Cells were stained with anti-chymase mAb or negative control IgG and chymase-positive cells were quantified as described in Materials and Methods. A minimum of 200 cells was scored in a double-blind manner. **A,** Data are expressed as total number of chymase positive cells. **B,** Results from a cord blood culture from another donor are expressed as percent chymase positive cells. Negative control staining was <1%. Similar results were obtained with two additional cord blood cultures from different donors. Data are means ± SD. *, $p < 0.05$, **, $p < 0.01$, compared to untreated controls.

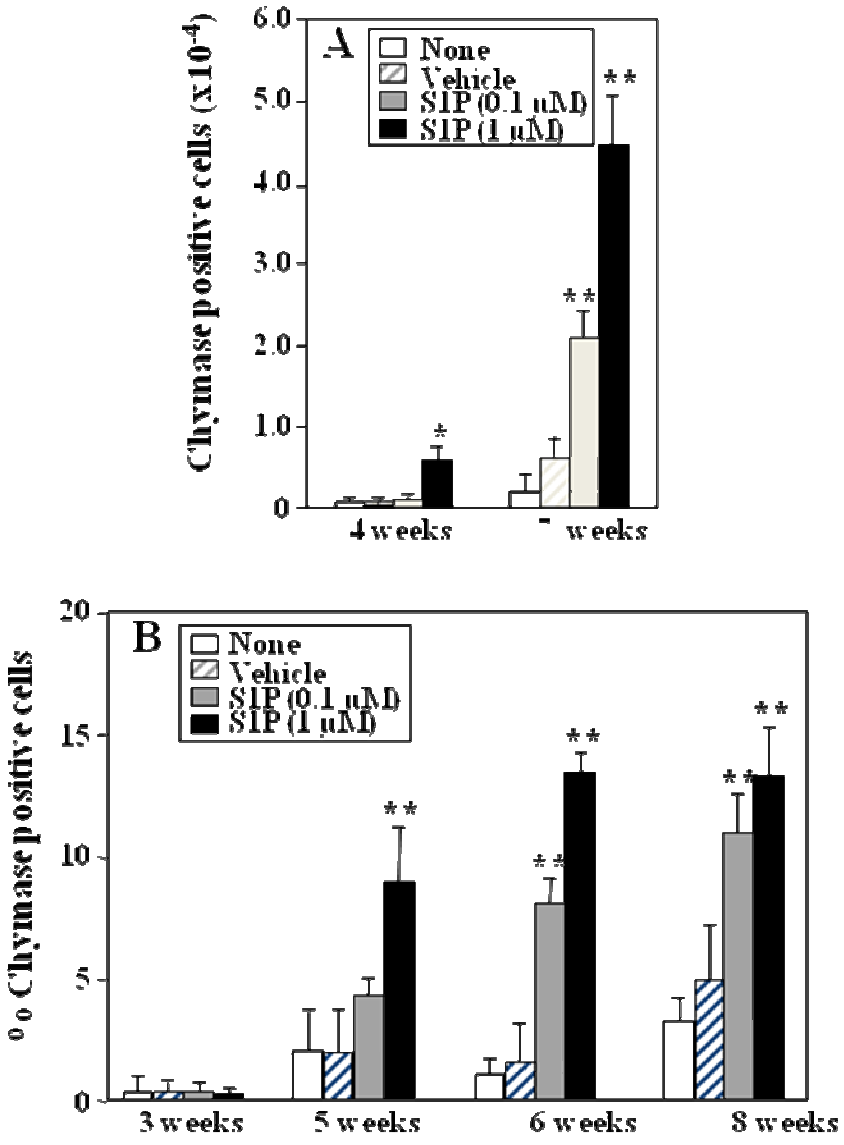
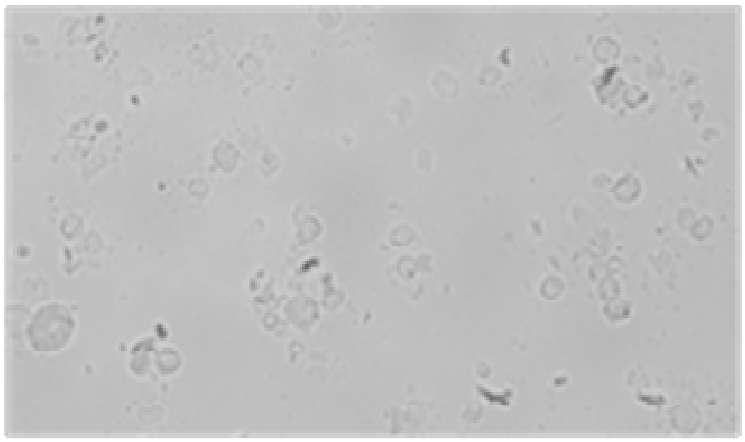

A
None
Vehicle
S1P (0.1 uM)
S1P (1 uM)
Chymase positive cells (x10-4)
6.0
5.0
4.0
3.0
2.0
1.0
0
*
**
**
4 weeks
B
None
Vehicle
S1P (0.1 uM)
S1P (1 uM)
% Chymase positive cells
20
15
10
5
0
**
**
**
**
3 weeks
5 weeks
6 weeks
8 weeks

Figure 5: Effect of S1P on chymase expression in developing human mast cells. Panels show representative fields of cells from a different donor stained with anti-chymase mAb and photographed under light microscopy at 200x magnification after treatment without (vehicle) (**A**) or with S1P (0.1 μM) (**B**) for 10 weeks. Similar results were obtained with two additional cord blood cultures from other donors.

A Vehicle



B S1P

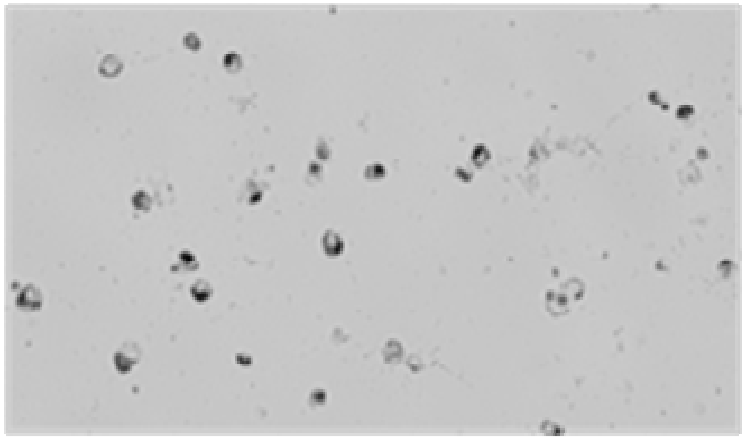
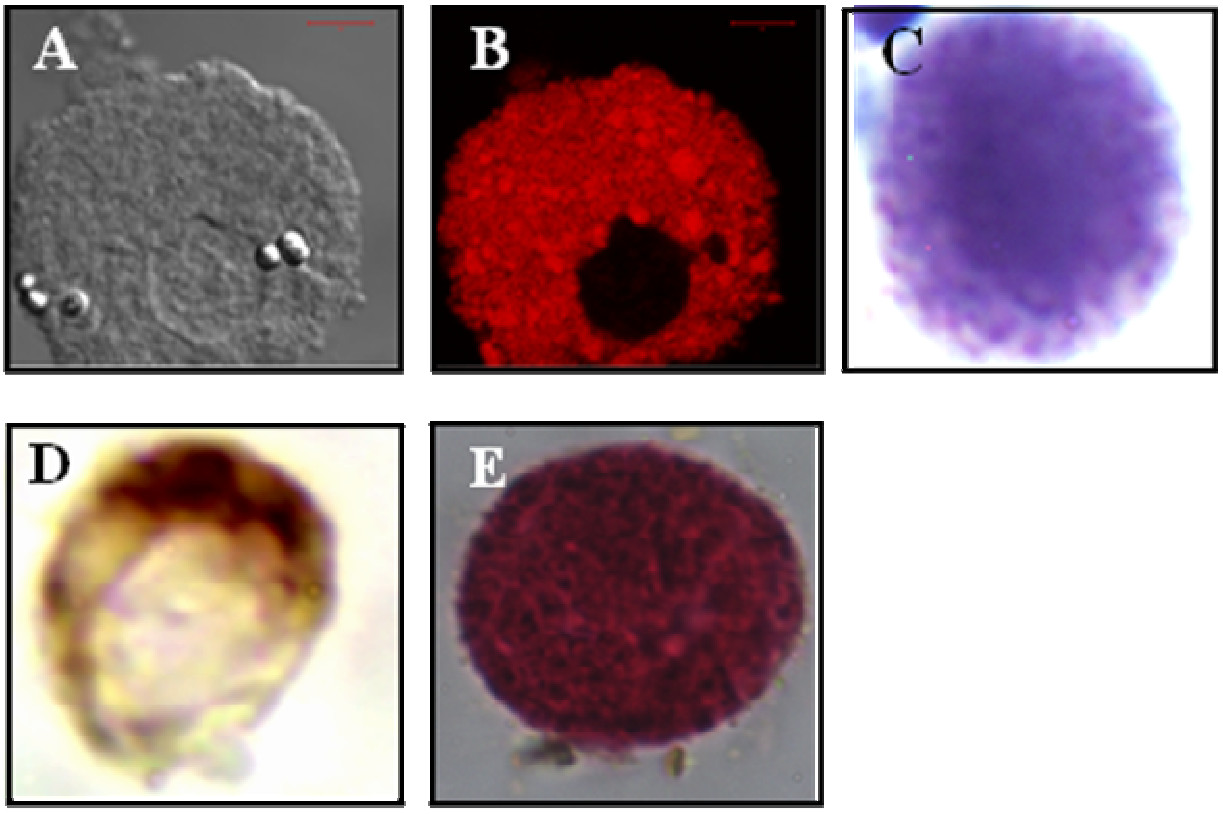
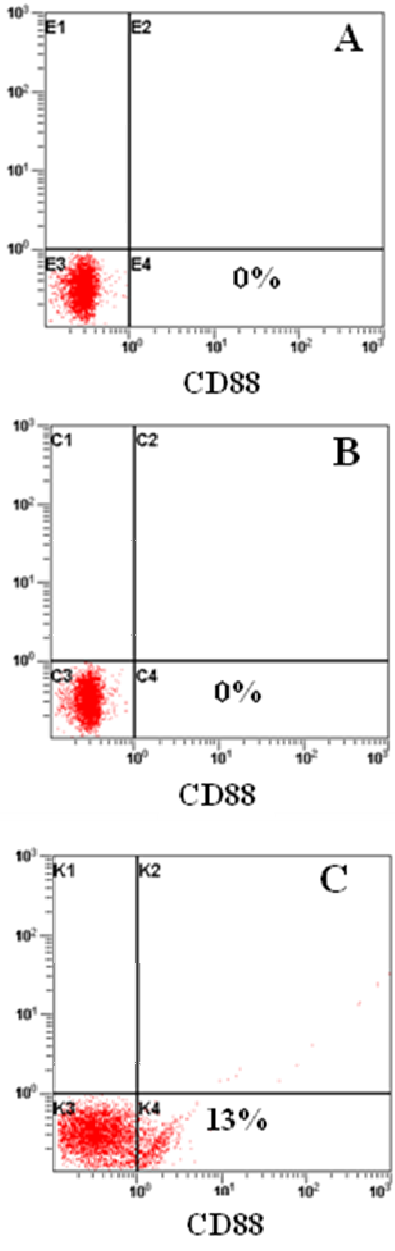


Figure 6: Histochemical characteristics of CB-MCs cultured in the presence of S1P. A-**E,** Cord blood mononuclear cells were cultured for 8 weeks with SCF and S1P (0.1 μM). Cells were visualized by confocal microscopy for DIC (**A**) and tryptase staining (**B**). Size bars, 5 μm. Toluidine blue (**C**) and chymase (**D**) staining were visualized by light microscopy. (**E**) Cells were stained for both chymase and tryptase. Representative individual cells from three experiments are shown.


A
B
C
D
E

In agreement with previous studies showing that surface CD88, the receptor for C5a (C5aR), is only expressed by mast cells double positive for tryptase and chymase (MC_{TC}) [93, 94], there was no detectable expression of CD88 on CB-MCs cultured for 8 weeks in the presence of SCF alone (Figure 7B), whereas in the presence of 1 μM S1P, 13% of mast cells expressed CD88 on the cell surface as determined by FACS analysis (Figure 7C). This result is consistent with the observation that 13.3% of the cells in this mast cell culture exposed to S1P are also chymase positive at that time (Figure 4B). Although similar levels of chymase expression were found in cultures from three donors, 29% and 35% of the mast cells from two other donors were chymase positive after culturing with 1 μM S1P for 8 weeks. This is the first demonstration that a serum-borne bioactive lipid can induce differentiation of CB-MC progenitors to mature chymase expressing mast cells.

Figure 7: CD88 cell surface expression. Cord blood mononuclear cells were cultured for 8 weeks with SCF in the absence (**B**) or presence of 1 μM S1P (**A,C**) and stained with rabbit anti-CD88 (**B,C**) or with non-immune rabbit IgG (**A**) followed by staining with Alexa Fluor 488-labeled secondary antibody and sorted by flow cytometry. Quadrants are indicated.


E1
E2
A
E3
E4
0%
CD8
C1
C2
B
C3
C4
0%
CD8
K1
K2
C
K3
K4
13%
CD8

2.3.2 S1P-induced secretion of IL-6 from cord blood-derived cultures is dependent on the presence of monocytes/macrophages

As IL-6 induces chymase protein expression in SCF-dependent CB-MCs [83-86], it was of interest to determine whether the effect of S1P on chymase expression in developing mast cells was due to production and secretion of IL-6. In agreement with previous studies [86, 94], IL-6 was not detected in supernatants of mast cells cultured for 1 week in the presence of SCF alone. However, cultures also treated with S1P secreted small amounts of IL-6 (Figure 8A). As secreted IL-6 is not stable for a period of 1 week [95], we next measured its secretion during the 24 h period after S1P addition to the culture medium. Interestingly, treatment with S1P induced secretion of significant amounts of IL-6 as early as 6 h compared to cultures treated with SCF alone in the absence or presence of vehicle (Figure 8B). Maximum IL-6 accumulation in the medium was observed at 10 h following addition of S1P and declined thereafter, consistent with its degradation by mast cell-derived proteases [95]. However, it should be noted that levels of IL-6 were still significantly elevated even 24 h after addition of S1P.

To determine whether IL-6 was derived from mast cells or from monocytes/macrophages that are also present in the hematopoietic precursor cultures, monocytes/macrophages were immunodepleted with anti-CD14 coated magnetic beads, as CD14 is a membrane-associated glycosylphosphatidylinositol-linked protein expressed at the surface of macrophages but not by mast cells. When these macrophage-depleted mast cells were cultured in the presence of S1P, there was no detectable production of IL-6 (Figure 9), suggesting that the monocytes/macrophages are the source of IL-6. Indeed,

macrophages isolated from cord blood cultures by virtue of their expression of CD14, secreted large amounts of IL-6 in response to S1P (Figure 10). Significant IL-6 secretion was evident within 5 h after addition of S1P, reaching maximum levels at around 10 h (Figure 10).

Figure 8: S1P enhances IL-6 secretion from cultures of cord blood-derived progenitors. A, Cord blood progenitors were cultured for 8 weeks with SCF (100 ng/ml) alone (open bar) or in the presence of S1P (0.1 and 1 μM, filled bars). Cells (10^6) were then stimulated again with SCF in the absence or presence of S1P for one week and IL-6 in the supernatants measured by ELISA. *, $p < 0.01$, compared to vehicle treated. **B**, Cord blood progenitors were cultured for 8 weeks with SCF (100 ng/ml) alone (None, circles) or in the presence of vehicle (triangles) or in the presence of 1 μM S1P (squares). Cells (10^6) were then stimulated again for the indicated times and IL-6 in the supernatants measured by ELISA. Similar results were obtained with additional cord blood cultures.

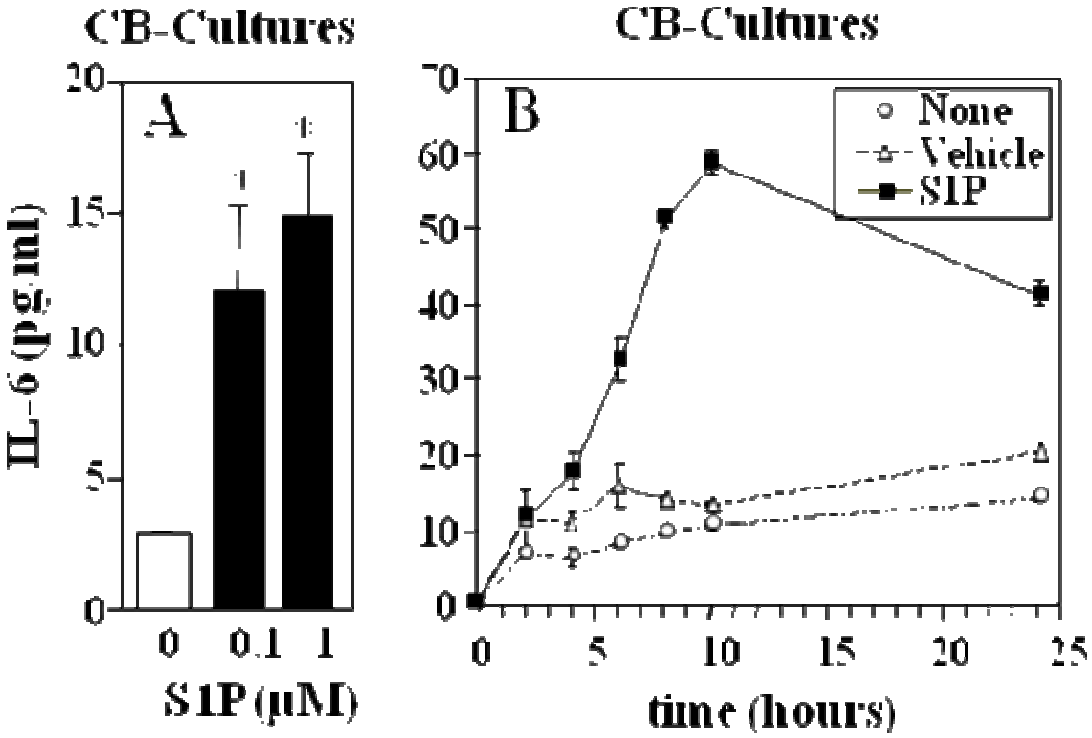

CB-Cultures
A
IL-6 (pg/ml)
20
15
10
5
0
0
0.1
1
S1P (μM)
CB-Cultures
B
None
Vehicle
S1P
70
60
50
40
30
20
10
0
0
5
10
15
20
25
time (hours)

Figure 9: S1P-induced secretion of IL-6 from cord blood cultures is not from purified CB-MCs. Cord blood progenitors were cultured for 8 weeks with SCF (100 ng/ml) alone, purified CB-MC (10^6) in which monocytes/macrophages were removed with anti-CD14 coated magnetic beads were treated without or with 1 μM S1P, as indicated. Supernatants were collected at the indicated times and IL-6 secretion determined by ELISA. Similar results were obtained with two additional cord blood cultures.

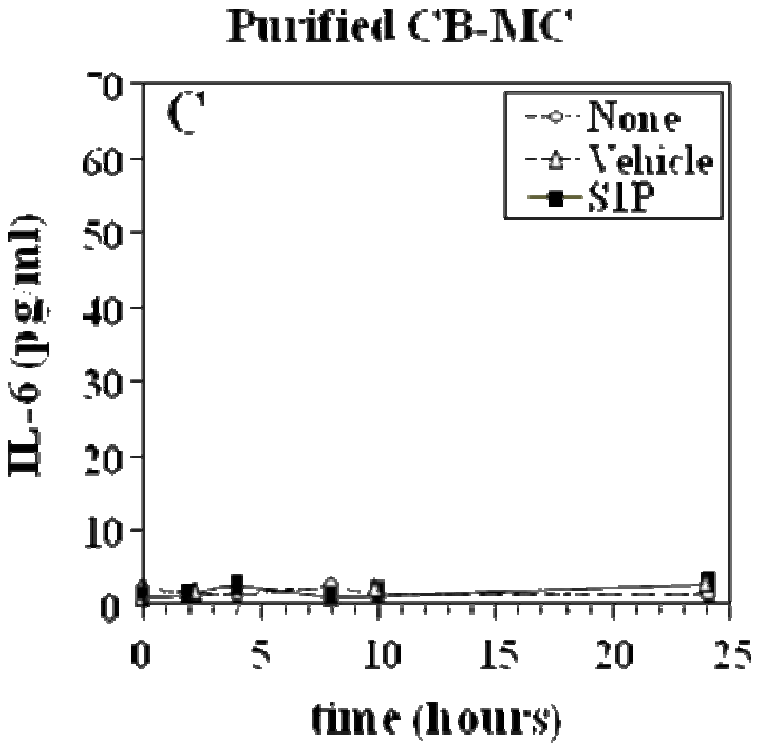
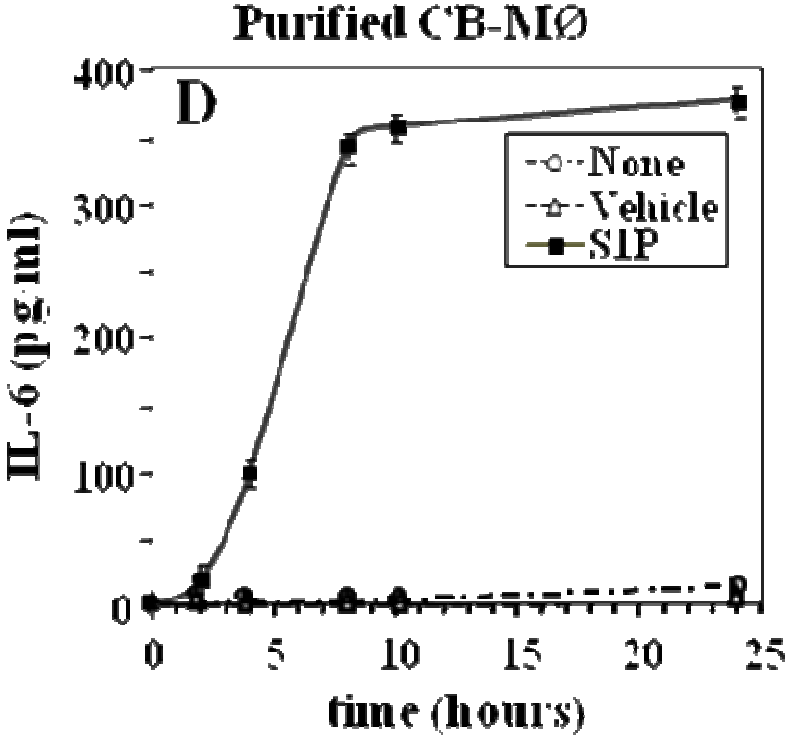

Purified CB-MC
C
None
Vehicle
S1P
IL-6 (pg/ml)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
time (hours)

Figure 10: S1P-induced secretion of IL-6 from cord blood cultures is dependent on the presence of monocytes/macrophages. Cord blood progenitors were cultured for 8 weeks with SCF (100 ng/ml) alone, purified macrophages (10^6) isolated with anti-CD14 coated beads were treated without or with 1 μM S1P, as indicated. Supernatants were collected at the indicated times and IL-6 secretion determined by ELISA. Similar results were obtained with two additional cord blood cultures.


Purified CB-MØ
D
None
Vehicle
S1P
IL-6 (pg/ml)
400
300
200
100
0
0
5
10
15
20
25
time (hours)

It was of interest to examine the fate of S1P added to CB-MCs. The culture medium only contains 1.6 nM S1P, as measured by LC-ESI-MS/MS. Five minutes after addition of 1 μM S1P to CB-MCs, there was no significant decrease in S1P levels; however, only one-third remained after 1 h (Figure 11). Although the concentration of exogenous S1P in the media decreases rapidly, even after 24 h, the S1P concentration is still significantly elevated (Figure 11). Interestingly, treatment of 8-week CB-MCs cultures with 1 μM S1P for 1 or 2 days was sufficient to significantly induce chymase expression (Figure 12). In agreement with the observation that S1P induced rapid secretion of IL-6 from cord blood cultures (Figure 8B), the presence of neutralizing anti-IL-6 antibody significantly decreased both IL-6- and S1P-induced chymase expression (Figure 13), further supporting a role of IL-6 in this process.

Figure 11: Time course of S1P disappearance. S1P levels in medium before and after addition (1 μM) to cord blood progenitors cultured for 8 weeks with SCF alone were determined at the indicated times by LC-ESI-MS/MS.

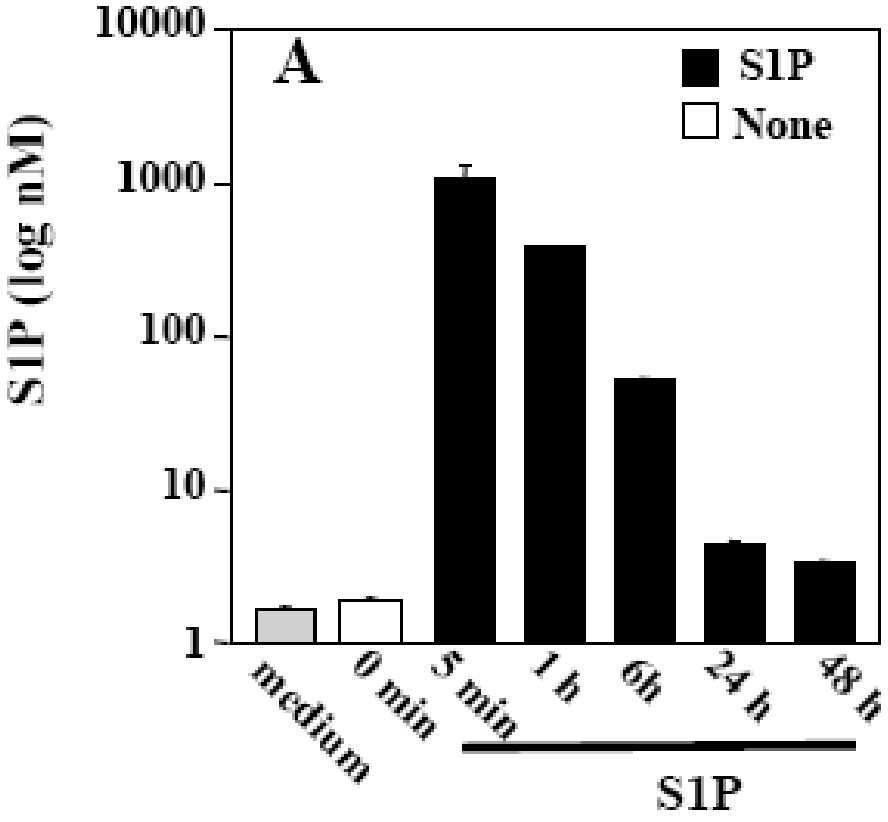

A
S1P
None
S1P (log nM)
10000
1000
100
10
1
medium
0 min
5 min
1 h
6h
24 h
48 h
S1P

Figure 12: Time course of S1P effects on chymase expression. Cord blood progenitors cultured for 8 weeks with SCF alone were treated without or with 1 μM S1P for the indicated times. The percent of chymase expressing MC was determined as described in Materials and Methods.

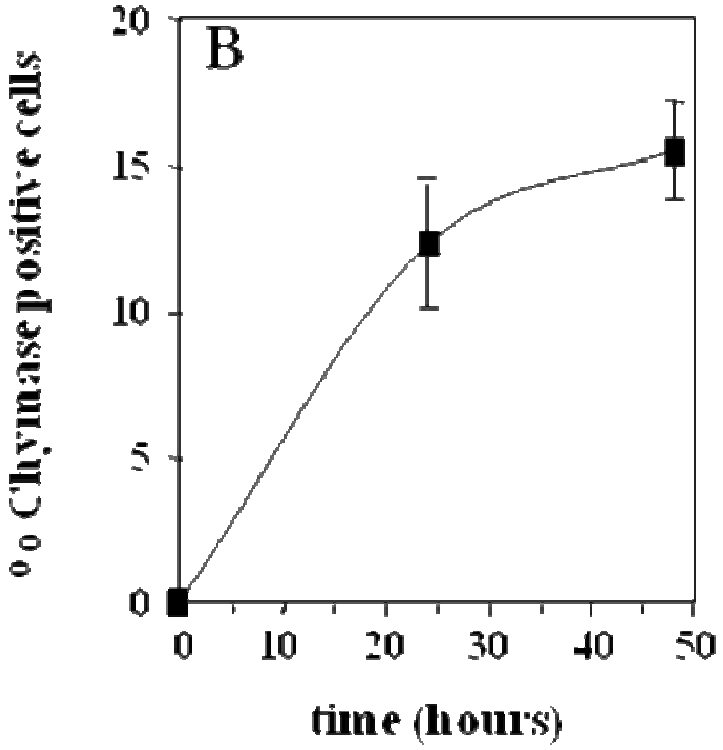
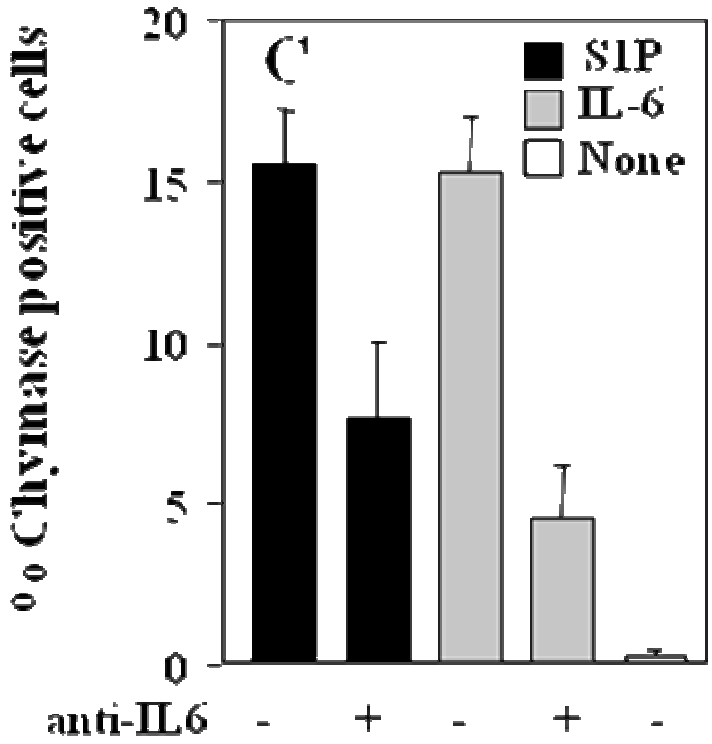

B
20
15
10
5
0
0
10
20
30
40
50
% Chymase positive cells
time (hours)

Figure 13: S1P-induced chymase expression is IL-6 dependent. Cord blood progenitors cultured for 8 weeks with SCF alone were treated without or with 1 μM S1P or 50 ng/ml IL-6 in the absence or presence of anti-IL-6 antibody for 48 h as indicated. The percent of chymase expressing MC was determined as described in Materials and Methods.


C
S1P
IL-6
None
% Chymase positive cells
20
15
10
5
0
anti-IL6 - + - + -

2.3.3 S1P enhances CCL2 release from purified CB-MCs but not from purified CB-macrophages

CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), is an important chemoattractant for monocytes/macrophages, the other cell type co0generated in cord blood cultures. Cord blood progenitor cultures produce and secrete large amounts of CCL2, which was enhanced by culturing in the presence of S1P (Figure 14A). Although cord blood cultures spontaneously secrete CCL2, treatment with S1P induced significant increases compared to cord blood cultures exposed to SCF alone, in the absence or presence of vehicle (Figure 14B). Importantly, S1P induced secretion of CCL2 from purified CB-MCs depleted of monocytes/macrophages (Figure 15). A significant increase was observed within 2 h after addition of S1P to purified CB-MCs (devoid of monocytes/macrophages) and increased thereafter (Figure 15). Levels of CCL2 remained elevated for at least 24 h after addition of exogenous S1P (Figure 15). Because these mast cells express $S1P_1$ and $S1P_2$ receptors [14], we next examined which of the receptors was involved in S1P-induced CCL2 secretion. The $S1P_2$ antagonist [96], JTE-013, markedly reduced CCL2 secretion in response to S1P, whereas VPC23019, an antagonist of $S1P_1$ [96], had no significant effect (Figure 16). In sharp contrast, although macrophages are capable of releasing large amounts of CCL2, no significant stimulation was observed in response to S1P (Figure 17).

Figure 14: S1P enhances CCL2/MCP-1 release from cultures of cord blood-derived progenitors. A, Cord blood progenitors were cultured for 8 weeks with SCF (100 ng/ml) alone (open bar) or in the presence of S1P (0.1 and 1 μM, filled bars). Cells (10^6) were then stimulated again with SCF in the absence or presence of S1P for one week. Supernatants were collected and CCL2/MCP-1 determined by ELISA. **B**, Cord blood progenitors were cultured for 8 weeks with SCF (100 ng/ml) alone (None, circles), or in the presence of vehicle (triangles) or 1 μM S1P (squares). Cells (10^6) were then stimulated again for the indicated times and CCL2 in the supernatants measured by ELISA. Similar results were obtained with three additional cord blood cultures.

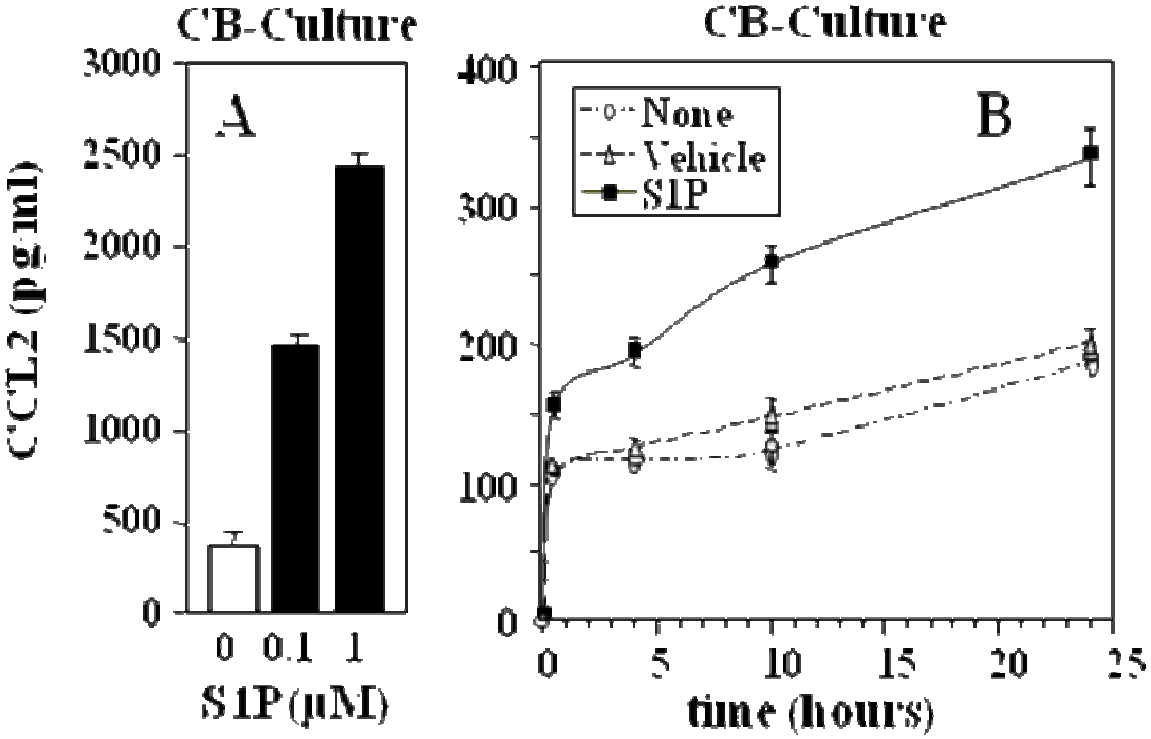

CB-Culture
A
CCL2 (pg/ml)
3000
2500
2000
1500
1000
500
0
0
0.1
1
S1P (μM)
CB-Culture
B
None
Vehicle
S1P
400
300
200
100
0
0
5
10
15
20
25
time (hours)

Figure 15: S1P enhances CCL2/MCP-1 release from purified CB-MCs. After 8 weeks in culture with SCF alone, purified CB-MC (10^6) in which monocytes/macrophages were removed with anti-CD14 coated beads were treated without or with 1 μM S1P. Supernatants were collected at the indicated times and CCL2/MCP-1 secretion determined by ELISA. Similar results were obtained with or two additional cord blood cultures.

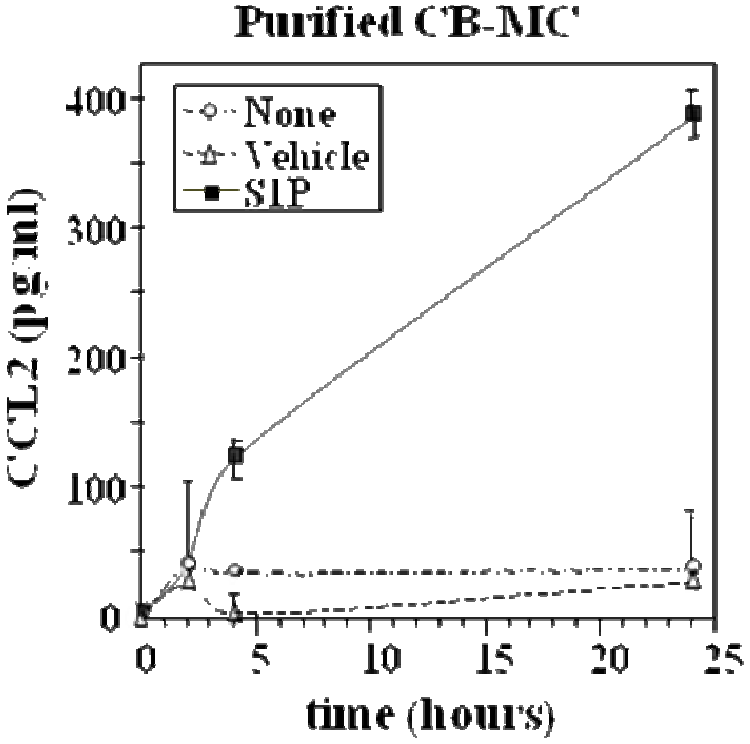

Purified CB-MC'
None
Vehicle
SIP
CCL2 (pg/ml)
400
300
200
100
0
0
5
10
15
20
25
time (hours)

Figure 16: S1P-enhanced CCL2/MCP-1 release from purified CB-MCs is $S1P_2$ dependent. After 8 weeks in culture with SCF alone, purified CB-MC (10^6) in which monocytes/macrophages were removed with anti-CD14 coated beads pretreated for 30 min with vehicle, 1 μM JTE-013, or 1 μM VCP23019 prior to stimulation without or with 1 μM S1P. Supernatants were collected after 24 h and CCL2/MCP-1 secretion determined by ELISA. Similar results were obtained with two additional cord blood cultures. *, $p < 0.01$, compared to S1P treatment.

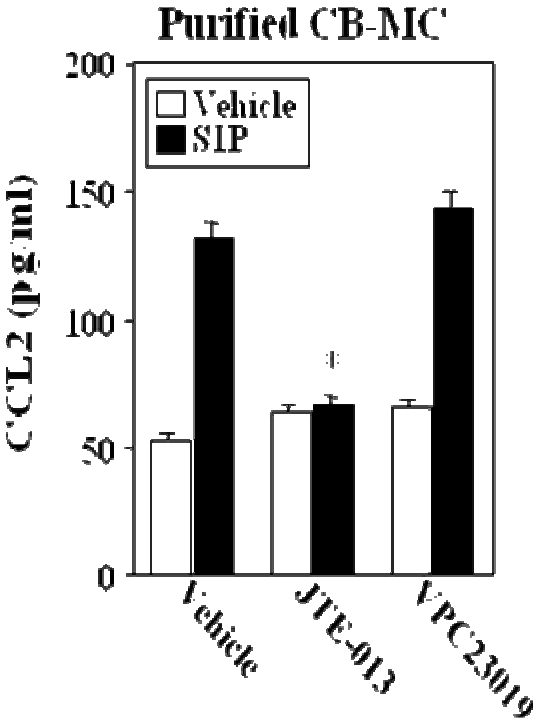
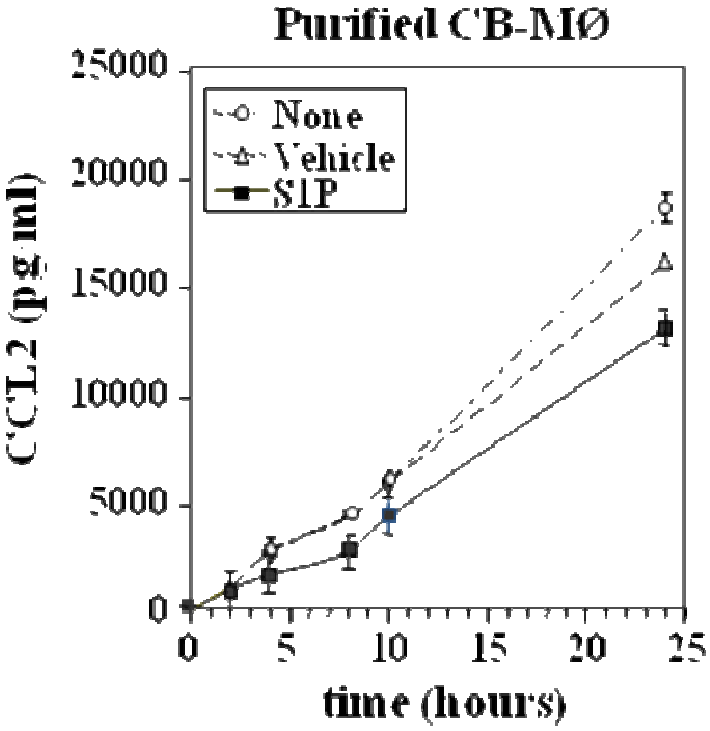

Purified CB-MC'
Vehicle
S1P
CCL2 (pg/ml)
200
150
100
50
0
Vehicle
JTE-013
VPC23019

Figure 17: S1P does not enhance CCL2/MCP-1 release from purified CB-macrophages. After 8 weeks in culture with SCF alone, purified macrophages (10^6) isolated with anti-CD14 coated beads were treated without or with 1 μM S1P. Supernatants were collected at the indicated times and secretion of CCL2/MCP-1 determined by ELISA.


Purified CB-MØ
None
Vehicle
S1P
CCL2 (pg/ml)
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
time (hours)

2.3.4 S1P triggers degranulation and induces functional features of CB-MCs expressing chymase

An important functional difference between double positive MC_{TC} and lung-like MC_T is that the former are also known to respond in an IgE-independent manner to a number of secretagogues, such as synthetic polyamines like compound 48/80, and the naturally occurring anaphylatoxin C5a [94]. To examine the functional characteristics of chymase expressing CB-MCs developed in the presence of S1P, their capacity to degranulate in response to antigen, C5a, and 48/80 was determined by β-hexosaminidase release. In agreement with previous studies [83], MC_T developed in the presence of SCF alone readily degranulated in response to crosslinking of FcεRI by antigen and substance P (a naturally occurring neurotransmitter) but did not respond to C5a or 48/80 (Figure 18). However, CB-MCs developed in the presence of S1P, which increases chymase and C5aR expression (Figure 4, 5, 7), degranulate in response to C5a (Figure 18), similar to skin-derived MC_{TC} [86]. Furthermore, these mast cells were also degranulated by compound 48/80 (Figure 18). As expected, ionomycin (a calcium ionophore that is a universal mast cell activator) and substance P induced similar degranulation in both phenotypes of mast cells (Figure 18). In addition, antigen, substance P, and ionomycin, but not C5a and 48/80, enhanced secretion of CCL2 from MC_T developed in the presence of SCF alone (Figure 19). Conversely, C5a and 48/80 only enhanced release of CCL2 from MC_{TC}, developed in the presence of S1P (Figure 19).

Figure 18: CB-MCs generated in the presence of S1P acquire functional features of skin MC_{TC}. After culturing for 8 weeks with SCF in the absence (open bars) or presence of S1P (filled bars), purified CB-MC were stimulated for 2 hours with Ag, C5a (1 μg/ml), compound 48/80 (1 μg/ml), substance P (1 μM), or ionomycin (1 μM). Degranulation was assessed by β-hexosaminidase release. Similar results were obtained with two additional cord blood cultures from other donors. *, $p < 0.01$, compared to vehicle treatment.

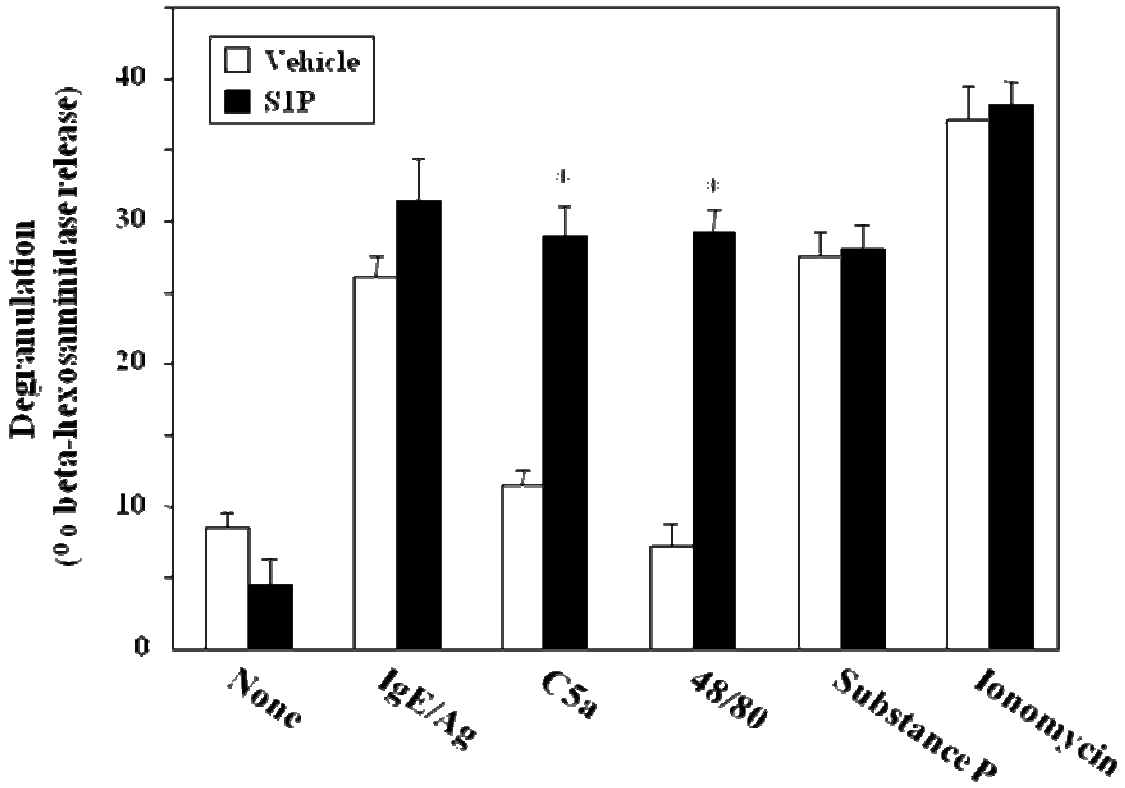
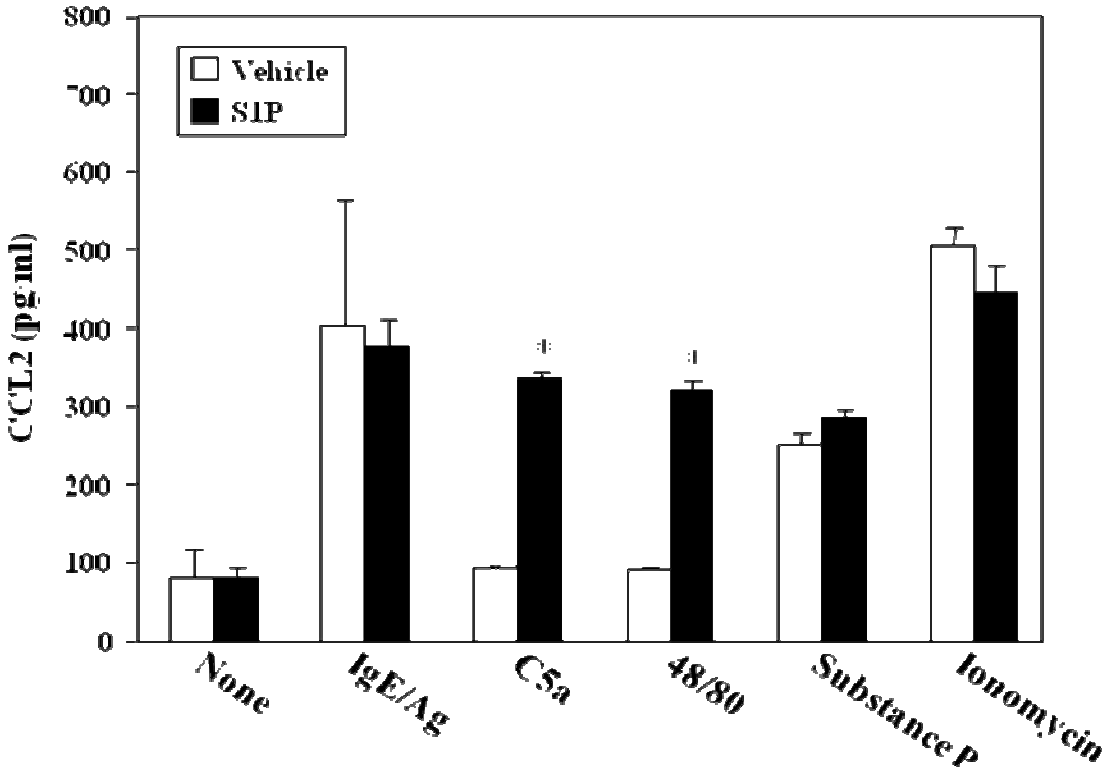

Vehicle
S1P
Degranulation
(% beta-hexosaminidase release)
40
30
20
10
0
*
*
None
IgE/Ag
C5a
48/80
Substance P
Ionomycin

Figure 19: S1P stimulates secretion of CCL2/MCP-1 from skin-like CB-MC$_{TC}$. After culturing for 8 weeks with SCF in the absence (open bars) or presence of S1P (filled bars), purified CB-MC were stimulated for 2 hours with Ag, C5a (1 μg/ml), compound 48/80 (1 μg/ml), substance P (1 μM), or ionomycin (1 μM). CCL2/MCP-1 in the supernatants was measured by ELISA. Similar results were obtained with two additional cord blood cultures from other donors. *, $p < 0.01$, compared to vehicle treatment.


800
700
600
500
400
300
200
100
0
CCL2 (pg/ml)
Vehicle
S1P
*
*
None
IgE/Ag
C5a
48/80
Substance P
Ionomycin

2.4 DISCUSSION

S1P has been added to the repertoire of mediators produced and released by mast cells that in turn regulate mast cell functions [4, 7, 8, 10-14]. FcεRI triggering has been shown to utilize SphK, the enzyme that produces S1P, to mobilize Ca^{2+} from internal stores, an event necessary for degranulation [7, 10]. Moreover, the balance between sphingosine and S1P determined by SphK is decisive for allergic responsiveness of mast cells [8]. Secreted S1P is able to bind and activate its receptors on mast cells. $S1P_1$ induces cytoskeletal rearrangements, leading to the movement of mast cells towards an antigen gradient; whereas $S1P_2$ is required for the degranulation response [11, 14]. S1P also increased expression of MCP-1, MIP-1α, MIP-1β, and MIP-2 in mast cells, all important modulators of monocyte, macrophage and eosinophil recruitment and inflammation [11, 14, 71]. Production of S1P in mast cells has grown more complex with the recent demonstration that both SphK1 and SphK2 are activated upon FcεRI engagement [12] and are important *in vivo* for mast cell-dependent anaphylactic responses in mice [13]. These findings together with the observation that SCF, an important growth factor required for mast cell survival and differentiation, also activates SphK1 and SphK2 [12], emphasize the important role of S1P generation in mast cell physiology.

Here we report that S1P also accelerates the generation of mast cells from hematopoietic progenitors and strikingly increases chymase expression. These CB-MC_{TC} have functional FcεRI and similar to skin MC_{TC}, are also activated by the anaphylatoxin C5a and the secretagogue 48/80. Thus, MC_{TC} are functionally distinguished from MC_T phenotypes of human mast cells, suggesting important differences that may affect their

participation in disease states. The ability of MC_{TC} to be activated by agents not associated with FcεRI and IgE suggest this cell type may have a greater role in innate immunity by responding to either innate or microbial danger signals. Although human mast cells can be generated from umbilical cord blood progenitors cultured in medium supplemented with SCF and varied accessory factors, including combinations of cytokines [79, 81, 97, 98], remarkably, the percent of mast cells expressing chymase is similar to what we found utilizing SCF with S1P alone. Interestingly, the responses of MC_{TC} to C5a and 48/80 are much greater than expected. There are several possible explanations for this. First, it is well established that mast cells of different maturity also differ in their histamine content and their ability to respond to cell activation [99]. Skin-like MC_{TC} release much more histamine in response to IgE/Ag than lung-derived MC_T [100]. Indeed, MC_{TC} granules generally are more uniformly electron dense, larger, and more numerous than MC_T granules [101]. Alternatively, a higher proportion of mast cells may be expressing C5aR than chymase, or smaller amounts of C5aR are needed for a functional response. It is also possible that C5a might activate MC_{TC} to release another factor that can activate MC_T through a pathway other than through C5aR.

Although LPA, a serum borne lysophospholipid structurally closely related to S1P, has been shown to accelerate mast cell proliferation and differentiation to tryptase expressing MC_T, interestingly, it had no effect on the small number of chymase expressing cells nor did it increase chymase activity [88]. The ability of S1P to induce expression of chymase and C5aR is most probably mediated via release of IL-6 from cord blood progenitor cultures that contain adherent macrophages. Indeed, highly purified

monocytes/macrophages from these cultures released IL-6 in response to S1P. Macrophages express multiple S1P receptors, of which $S1P_1$ and $S1P_2$ predominate [102-104]. In agreement, inhibiting $S1P_1$ and $S1P_2$ with specific antagonists significantly reduced S1P-stimulated IL-6 release from macrophages (Figure 20). Of note, S1P stimulated secretion of CCL2 from mast cells independent of the presence of macrophages.

Our data suggest crucial roles for S1P in regulating development of hematopoietic progenitors into functionally mature mast cells expressing chymase and reveal a complex interplay between macrophages and mast cells during the development of fully differentiated mast cells (Figure 21). According to this model, S1P (possibly from the blood) induces secretion of IL-6 from monocytes/macrophages and CCL2 from mast cells. IL-6 in turn may act on progenitors, enhancing the mitogenic and survival effects of SCF (most likely from fibroblasts) and promoting development and maturation of mast cells and inducing chymase and C5aR expression [83]. In addition, S1P induces CCL2 release from mast cells to recruit monocytes/macrophages to their vicinity, thereby enhancing the interaction between these different types of cells. In this regard, an elegant study in mice demonstrated that adult mast cell progenitors are derived directly from multipotential progenitors instead of, as previously proposed, common myeloid progenitors or granulocyte macrophage progenitors [105]. Moreover these mast cell-committed progenitors can give rise to both connective tissue-type and mucosal-type mast cells, which is determined by factors present in the site of differentiation [105]. Thus, S1P can regulate the phenotype and therefore the responsiveness of mast cells. This suggests that S1P present in the serum at high concentrations is capable of shaping a given physiological response. It is interesting to

speculate once this alteration in mast cell phenotype has occurred and the inflammatory response has been resolved whether mast cell phenotype reverses to MC_T or rather remains a more responsive MC_{TC} phenotype.

Figure 20: $S1P_1$ and $S1P_2$ both play a role in S1P-enhanced secretion of IL-6 from purified CB-macrophages. After 8 weeks in culture with SCF alone, purified macrophages (10^6) isolated with anti-CD14 coated beads were pretreated for 30 min with vehicle, 1 μM JTE-013, or 1 μM VCP23019 prior to stimulation without or with 1 μM S1P. Supernatants were collected after 24 h and secretion of IL-6 determined by ELISA.

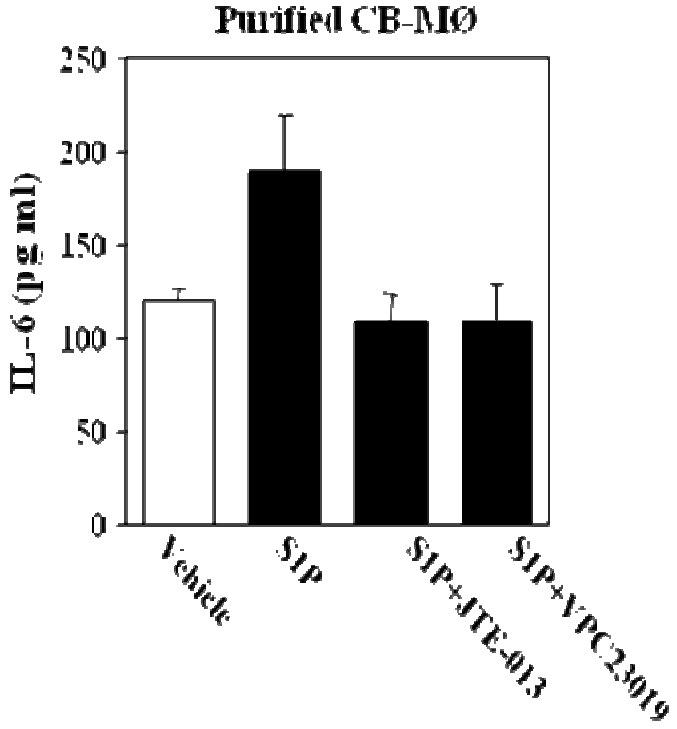
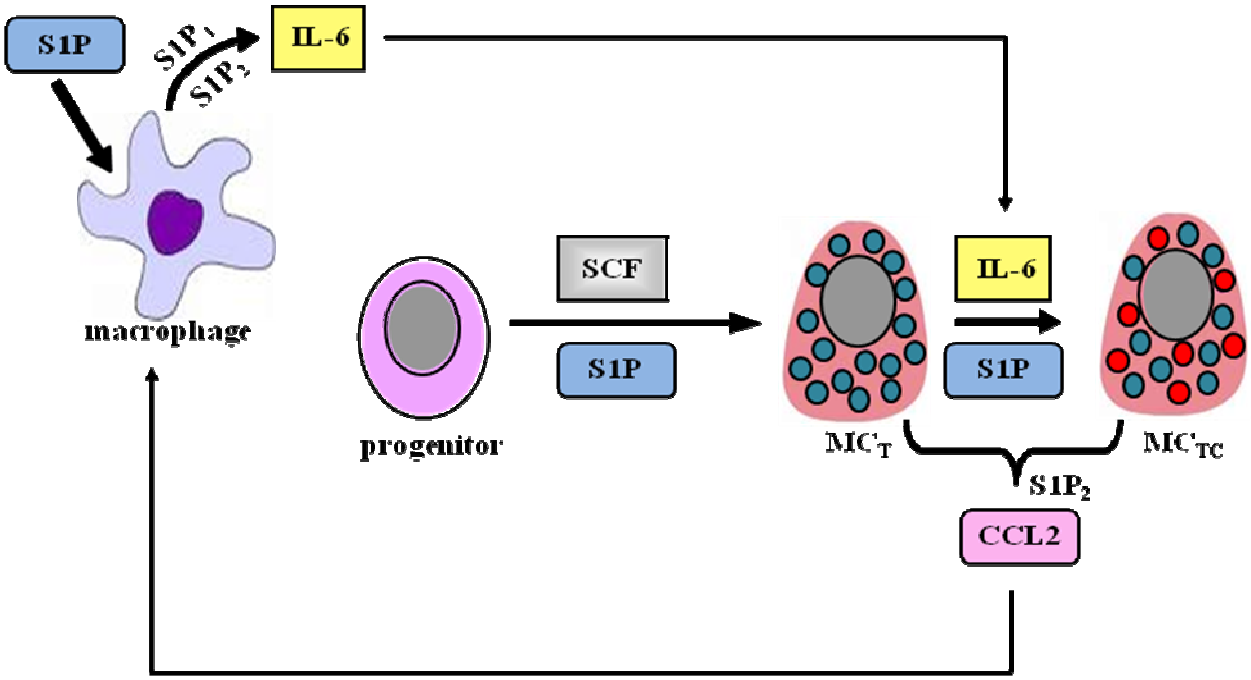

Purified CB-MØ
IL-6 (pg ml)
250
200
150
100
50
0
Vehicle
S1P
S1P+JTE-013
S1P+VPC23019

Figure 21: Proposed model of human mast cell development and the involvement of S1P. S1P stimulates release of IL-6 by monocytes/macrophages. In turn, IL-6 can act on developing mast cells at different stages of development, promoting proliferation and inducing chymase expression. S1P also enhances CCL2 secretion from mast cells, which is a chemoattractant for monocytes/macrophages, further enhancing crosstalk between monocytes/macrophages and mast cells in response to S1P. For simplicity, a multipotential progenitor capable of developing into MC_T and MC_{TC} is depicted.


S1P
S1P1
S1P2
IL-6
macrophage
progenitor
SCF
S1P
IL-6
S1P
MCT
MCTC
S1P2
CCL2

We found that S1P can also induce release of IL-6 from macrophages. Interestingly, previous results have suggested that SphK1, which produces S1P, plays a key role in the generation and release of proinflammatory mediators from human macrophages triggered by anaphylatoxins [47] and in neutropenia, peritonitis, and cytokine production *in vivo* [106]. Anaphylatoxin C5a, one of the complement fragments produced by activation of the complement system, is involved in a variety of disorders in which mast cells play critical roles, including septic shock and adult respiratory distress syndrome. Our finding that S1P enhances mast cell expression of C5aR and their ability to respond to C5a further support the notion of a potential role of S1P in anaphylatoxin-triggered inflammatory responses *in vivo* [47, 106].

Chymase, a chymotrypsin-like serine protease that is only secreted from MC_{TC}, has been associated with sepsis in various mouse models [107-109]. It has been suggested that increased intracellular chymase activity leads to enhanced microbiocidal activity directly or may function indirectly. Extracellularly, mast cell chymase can degrade endothelin-1, a potent constrictor of blood vessels that has been implicated in vascular changes associated with sepsis [110] and cleave chemokine precursors to generate activated chemokines that recruit neutrophils to bacterial infections [109]. Thus, murine mast cells, which express at least four chymase proteins, have the potential to help [108, 109], rather than harm. Nonetheless, earlier studies found that mice with mast cells deficient in chymases usually survive peritonitis induced by cecal ligation and puncture better than wild-type mice [107]. The serine peptidases seem to increase mortality by cleaving survival-enhancing cytokines, such as IL-6 [107]. Similarly, it has been demonstrated that a variety of cytokines

produced by cultured human skin MC_{TC}, including IL-5, IL-6, IL-13, and TNFα, are cleaved by mast cell peptidases, primarily chymase [95]. However, there is only a single chymase gene in humans [111]. Recently, it was demonstrated that lipopolysaccharide upregulates chymase expression in human mast cells, suggesting that a gram negative bacterial infection may induce mast cells to express a unique composition of proteases beneficial for controlling and eliminating the infection [112]. Although chymase expression has been reported to be elevated in individuals dying from anaphylaxis [113], its functions in sepsis and anaphylaxis are still not well understood [111, 114]. Our results demonstrate an important role for S1P in regulating development of functionally mature chymase expressing human mast cells and their functions.

CHAPTER 3: A SPECIFIC SPHINGOSINE KINASE 1 INHIBITOR ATTENUATES AIRWAY HYPERRESPONSIVENESS AND INFLAMMATION IN A MAST CELL-DEPENDENT MODEL OF ALLERGIC ASTHMA

3.1 INTRODUCTION

Allergic asthma is a complex disease characterized by airway inflammation and airway hyperresponsiveness (AHR) that is becoming increasingly widespread in developed nations [115]. Mast cells are key effector cells that are increased in airways of asthmatics and can contribute to multiple features of allergic inflammation by secreting a vast array of inflammatory mediators that exacerbate vasodilation and vascular permeability, airway smooth muscle contraction, mucus secretion, and immune cells recruitment [45].

Sphingosine-1-phosphate (S1P) is a new addition to the growing list of inflammatory mediators secreted by activated mast cells that is now emerging as a regulator of multiple aspects of both innate and adaptive immunity [87, 116]. S1P aggravates antigen-induced airway inflammation in mice [117] and its levels are elevated in the bronchoalveolar lavage (BAL) fluid of allergen challenged patients with allergic asthma [67]. The majority of actions of S1P in innate and adaptive immunity are mediated by five specific S1P receptors, denoted $S1P_{1\text{-}5}$ [116]. However, recent studies demonstrated that S1P also has important intracellular actions required for activation of the transcription factor NF-κB important in inflammatory and immune responses. [18, 118].

Crosslinking of the high affinity IgE receptor (FcεRI) on mast cells activates sphingosine kinase 1 (SphK1) [10, 11, 119] and possibly also SphK2 [12, 13] leading to

rapid increases in intracellular S1P and its subsequent secretion [11, 12]. Although it has long been recognized that SphKs are involved in mast cell activation [7], the importance of each of the SphK isoenzymes is still a matter of debate. Whereas silencing of SphK1 but not SphK2 impaired FcεRI-mediated mast cells activation [10, 11] [14], [120], in sharp contrast, calcium influx, cytokine production, and degranulation were abrogated in mast cells derived from *Sphk2* and not from *Sphk1* knockout mice [13, 119]. Furthermore, studies of allergic responses in isotype-specific SphK knockout mice have also yielded conflicting results [121]. In the present study, we utilized a mast cell- and IgE-dependent murine model of chronic asthma [122, 123] to investigate the role that SphK1 and S1P play *in vivo* in mast cell-mediated allergic responses.

3.2 MATERIALS AND METHODS

3.2.1 Reagents and Antibodies

Dinitrophenyl-human serum albumin (DNP-HSA, Ag), ionomycin, chicken ovalbumin (OVA), Acetyl-β-methylcholine chloride (Methacholine), and type 1 DNase were purchased from Sigma (St. Louis, MO). FBS, L-glutamine, penicillin, streptomycin, sodium pyruvate, and HEPES were from Biofluids (Rockville, MD). Hyaluronidase was from Worthington Biochemical (Lakewood, NJ) and complete RPMI (cRPMI) 1640 medium was from Invitrogen Life Technologies (Carlsbad, CA). Human cytokine ELISA kits were from BD Biosciences (San Diego, CA), murine cytokine ELISA kits were from Peprotech (Rocky Hill, NJ), and histamine ELISA kits was from Neogen (Lexington, KY). Anti-phospho-IKKalpha/Beta, anti- phospho-IκBalpha, anti-phospho-ERK1/2, and total ERK2 were from Cell Signaling (Boston, MA). Anti-p65 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Protocol 10% Neutral buffered formalin was from Fisher (Pittsburgh, PA), Diff-Quik stain set was from Siemens Healthcare Diagnostics (Deerfield, IL), and Dako LSAB+ kit was Dako North America (Carpinteria, CA).

3.2.2 Culture of human mast cells

All protocols involving human tissues were approved by the human studies Internal Review Board at Virginia Commonwealth University (VCU). Human skin-derived mast cells were dispersed from human skin tissue obtained after breast reduction, mastectomy for breast cancer, or from abdominoplasties through the National Disease Research

Interchange (Philadelphia, PA) or the Cooperative Human Tissue Network of the National Cancer Institute (Columbus, OH). Subcutaneous fat was removed by blunt dissection and residual tissue was cut into 1- to 2-mm fragments, which were incubated in a solution of Hanks balanced salt solution (HBSS) containing 1.5 mg/mL type 2 collagenase, 0.7 mg/mL hyaluronidase, 0.3 mg/mL type 1 DNase, 1% fetal calf serum (FCS), and 1 mM $CaCl_2$ for 2 hours at 37°C with constant shaking. The dispersed cells were separated from residual tissue by filtration through a No. 80 mesh sieve and suspended in HBSS containing 1% FCS and 10 mM HEPES. The remaining tissue was subjected to an additional digestion as above, and combined with the cells from first digestion. Cells were resuspended in HBSS, layered over a Percoll cushion, and centrifuged at 700*g* at room temperature for 20 minutes. Nucleated cells were collected from the buffer/Percoll interface. Percoll gradient-enriched cells were resuspended at a concentration of 1×10^6 cells/mL in serum-free X-VIVO 15 medium (Lonza, Walkersville, MD) containing 100ng/ml recombinant human SCF. The culture medium was changed weekly and cells were split every 4–5 days or when they reached a concentration of ~2×10^6 cells/mL. Cultures of human skin-derived mast cells were maintained for up to 3 months and were ~100% mast cells.

3.2.3 Culture of murine mast cells

Murine bone marrow-derived mast cells (BMMCs) were isolated and derived from mice by culture in complete RPMI (cRPMI) 1640 containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 1

mM HEPES, supplemented with IL-3–containing supernatant from WEHI-3 cells and stem cell factor (SCF)-containing supernatant from BHK-MKL cells. The final concentration of IL-3 and SCF was adjusted to 1 ng/ml and 10 ng/ml, respectively, as measured by ELISA. BMMCs were cultured at 3-5 × 10^5 cell/mL, washed, and incubated at 37˚C for 4-6 hours in cRPMI without cytokines prior to assays. Mast cell viability was determined by trypan blue staining.

3.2.4 Degranulation and ELISA

Human skin-derived mast cells (10^6) in the presence of soybean trypsin inhibitor (SBTI; 100μg/ml) were sensitized overnight with 1 μg/ml anti-DNP IgE overnight, washed to remove unbound IgE, and then stimulated with 30 ng/ml DNP-HSA (Ag) at 37˚C for 24 hours. Degranulation was measured by β-hexosaminidase assays as described previously. Murine BMMCs (10^6) were sensitized overnight with 0.5 μg/ml anti-DNP IgE overnight, washed to remove unbound IgE, and then stimulated with 20 ng/ml DNP-HSA (Ag) at 37˚C for 24 hours. SK1-I was added at the indicated concentrations during crosslinking. Degranulation was measured by β-hexosaminidase release, expressed as a percentage of the total cellular β-hexosaminidase released into the medium, or by histamine release determined by ELISA. Cytokine and chemokine release from these cells were measured by ELISA.

3.2.5 Mice

Female C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and kept at the animal care facilities at Virginia Commonwealth University (Richmond, VA) under standard temperature, humidity, and timed light conditions, and were provided with mouse chow and water *ad libitum*. All experiments were performed in compliance with the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Resources, National Research Council, published by the National Academy Press (revised 1996), and with approval from the VCU institutional animal care and use committee.

3.2.6 Induction of allergic inflammation and AHR

Eight weeks old C57BL/6 mice were sensitized by intraperitoneal (i.p.) injection of 100 μl PBS or OVA (50 μg) on days 1, 3, 5, and 7. Mice were challenged by intranasal (i.n.) injection of 20 μl PBS or OVA (200 μg) on days 22, 25, and 28. Mice were assessed for airway hyperresponsiveness (AHR) and airway inflammation 24 hours after the last i.n. challenge. SK1-I (5 mg/kg in PBS) or vehicle (PBS) was administered i.n. 1 hour prior to OVA sensitization and challenge (SK1-I group 1) or prior to OVA challenge only (SK1-I group 2).

3.2.7 Measurement of airway reactivity

Mice were anesthetized by i.p. injection of 206.7 mg/kg of ketamine and 41.7 mg/kg of xylazine, were ventilated after tracheotomies, and measurements of baseline lung function were made with the FlexiVent (Scireq, Montreal, QC, Canada). Mice were

exposed to aerosols containing increasing doses (0, 10, 25, 50, 100 mg/mL) of acetyl-β-methylcholine chloride (Methacholine) and resistance (R), Compliance (C), Newtonian resistance (Rn), and tissue damping (G) were measured using the FlexiVent software version 5.3 (Scireq, Montral, Quebec, Canada). Results are expressed as relative increases over baseline values.

3.2.8 BAL fluid collection

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

3.2.9 Lung histology

Following lavage, lungs were inflated through the trachea, removed, and fixed in 10% neutral buffered formalin. The formalin-fixed tissues were embedded in paraffin and 5 millimmeter sections stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS). A Nikon ECLIPSE E800M microscope equipped with a Diagnostic Instruments Spot RT CCD camera was used to photograph the sections. Total lung inflammation was assessed as the severity of perivascular infiltration and was scored semi-quantitatively for the following features: 0, normal; 1, few cells; 2, rings of inflammatory cells 1 cell layer

deep; 3, rings of inflammatory cells 2–4 cells deep; 4, rings of inflammatory cells ≥4 cells deep.

In some experiments, lung sections were stained with anti-p65 antibody and visualized with a Dako LSAB+ kit.

3.2.10 Cytokine and chemokine measurements

The following cytokines and chemokines in BAL fluid were measured with a Bioplex Array Reader (LUMINEX 100, Bio-Rad Laboratories, Hercules, CA) using a custom mouse Bioplex 8 panel (Bio-Rad Laboratories, Hercules, CA): IL-4, IL-5, IL-6, IL-13, Eotaxin, IFN-γ, CCL2/MCP-1, and TNF-α, according to the manufacturer's instructions. The Bioplex cytokine assay is a magnetic bead-based assay designed for the quantitative measurement of multiple cytokines in a single well. Briefly, 50 µl of cytokine/chemokine standards or samples (supernatants from BAL) were incubated with 50 µl of anti-cytokine/chemokine conjugated beads in 96-well filter plates for 30 min at room temperature with shaking. Plates were then washed by vacuum filtration three times with 100 µl of Bio-Plex wash buffer, 25 µl of diluted detection antibody were added, and plates were incubated for 30 min at room temperature with shaking. After three filter washes, 50 µl of streptavidin-phycoerythrin was added, and the plates were incubated for 10 min at room temperature with shaking. Finally, plates were washed by vacuum filtration three times, beads were suspended in Bio-Plex assay buffer, and samples were analyzed on a Bio-Rad 96-well plate reader using the Bio-Plex Suspension Array System and Bio-Plex Manager software (Bio-Rad Laboratories, Hercules, CA).

3.2.11 Mass spectrometry

Lipids were extracted from lung tissues and serum by the Lipidomics Core at VCU. Internal standards were added (0.5 nmol each, Sphingolipid Mixture II/LM-6005, Avanti Polar Lipids), lipids extracted, and sphingolipids quantified by liquid chromatography, electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS; 4000 QTRAP; ABI), as described [90].

3.2.12 Statistical analysis

Statistical significance was determined with the Student's t test for unpaired samples. *In vitro* experiments were repeated at least three times in triplicate with consistent results. *In vivo* experiments were repeated four times and each experimental group consisted of at least seven mice.

3.3 RESULTS

3.3.1 A specific SphK1 inhibitor attenuates activation of human mast cells

Although many studies using siRNA to downregulate SphK1 indicate that S1P formed by its activation is pivotal in IgE-mediated mast cell degranulation and secretion of pro-inflammatory cytokines [10, 11, 14, 119], others using mast cells derived from knockout mice concluded that SphK2 rather than SphK1 is indispensable for these mast cell functions [13]. To clarify this controversy, we examined the effect of a specific SphK1 inhibitor, SK1-I, which does not affect SphK2 activity [120], on mast cell functions. SK1-I drastically inhibited degranulation of human skin-derived mast cells triggered by FcεR1 crosslinking with antigen in a dose-dependent manner (Figure 22A). These concentrations of SK1-I had no effect on mast cell viability (Figure 22B) or degranulation induced by ionomycin (Figure 22A). In agreement with our previous results [14], S1P potently induced degranulation of human mast cells, which as expected, was not altered by inhibition of SphK1 (Figure 22A). In addition, SK1-I significantly reduced antigen-induced secretion of the cytokines, TNF-α (Figure 23) and IL-6 (Figure 24), whereas the secretion of these cytokines in response to S1P or ionomycin was unaffected. Similarly, SK1-I only reduced antigen-induced but not S1P- or ionomycin-induced secretion of CCL2/MCP-1 (Figure 25), an important chemokine that plays a major role in a mast cell-dependent model of allergic asthma [124].

Figure 22: Inhibition of SphK1 reduces activation of human mast cells. IgE **s**ensitized skin-derived mast cells were treated without (open bars) or with SKI-1 (2.5 μM, grey bars; 5 μM, black bars) and then stimulated with vehicle, 30 ng/ml Ag (IgE/Ag), 100 nM S1P, or 1 μM ionomycin. **A,** Degranulation was determined by β-hexosaminidase release and **B,** viability by trypan blue exclusion. Data are the means ± SD of triplicate determinations. *P<0.01. Similar results were obtained using cells from 2 different donors.

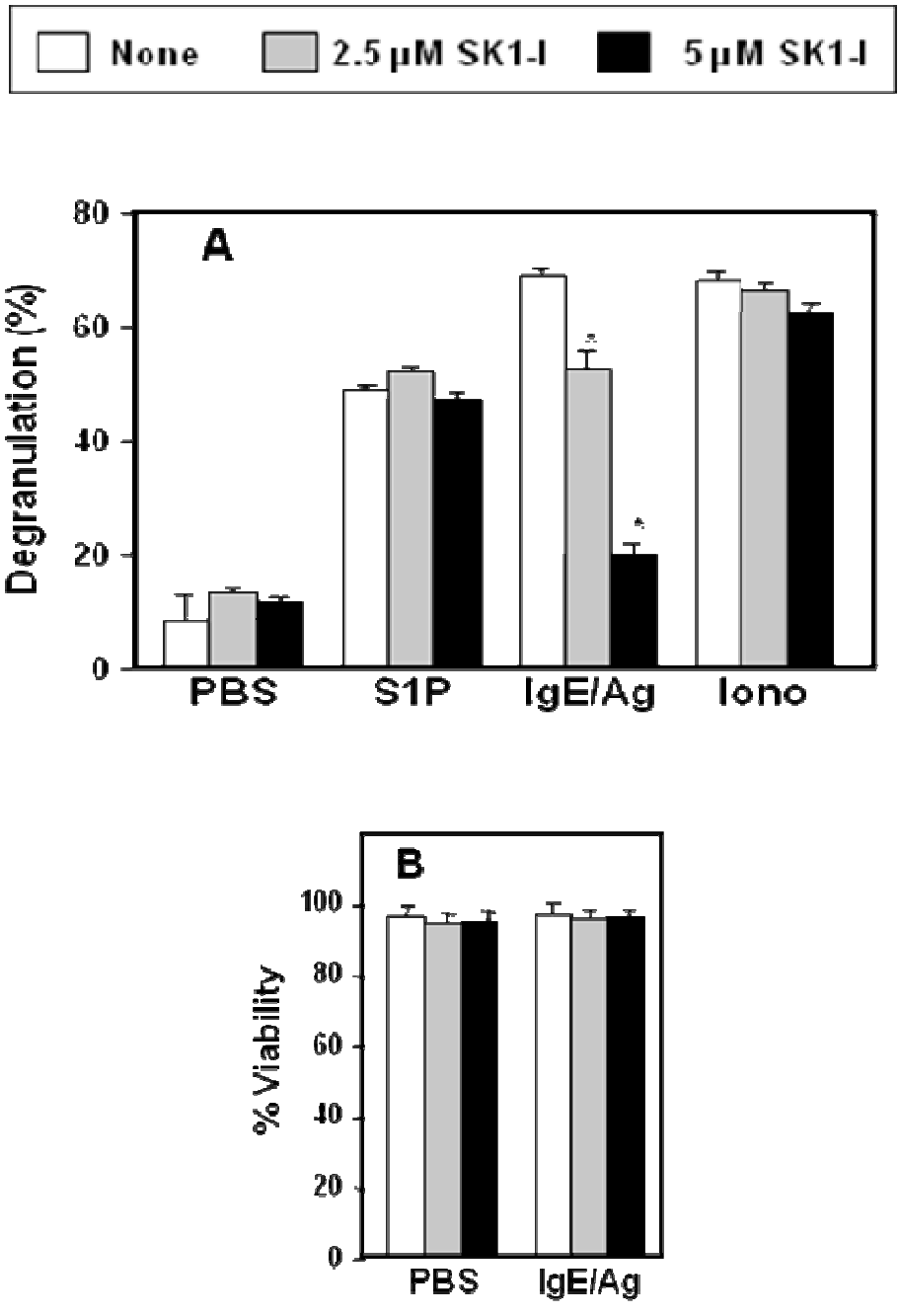

None
2.5 µM SK1-I
5 µM SK1-I
A
Degranulation (%)
80
60
40
20
0
PBS
S1P
IgE/Ag
Iono
B
% Viability
100
80
60
40
20
0
PBS
IgE/Ag

Figure 23: Inhibition of SphK1 reduces secretion of TNF-α from human mast cells. IgE **s**ensitized skin-derived mast cells were treated without (open bars) or with SKI-1 (2.5 μM, grey bars; 5 μM, black bars) and then stimulated with vehicle, 30 ng/ml Ag (IgE/Ag), 100 nM S1P, or 1 μM ionomycin for 24 hours. Secretion of TNF-α was measured by ELISA. Data are the means ± SD of triplicate determinations. *P<0.01. Similar results were obtained using cells from 2 different donors.

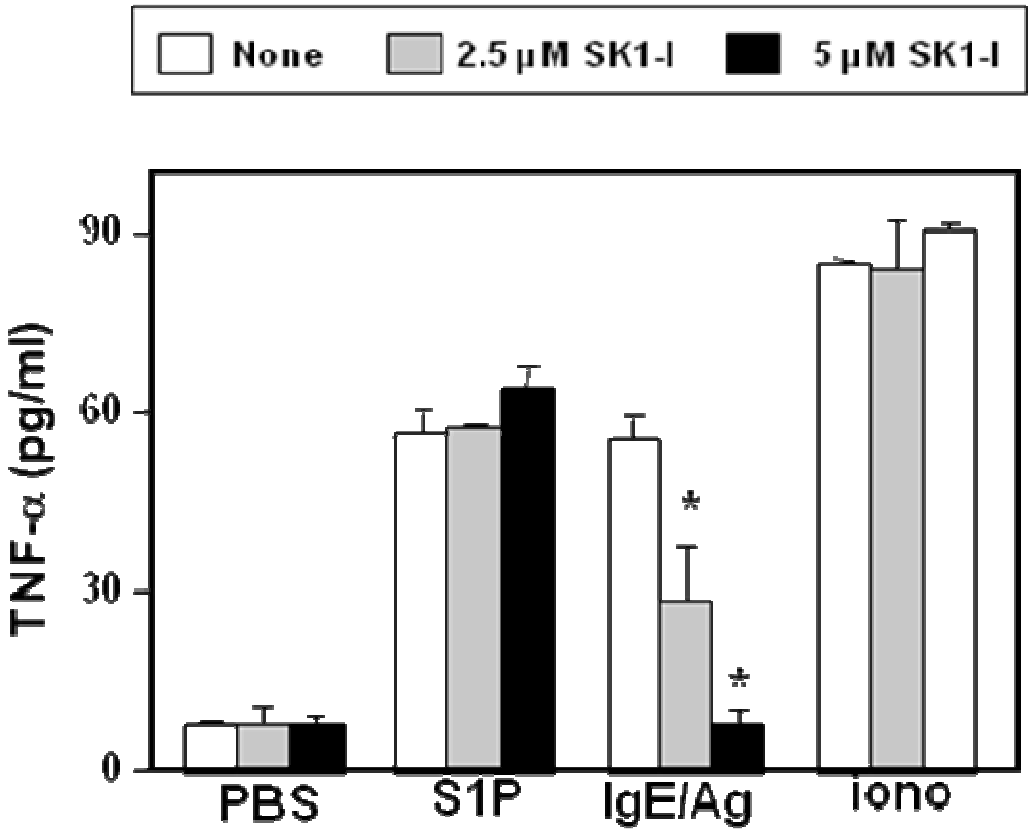

None
2.5 μM SK1-I
5 μM SK1-I
TNF-α (pg/ml)
90
60
30
0
*
*
PBS
S1P
IgE/Ag
Iono

Figure 24: Inhibition of SphK1 reduces secretion of IL-6 from human mast cells. IgE **s**ensitized skin-derived mast cells were treated without (open bars) or with SKI-1 (2.5 μM, grey bars; 5 μM, black bars) and then stimulated with vehicle, 30 ng/ml Ag (IgE/Ag), 100 nM S1P, or 1 μM ionomycin for 24 hours. Secretion of IL-6 was measured by ELISA. Data are the means ± SD of triplicate determinations. *P<0.01. Similar results were obtained using cells from 2 different donors.

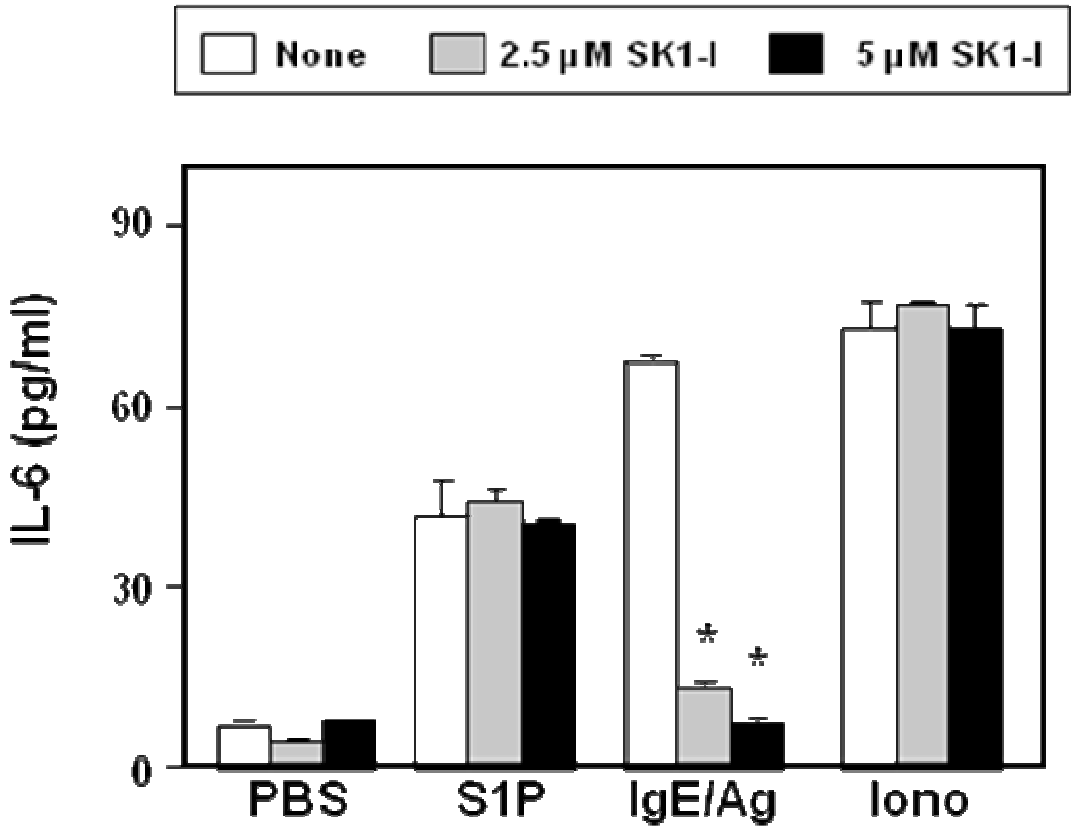
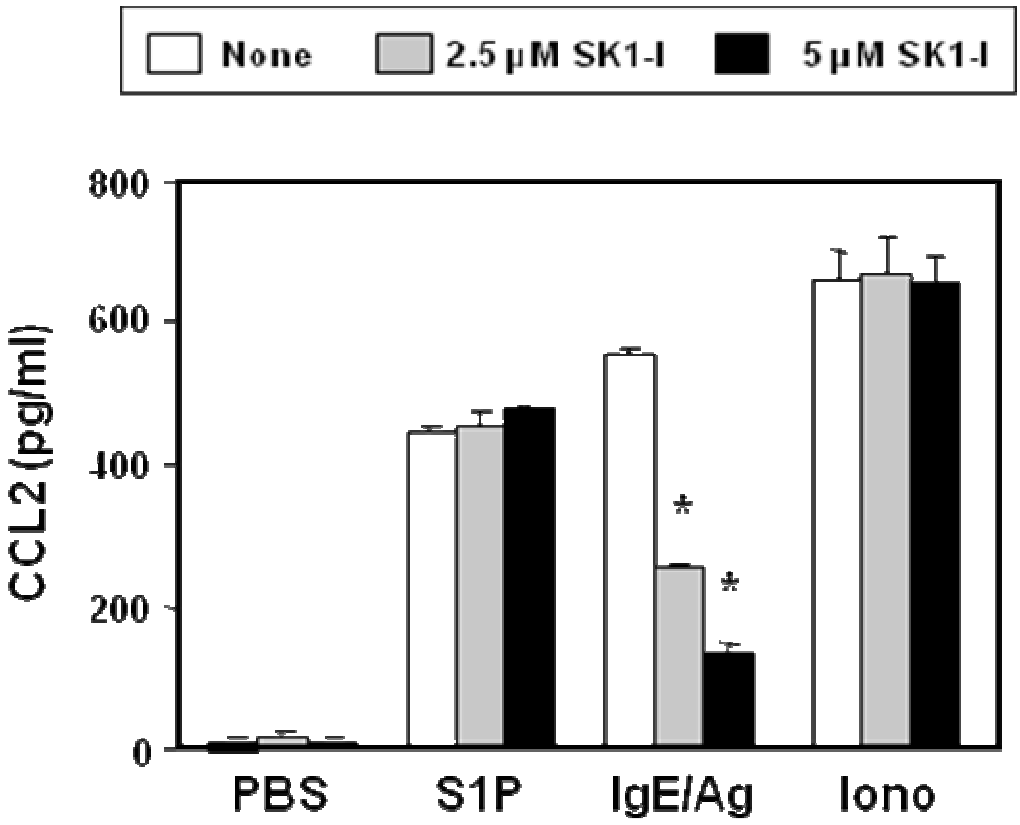

None
2.5 μM SK1-I
5 μM SK1-I
IL-6 (pg/ml)
90
60
30
0
*
*
PBS
S1P
IgE/Ag
Iono

Figure 25: Inhibition of SphK1 reduces secretion of CCL2/MCP-1 from human mast cells. IgE **s**ensitized skin-derived mast cells were treated without (open bars) or with SKI-1 (2.5 μM, grey bars; 5 μM, black bars) and then stimulated with vehicle, 30 ng/ml Ag (IgE/Ag), 100 nM S1P, or 1 μM ionomycin for 24 hours. Secretion of CCL2 was measured by ELISA. Data are the means ± SD of triplicate determinations. *P<0.01. Similar results were obtained using cells from 2 different donors.


None
2.5 µM SK1-I
5 µM SK1-I
CCL2 (pg/ml)
800
600
400
200
0
*
*
PBS
S1P
IgE/Ag
Iono

3.3.2 Inhibition of SphK1 in murine mast cells reduces IgE-mediated degranulation, cytokine release, and NF-κB activation

We also investigated the effects of inhibition of SphK1 on functions of murine bone marrow-derived mast cells. Similar to human mast cells, treatment of murine mast cells with SK1-I greatly reduced their degranulation (Figure 26A) without affecting viability (Figure 26B). Moreover, SK1-I also significantly reduced secretion of the cytokines TNF-α (Figure 27A) and IL-6 (Figure 27B), and IL-13 (Figure 27C) and the chemokine MIP-1α (Figure 27D). Altogether, these data substantiate the notion that SphK1 is generally important for mast cell functions.

We recently showed that S1P formed by SphK1 plays a critical role in TNF-α-induced activation of the master transcription factor NF-κB which regulates expression of many important pro-inflammatory cytokines [18]. As IL-6 and TNF-α production in mast cells is dependent on NF-κB [125, 126], it was of interest to determine whether the inhibitory effect of SKI-1 on production of these cytokines was related to NF-κB activation. To test this, we analyzed phosphorylation of the inhibitor of NF-κB (IκBα), as the common pathway leading to NF-κB activation requires its phosphorylation by phosphorylated IκB kinase (IKK) and degradation. This is a key step in release of NF-κB subunits (p50, p65), which then translocate from the cytosol to the nucleus and initiate cytokine gene transcription. Treatment of mast cells with SKI-1 nearly abolished phosphorylation of IKK and IkBα triggered in response to FcεRI ligation, without affecting

ERK activation (Figure 28), suggesting that SphK1 is also important for NF-κB activation downstream of FcεRI crosslinking.

Figure 26: Inhibition of SphK1 reduces murine mast cell activation. Sensitized BMMCs were treated without (open bars) or with SKI-1 (10 μM, black bars) and then stimulated with vehicle (PBS) or with 20 ng/ml Ag (IgE/Ag). **A,** Secretion of histamine was measured by ELISA and **B,** viability by trypan blue exclusion. Data are the means ± SD of triplicate determinations. *P<0.01.

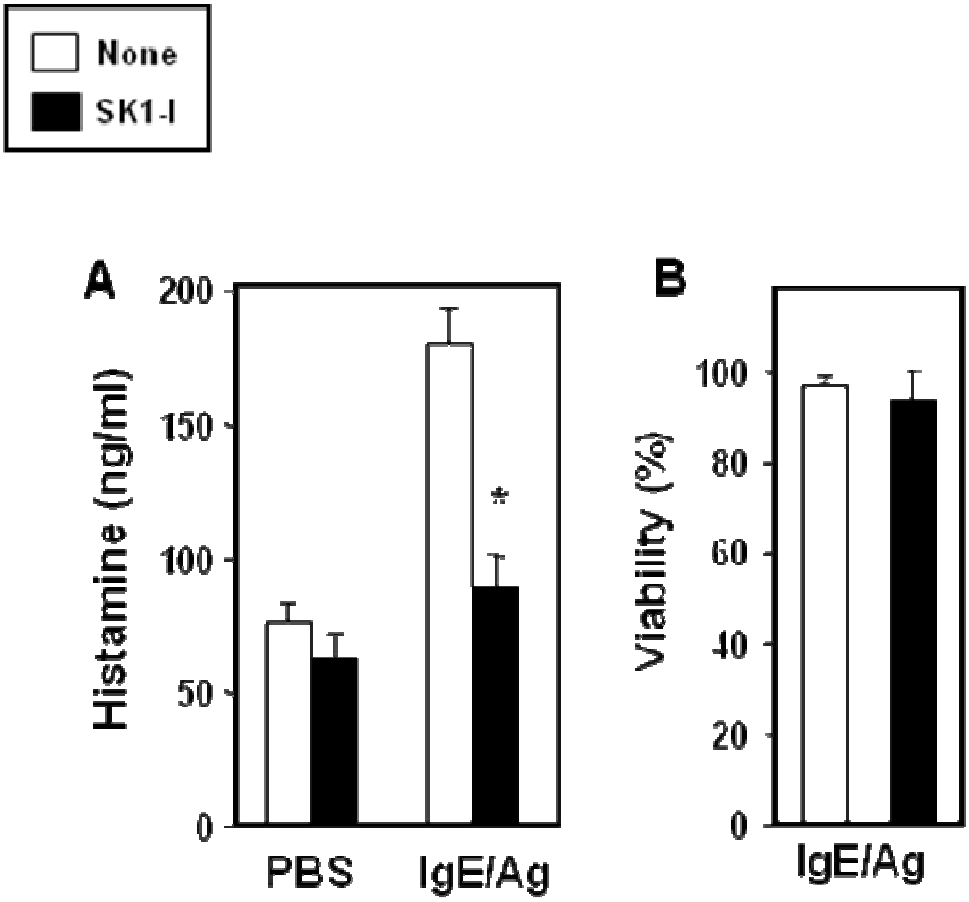

None
SK1-I
A
Histamine (ng/ml)
200
150
100
50
0
*
PBS
IgE/Ag
B
Viability (%)
100
80
60
40
20
0
IgE/Ag

Figure 27: Inhibition of SphK1 dampens cytokine release from murine mast cells. Sensitized BMMCs were treated without (open bars) or with SKI-1 (10 μM, black bars) and then stimulated with vehicle (PBS) or with 30 ng/ml Ag (IgE/Ag). Secretion of (**A**) TNF-α, (**B**) IL-6, (**C**) IL-13, and (**D),** MIP-1α were measured by ELISA. Data are the means ± SD of triplicate determinations. *P<0.01.

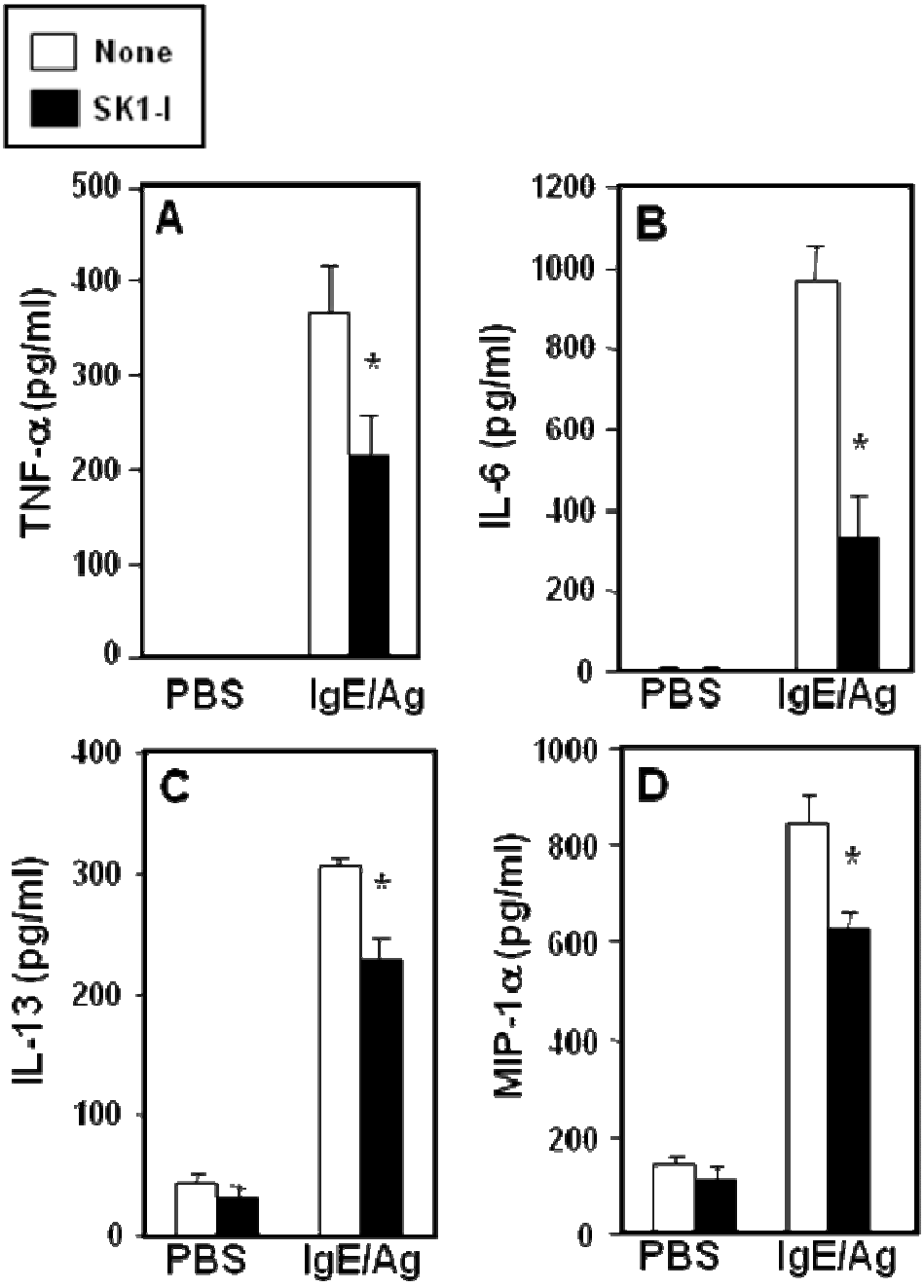
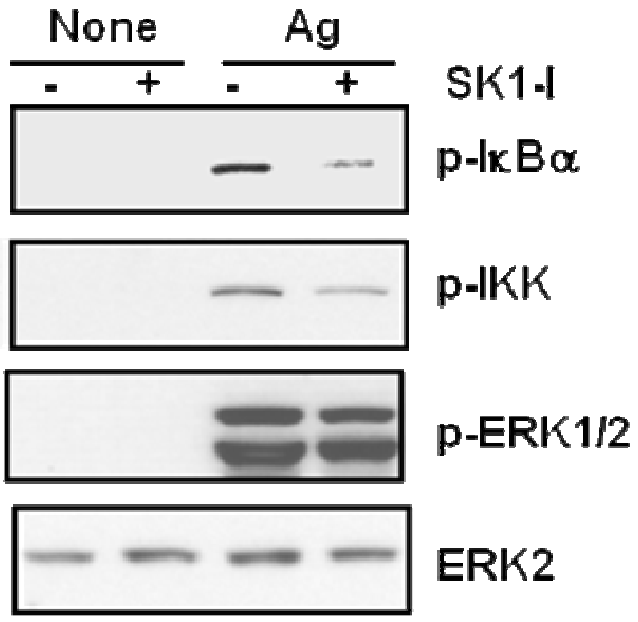

None
SK1-I
A
TNF-α (pg/ml)
500
400
300
200
100
0
PBS
IgE/Ag
*
B
IL-6 (pg/ml)
1200
1000
800
600
400
200
0
PBS
IgE/Ag
*
C
IL-13 (pg/ml)
400
300
200
100
0
PBS
IgE/Ag
*
D
MIP-1α (pg/ml)
1000
800
600
400
200
0
PBS
IgE/Ag
*

Figure 28: Inhibition of SphK1 nearly abolishes antigen-induced NF-κB activation in murine mast cells. IgE-sensitized mast cells were treated without or with Ag for 5 min and cell lysates were immunoblotted with antibodies against p-IkBα, p-IKK, p-ERK1/2 and total ERK2 as a loading control.

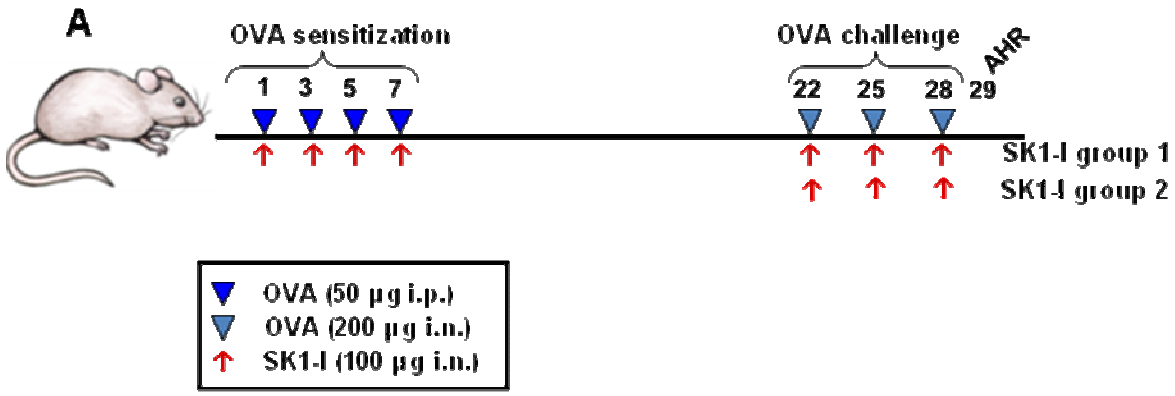

None
Ag
- + - +
SK1-I
p-IκBα
p-IKK
p-ERK1/2
ERK2

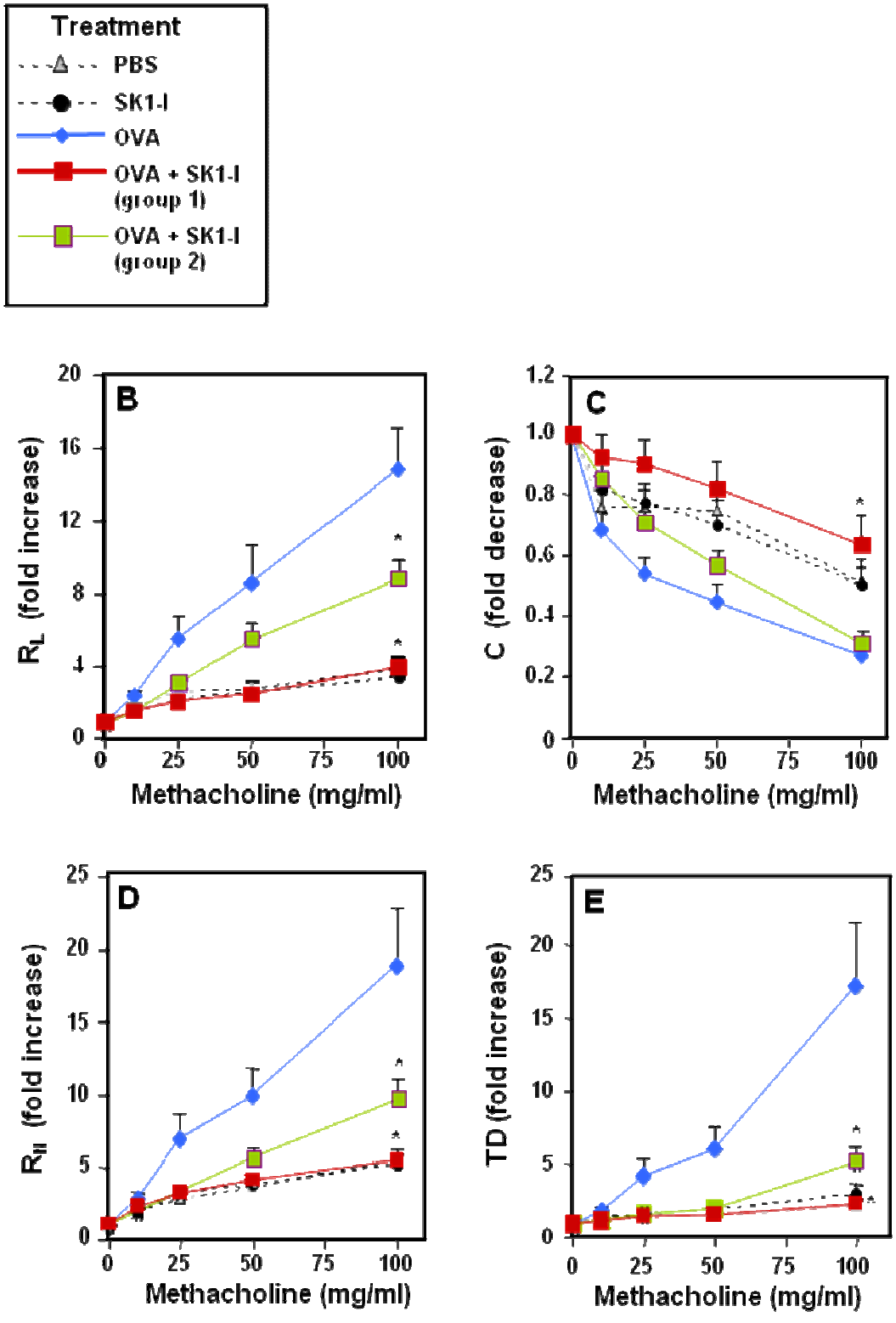
3.3.3 SK1-I treatment reduces development of mast cell dependent airway hyperresponsiveness to methacholine

Having established that SK1-I inhibits *in vitro* mast cell activation, it was next important to examine the effects of SphK1 inhibition on mast cell functions and allergic responses *in vivo*. Previous studies of the role of SphK1 in mouse models of allergic responses all utilized ovalbumin (OVA) antigen sensitization with alum as an adjuvant [127-129]. However, mast cells and IgE are not essential for the development of airway allergic inflammation with this type of protocol [122, 130, 131]. Therefore, we examined the effects of SK1-I in the development of AHR in mice after sensitization with OVA without alum, a chronic allergic asthma model that is significantly mast cell–dependent [122, 123] [132]. In this protocol, mice were sensitized with OVA i.p. and challenged with OVA i.n. as shown in Figure 29A. In OVA-sensitized mice, AHR to methacholine was significantly increased in OVA challenged mice compared to mice challenged with PBS only (Figure 29B). Because SK1-I is water soluble, it was administered i.n. in PBS and had no effect on AHR to methacholine in unsensitized mice (Figure 29B). However, administration of SK1-I i.n. one hour prior to both sensitization and challenge (SK1-I group 1), significantly reduced AHR to methacholine. Lung resistance (Figure 29B), lung compliance, the ease with which lungs can be extended (Figure 29C), Newtonian resistance, a measure of central airway resistance (Figure 29D), and tissue damping (Figure 29E) were all significantly attenuated compared to OVA-sensitized mice. Next it was of interest to examine whether later SK1-I treatment only during the challenge phase (SK1-I group 2) would be able to attenuate AHR induced by OVA. Interestingly, this treatment

significantly reduced lung resistance (Figure 29B), Newtonian resistance (Figure 29D), and tissue damping (Figure 29E). However, lung compliance was significantly different only at lower doses of methacholine (10, 25 mg/ml) compared to OVA-sensitized mice, but not at higher doses (50, 100 mg/ml; Figure 29C). These data suggest that inhibition of SphK1 can attenuate development of mast cell-dependent AHR.

Figure 29: Effect of SK1-I on development of OVA-induced mast cell-dependent AHR. A, Mast cell- and IgE-dependent allergic asthma model. Mice were sensitized and challenged with OVA administered i.p. (50 µg) and i.n. (200 µg), respectively, on the indicated days. PBS or SK1-I (100 µg) were administered intranasally 1 hour prior to OVA sensitization and challenge (group 1) or only prior to OVA challenges (group 2). Unsensitized mice received either PBS or SK1-I on days 1, 3, 5, 7, 22, 25, and 28. Airway responses to methacholine were measured on day 29, 24 hours after the last intranasal OVA or PBS challenge. **B,** lung resistance, **C,** compliance, **D,** Newtonian resistance, and **E,** tissue damping were measured with Flexivent apparatus. * P< 0.05, compared to OVA-challenged mice. Data are means ± SEM from at least 7 mice in each group and are shown as fold changes.




Treatment
PBS
SK1-I
OVA
OVA + SK1-I (group 1)
OVA + SK1-I (group 2)
B
R_L (fold increase)
Methacholine (mg/ml)
C
C (fold decrease)
Methacholine (mg/ml)
D
R_H (fold increase)
Methacholine (mg/ml)
E
TD (fold increase)
Methacholine (mg/ml)

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

As expected, OVA challenged mice displayed extensive inflammatory infiltrates into perivascular areas of the lung, whereas cellular infiltrates were nearly absent in PBS challenged mice as evidenced by H&E staining (Figure 30) as well as by semi-quantitative inflammatory scoring (Figure 31). Interestingly, treatment with SK1-I during sensitization and challenge markedly attenuated OVA-induced inflammatory infiltrates. Moreover, even treatment with SK1-I only during challenge also significantly reduced infiltration of inflammatory cells (Figure 30, 31). Similarly, significant increase in mucus production and goblet hyperplasia were evident by periodic acid-Schiff (PAS) staining only in OVA challenged mice which was greatly reduced by SK1-I treatment throughout and to a lesser but significant extent in the mice treated only during the challenge and fewer of the smaller bronchioles were positively stained (Figure 32).

Consistent with these histology findings, IgE/Ag-challenge significantly increased infiltration of inflammatory cells, especially eosinophils and neutrophils (Figure 33). Treatment with SK1-1 throughout sensitization and challenge drastically reduced eosinophilia, whereas treatment with SK1-I only during the challenge had no significant effect on eosinophil infiltration (Figure 33). Taken together, these results indicate that SphK1 and S1P play an important role in progression of mast cell-dependent airway inflammation.

Figure 30: Inhibition of SphK1 attenuates airway immune cell infiltration and mucus secretion. Mice were sensitized, challenged, and treated as described in Fig. 3. At day 29, lung sections were fixed and stained with hematoxylin and eosin (H&E), scale bar 100 μm and photographed under light microscopy at 100x magnification. Selected areas are shown at higher magnification. Prominent infiltrates of inflammatory cells are present near the airways in OVA-sensitized and challenged mice but not in SK1-I-treated mice.

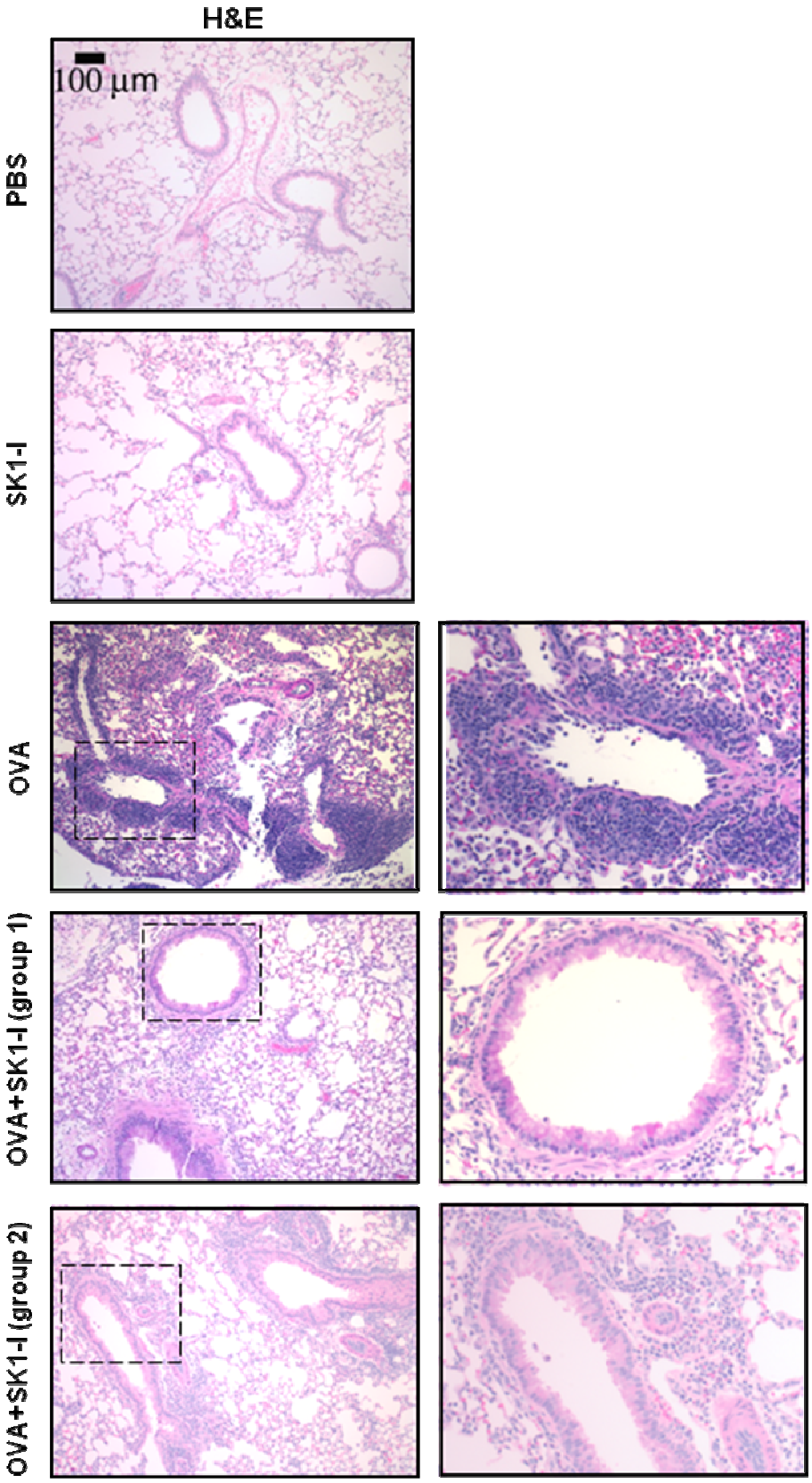

H&E
100 µm
PBS
SK1-I
OVA
OVA+SK1-I (group 1)
OVA+SK1-I (group 2)

Figure 31: Inhibition of SphK1 reduces perivascular inflammation. Mice were sensitized, challenged, and treated as described in Fig. 3. At day 29, lung sections were fixed and stained with hematoxylin and eosin (H&E). Prominent infiltrates of inflammatory cells are present near the airways in OVA-sensitized and challenged mice but not in SK1-I-treated mice. Perivasuclar inflammation was scored as described in Materials and Methods. Data are means ± SEM. * $P < 0.05$, compared to OVA-challenged mice.

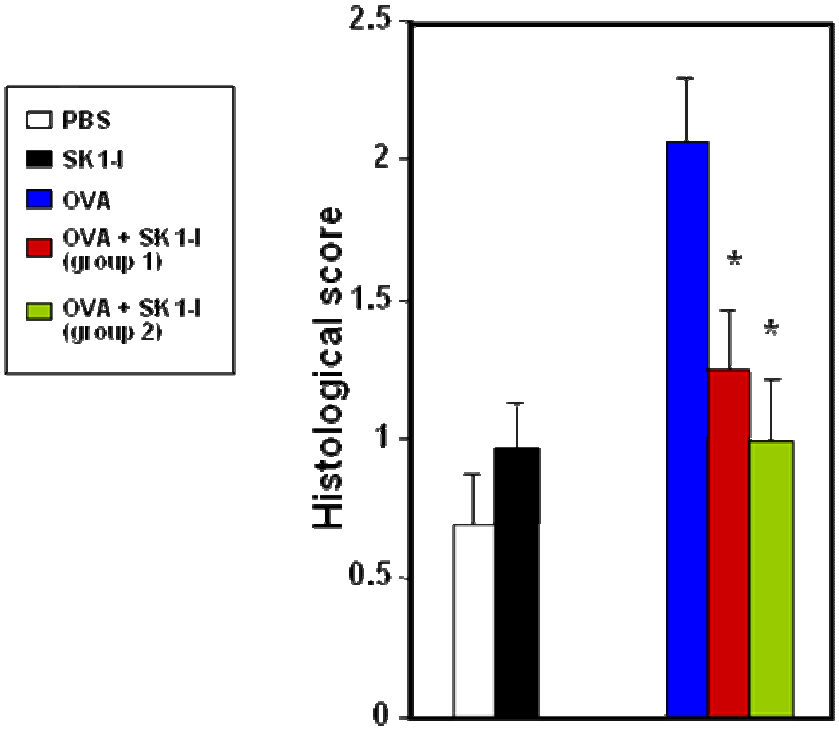

PBS
SK1-I
OVA
OVA + SK1-I (group 1)
OVA + SK1-I (group 2)
Histological score
2.5
2
1.5
1
0.5
0
*
*

Figure 32: Inhibition of SphK1 diminishes mucus secretion in the airways. Mice were sensitized, challenged, and treated as described in Fig. 3. At day 29, lung sections were fixed and stained periodic acid/alcian blue/Schiff (PAS) and photographed under light microscopy at 200x magnification. Scale bar 50 μm.

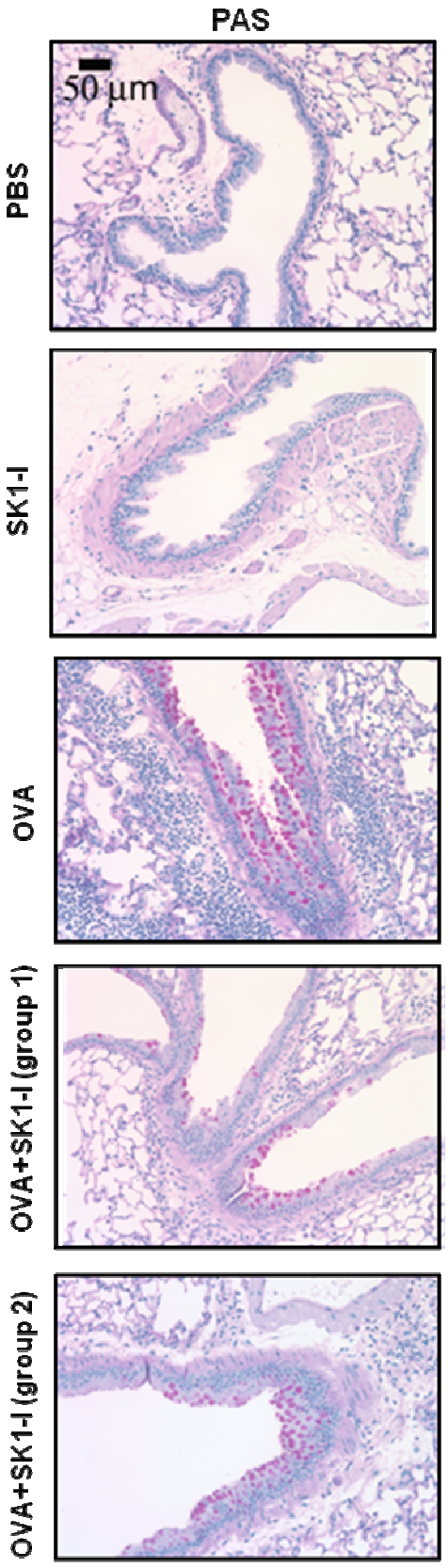
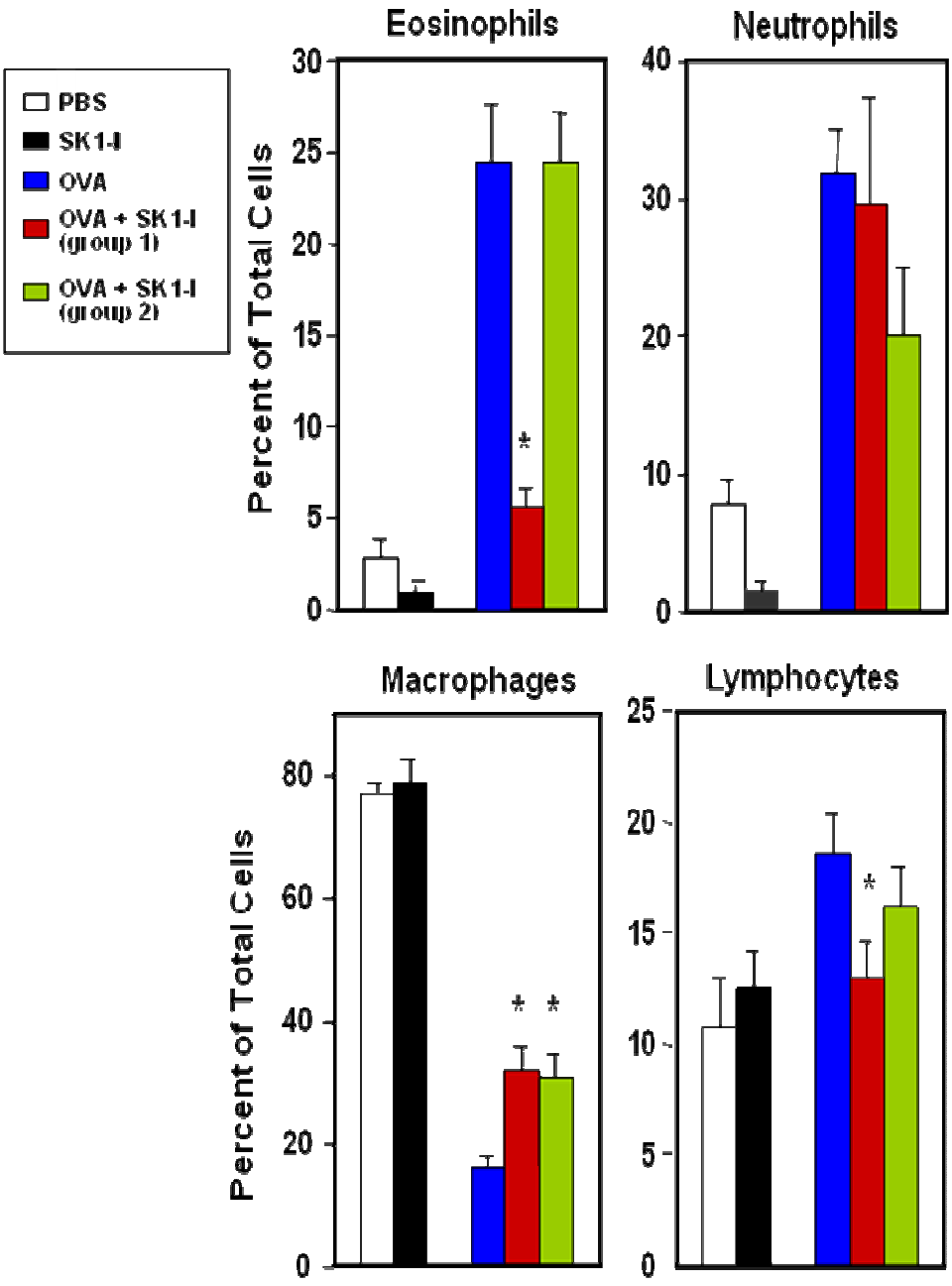

PAS
50 µm
PBS
SK1-I
OVA
OVA+SK1-I (group 1)
OVA+SK1-I (group 2)

Figure 33: SK1-I treatment reduces airway eosinophilia. Mice were sensitized, challenged, and treated as described in Fig. 3. BAL fluid was collected on day 29 and the percentages of eosinophils, neutrophils, macrophages, and lymphocytes were determined. * $P < 0.05$, compared to OVA-challenged mice. Data are means ± SEM from at least 7 mice in each group.


PBS
SK1-I
OVA
OVA + SK1-I (group 1)
OVA + SK1-I (group 2)
Eosinophils
Neutrophils
Macrophages
Lymphocytes
Percent of Total Cells
Percent of Total Cells
0
5
10
15
20
25
30
0
10
20
30
40
0
20
40
60
80
0
5
10
15
20
25
*
*
*
*
*

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

It has previously been shown that S1P levels are increased in BAL fluid of asthmatics after ragweed challenge [67]. Therefore, it was of interest to determine changes in S1P levels following OVA challenge and the effects of SphK1 inhibition. Indeed, levels of S1P were significantly increased in both lung and serum after OVA challenge (Figure 34A, 35A), whereas dihydro-S1P, which is present at much lower levels, was elevated only in the serum (Figure 35A). Consistent with its effect on lung inflammatory responses, administration of SK1-I during sensitization and challenge markedly reduced these elevations of S1P in both the lung and in the circulation (Figure 34A, 35A), whereas treatment with SK1-I only during the OVA challenge was less efficacious and only reduced S1P levels in the lung. Administration of SK1-I intranasally during both sensitization and challenge resulted in higher levels of SK1-I than when administered only during the challenge not only in the lung (Figure 34B) but also in the serum (Figure 35B). Surprisingly, however, SK1-I levels in the lungs and serum of group 1 animals were greater than non-OVA treated mice even though the amount of SK1-I inhaled was identical, suggesting that either OVA administration or the inflammation itself increased retention or uptake of SK1-I (Figure 34B, 35B).

Figure 34: Effect of SK1-I on S1P levels in lung. Mice were sensitized, challenged, and treated as described in Fig. 3. On day 29, lungs**,** were collected and levels of S1P, dihydro-S1P (DHS1P), and SK1-I were determined by LC-ESI-MS/MS. † $P< 0.05$, compared to PBS-treated mice. * $P< 0.05$, compared to OVA-challenged mice.

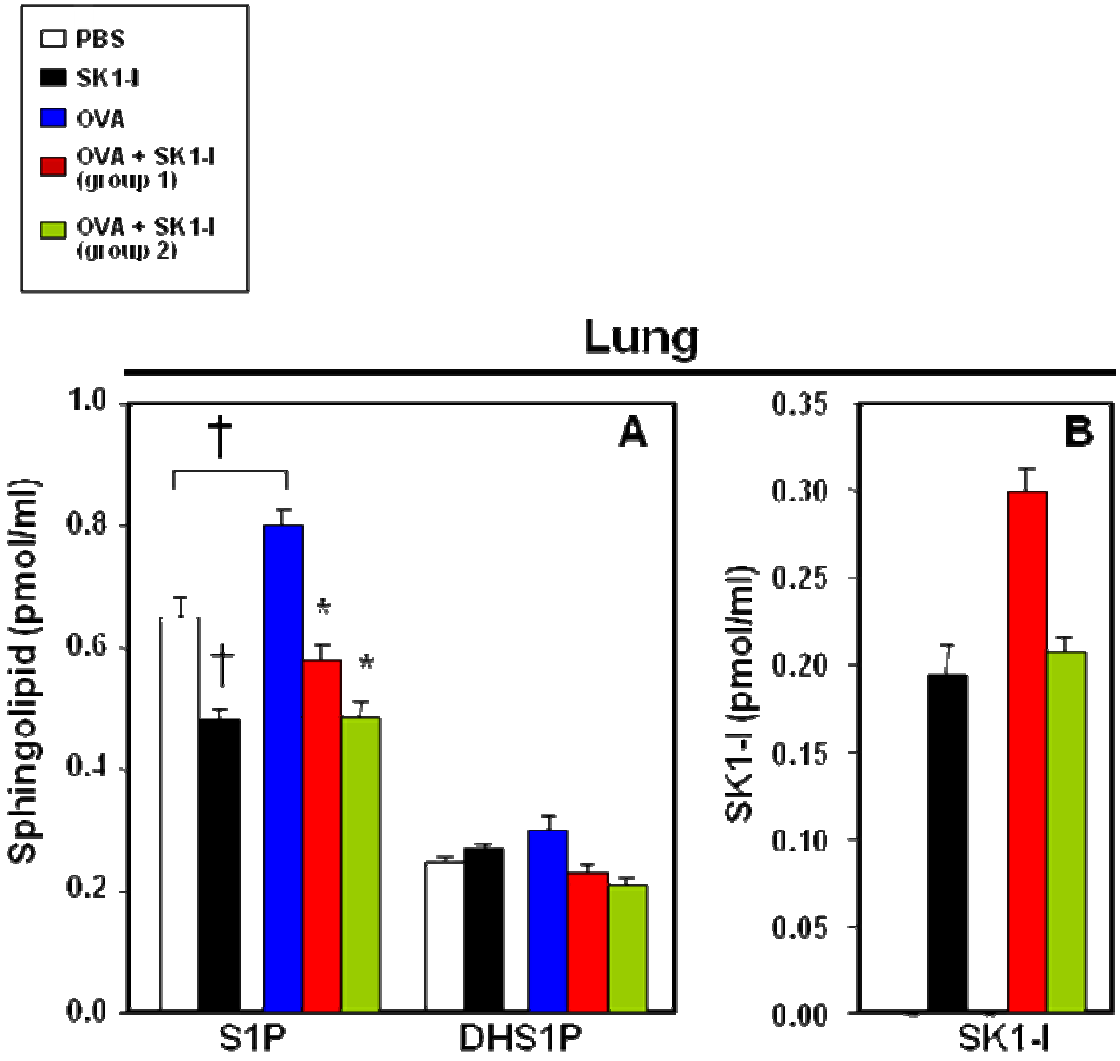
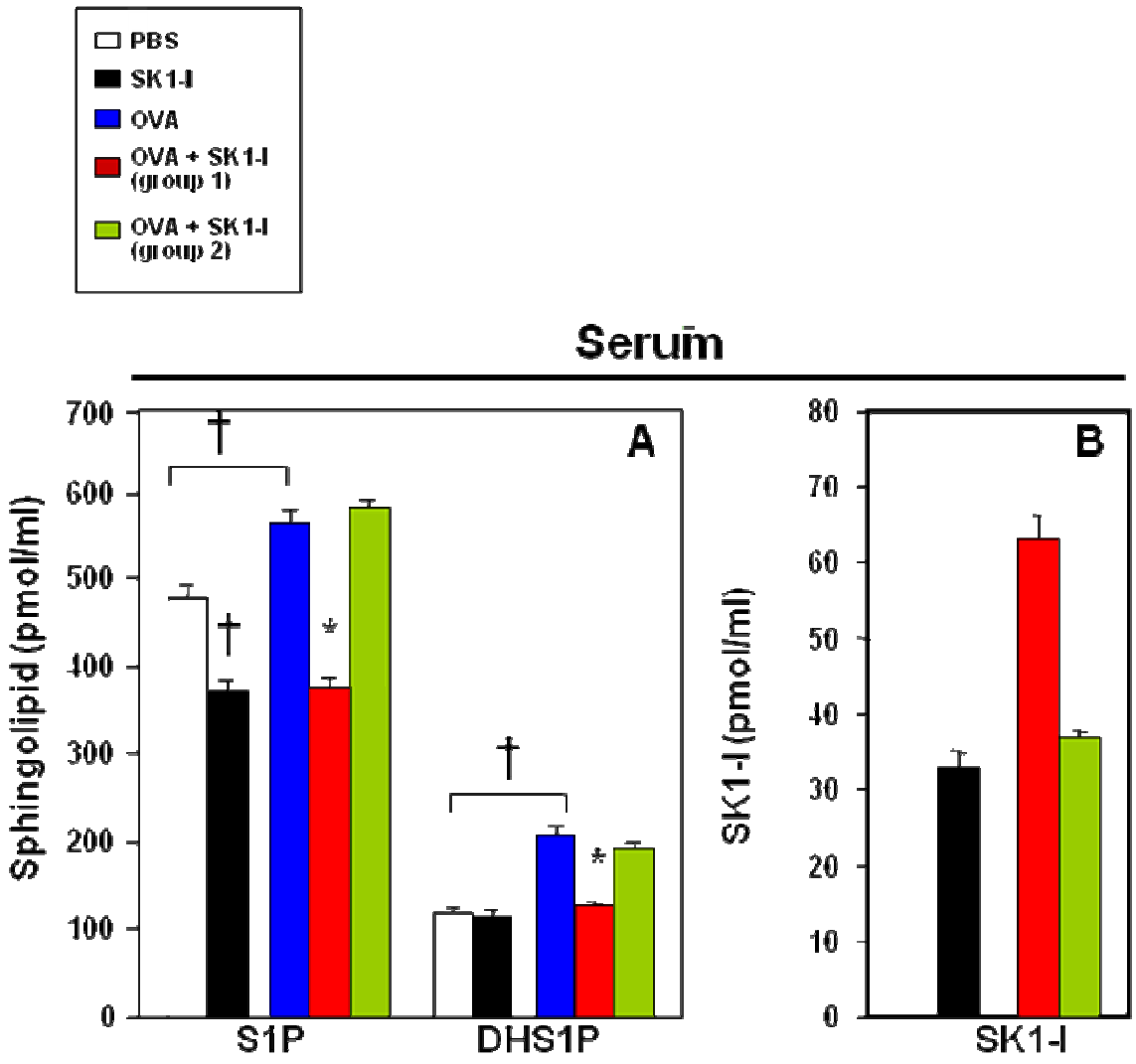

PBS
SK1-I
OVA
OVA + SK1-I (group 1)
OVA + SK1-I (group 2)
Lung
Sphingolipid (pmol/ml)
1.0
0.8
0.6
0.4
0.2
0.0
†
†
*
*
A
S1P
DHS1P
SK1-I (pmol/ml)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
B
SK1-I

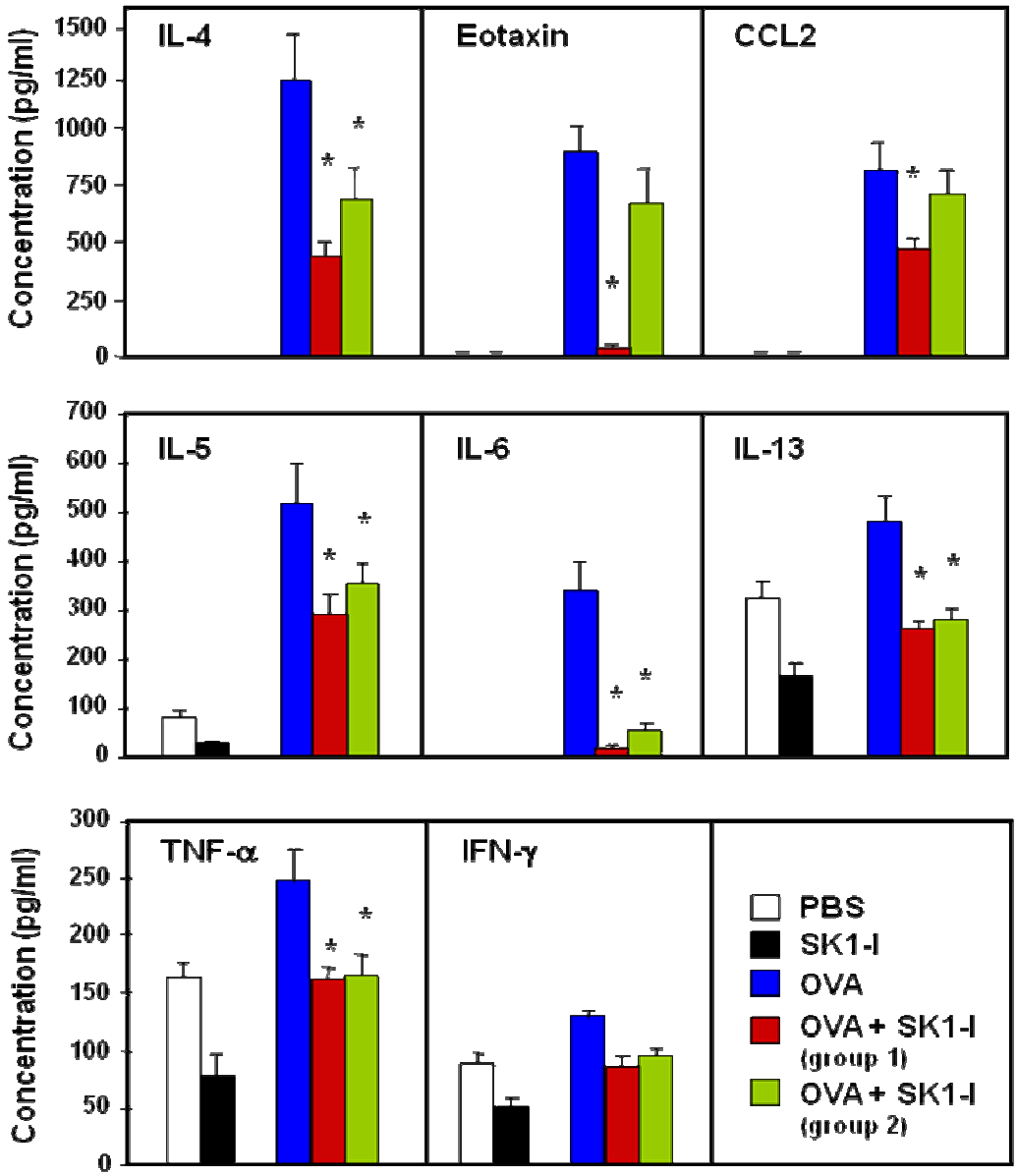
Figure 35: Effect of SK1-I on S1P levels in serum. Mice were sensitized, challenged, and treated as described in Fig. 3. On day 29, serum was collected and levels of S1P, dihydro-S1P (DHS1P), and SK1-I were determined by LC-ESI-MS/MS. † $P < 0.05$, compared to PBS-treated mice. * $P < 0.05$, compared to OVA-challenged mice.


PBS
SK1-I
OVA
OVA + SK1-I (group 1)
OVA + SK1-I (group 2)
Serum
A
B
Sphingolipid (pmol/ml)
SK1-I (pmol/ml)
700
600
500
400
300
200
100
0
80
70
60
50
40
30
20
10
0
†
*
S1P
DHS1P
SK1-I

3.3.6 Inhibition of SphK1 decreases cytokines and chemokines

Because inhibition of SphK1 has been shown to greatly reduce production of cytokines and chemokines secreted from activated mast cells [11, 14, 119, 127, 128], we next examined effect of SK1-I administration on relevant chemokine and cytokine levels in the BAL fluid. In agreement with previous studies (reviewed in [133]), cytokines including T_H2-type IL-4 and IL-13, which have been implicated in the induction of AHR associated with allergic inflammation in the lungs, IL-5 that contributes to eosinophilia, the chemokines eotaxin and CCL2 which are also involved in eosinophilia and diverse types of inflammatory cell recruitment, respectively, were all significantly elevated in OVA challenged mice (Figure 36). Notably, all of these increases were greatly diminished in both SK1-I treatment groups. Similarly, levels of the pleiotropic cytokine IL-6 and TNF-α, whose release from activated mast cells is dependent on SphK1 [14, 119, 127], were elevated in the OVA sensitized mice and were also significantly reduced by treatment with SK1-I. As was observed by others [131], levels of the T_H1-type cytokine IFN-γ were not elevated following OVA challenge. Taken together, these data demonstrate that SphK1 is involved in the regulation of numerous cytokines and chemokines and thus helps to perpetuate pulmonary inflammation.

Figure 36: SK1-I reduces OVA-induced pulmonary cytokines and chemokines. Mice were sensitized, challenged, and treated as described in Fig. 3. On day 29, BAL fluid was collected and levels of the indicated cytokines and chemokines were measured by Bioplex assay. Data are means ± SEM from at least 7 mice in each group. * $P < 0.05$, compared to OVA-challenged mice.


IL-4
Eotaxin
CCL2
IL-5
IL-6
IL-13
TNF-α
IFN-γ
Concentration (pg/ml)
PBS
SK1-I
OVA
OVA + SK1-I (group 1)
OVA + SK1-I (group 2)

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

As noted above, SphK1 is required for optimal NF-κB activation and proinflammatory cytokine production upon FcεRI triggering of mast cells. Therefore, it was of interest to examine the activation of NF-κB *in vivo* in mast cell dependent allergic responses. OVA challenge induced a marked increase in staining of the p65 subunit of NF-κB in the infiltrated inflammatory cells and bronchial epithelial cells that was nearly absent in unchallenged mice treated with PBS or SK1-I (Figure 37). The increase of p65 staining was dramatically reduced in OVA challenged mice treated with SK1-I. Similarly, OVA challenge induced phosphorylation of p65 (serine 536), known to be important for its transcriptional activity, was decreased by SK1-I treatment (Figure 38).

Figure 37: Inhibition of SphK1 reduces global expression of NF-κB in the lungs of OVA-challenged mice. Mice were sensitized, challenged, and treated as described in Fig. 3. At day 29, lung sections were fixed and stained with anti-p65 antibody and photographed under light microscopy at 200x magnification. Scale bar 50 μm.

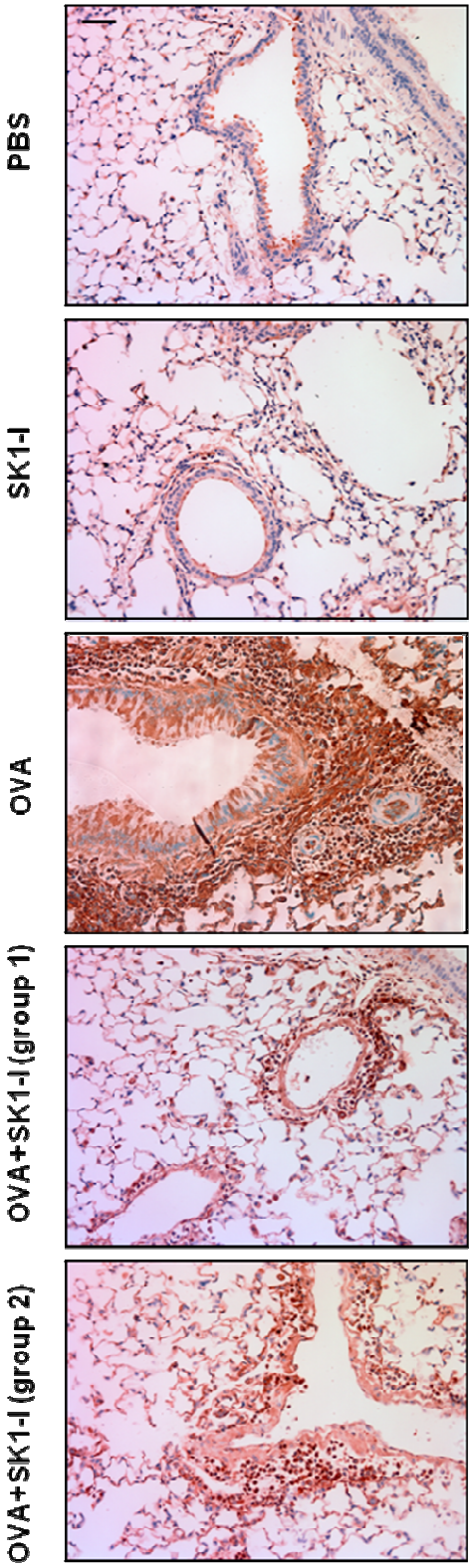
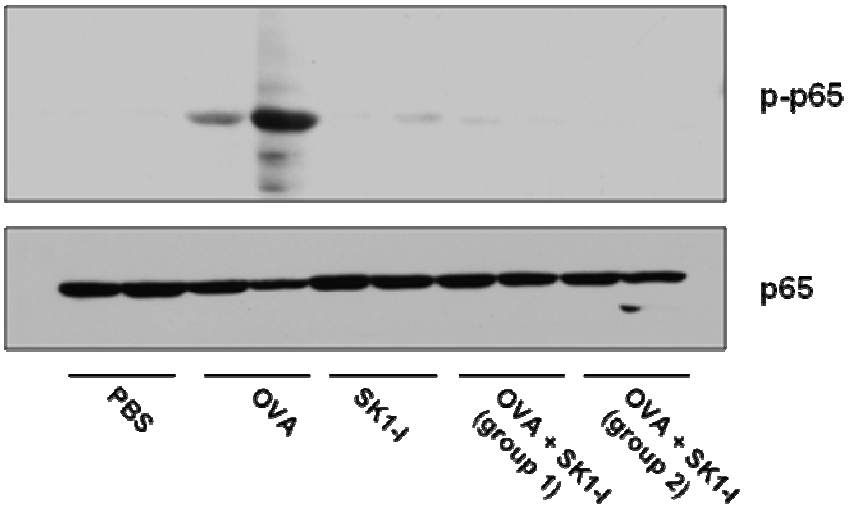

PBS
SK1-I
OVA
OVA+SK1-I (group 1)
OVA+SK1-I (group 2)

Figure 38: Inhibition of SphK1 attenuates activation of NF-κB in the lungs of OVA-challenged mice. Mice were sensitized, challenged, and treated as described in Fig. 3. At day 29, lung sections were homogenized and equal amounts of proteins were analyzed by immunoblotting with anti-p-p65 antibody. Blots were stripped and blotted with p65 antibody to demonstrate equal loading and transfer.


p-p65
p65
PBS
OVA
SK1-I
OVA + SK1-I (group 1)
OVA + SK1-I (group 2)

3.4 DISCUSSION

S1P has emerged as an important regulator of mast cell effector functions and pathogenesis of allergic disease [116, 121]. S1P produced in allergically-stimulated mast cells is involved in their degranulation, cytokine and chemokine production, and migration towards sites of inflammation. Here we have shown that specifically inhibiting SphK1 with SK1-I effectively attenuated degranulation of both human and murine mast cells and also inhibited secretion of cytokines and chemokines that contribute to the pathophysiology of allergic disease. Our results support the notion that SphK1 is the key SphK isoenzyme involved in FcεRI mediated mast cell activation. Surprisingly however, BMMCs derived from SphK1 knockout mice had normal responses whereas silencing SphK1 markedly impaired BMMC functions [119], leading to the suggestion that this is due to a compensation mechanism during development of the mice with a deletion of this important gene [119].

TNF-α and IL-6 expression in response to FcεRI ligation is strictly dependent on NF-κB [134]. Indeed, we found that inhibition of SphK1 nearly abolished antigen-induced phosphorylation of both IKK and IkBα, key players in the NF-κB pathway, and secretion of these proinflammatory cytokines, suggesting SphK1 is also important for NF-κB activation downstream of FcεRI crosslinking. Similar effects were found by downregulation of SphK1, but not SphK2, in BMMC [119].

S1P levels are elevated in human asthmatics [67] and recent studies have implicated S1P and SphK1 in the pathogenesis of chronic asthma based on animal models of allergic airway inflammation [117, 127-129, 135, 136]. In mice, the administration of

S1P aggravates antigen-induced airway inflammation [117] and bronchial hyperresponsiveness [136]. Conversely, treatment of OVA challenged mice with a pan SphK inhibitor or with siRNA targeted to SphK1 reduced pulmonary infiltration of inflammatory cells, eosinophilia, cytokine and chemokine secretion, and AHR [127, 135]. In contrast, although SphK1 deficiency in mice decreased allergen-induced airway inflammation surprisingly however it increased pulmonary vascular hyperresponsiveness [128]. Moreover, treatment of OVA challenged mice with SKI-II, another SphK1 inhibitor, ameliorated bronchial smooth muscle hyperresponsiveness, yet had no effect on other features of airway inflammation [129]. Some of these apparent controversies regarding the roles of S1P and SphK1 in various features of mouse models of asthma probably reflect the pleiotropic actions of S1P in immune regulation and its diverse roles in many types of immune cells in addition to the regulation of the effector functions of mast cells. Furthermore, the majority of these reports utilized an allergic model with alum as an adjuvant, which can itself enhance T_H2 responses and thus mask important functions of mast cells [137].

Using a murine model of allergic asthma that is strictly mast cell-dependent [122, 131], we found that inhibition of SphK1 suppressed development of AHR, chronic inflammation including infiltration of eosinophils, and airway epithelial goblet cell hyperplasia. This suppressive action of SK1-I is consistent with previous findings that SphK1 expression is enhanced by OVA challenge [70], particularly around bronchial epithelial walls [135], and that S1P is important for recruitment of inflammatory cells, including mast cells, lymphocytes, and eosinophils to sites of inflammation (reviewed in

[87, 116]) and has also been implicated in production of MUC5AC and in goblet cell hyperplasia [135, 138]. SK1-I also suppressed OVA-induced secretion of the T_H2 cytokines, IL-4, IL-5, and IL-13, and the chemokines, eotaxin and CCL2, that orchestrate the inflammatory response in chronic asthma. Furthermore, inhibition of SphK1 with SK1-I suppressed the pro-inflammatory cytokines TNF-α and IL-6 that amplify inflammation, probably by inhibition of the activation of NF-κB. In this regard, intracellular S1P produced by SphK1 has recently been implicated as a critical regulator of NF-κB either as a required cofactor for the K63-linked polyubiquitylation of RIP1 by TRAF2 [18], a key step leading to activation of NF-kB, or for activation of protein kinase Cδ (PKCδ), which promotes IκB kinase and NF-κB activation [118].

Interestingly, SK1-I was effective even when only administered during the OVA challenge, which has therapeutic implications for treatment of allergic patients. It is likely that SK1-I was so effective at reducing lung inflammation because it is water soluble and could be administered intranasally. This maintained the SK1-I at a high enough concentration in the lungs to inhibit SphK1 and S1P production. Moreover, we demonstrated that inhalation of SK1-I not only prevented the OVA-induced increase in S1P levels in the lungs but also in the circulation. Therefore, modulating the production of S1P by specific targeting of SphK1 deserves consideration as a potential therapeutic approach to control chronic airway diseases and other mast cell-mediated allergic reactions.

CHAPTER 4: CONCLUSIONS

4.1 S1P modulates immune responses

S1P is an important regulator of mast cell functions, including degranulation, mediator production and migration towards sites of inflammation [116]. In these studies, we demonstrate that chronic exposure to S1P can influence the differentiation and responsiveness of mast cells. These hyperresponsive mast cells express chymase and tryptase, important intragranular proteases thought to play both pro-inflammatory (promoting bronchial hyperresponsiveness and influx of neutrophils and eosinophils, degrading proteins that regulate coagulation, and activating proteins that modulate extracellular matrix remodeling) and anti-inflammatory roles (degrading pro-inflammatory cytokines and chemokines, inducing vasoconstriction by cleavage of angiotensin I, targeting extracellular matrix proteins, inactivating toxic peptidases) in asthma and allergic rhinitis depending on the tissue and context in which they are released [5, 139]. Thus, changes in S1P levels can have drastic effects in the surrounding tissue.

Normally, levels of S1P in tissues or inside cells remains low due to its degradation and/or dephosphorylation by S1P lyase and S1P phosphatases, respectively [58, 140, 141]. However, dysregulation or local increases in S1P levels occur in pathophysiological conditions such as acute inflammation [142], asthma [67], and rheumatism [143, 144], and therefore may alter immune responses. The mechanisms by which S1P levels are elevated are not fully understood, but mast cells and red blood cells are likely sources. Indeed, mast cell numbers are known to increase significantly in tissues during inflammatory conditions

such as in the joints with arthritis or in the lungs of patients with allergic asthma [66, 145, 146]. In asthmatics, this is accompanied by an increase in circulating progenitor cells [147].

During early stages of inflammation, increases in S1P correlate with mast cell degranulation [13]. Once activated, mast cells secrete S1P into the surroundings, which can signal in an autocrine and paracrine fashion to induce cytokine and chemokine release and promote migration to target sites. Maintenance of S1P gradients established within the tissues are critical for recruitment of progenitors, homing of lymphocytes, local S1P production, and regulation of S1P receptors in the area in order to mount an appropriate response [8, 11, 32, 71, 148-150]. S1P can also shift T-cell responses to favor T_H2, which orchestrate inflammation in asthma, over T_H1 responses. Indeed, we found that inhibition of SphK1 drastically reduced T_H2 cytokines in the BAL fluid of OVA challenged mice (Figure 36) Additionally, $S1P_2$ expression is enhanced during mast cell activation and S1P via $S1P_2$ further enhances degranulation and induces vascular permeability [40, 151, 152]. In later, resolving stages of inflammation, S1P may act to dampen inflammation and restore homeostasis. S1P regulates the development of mast cells from recruited progenitors, shifting their phenotype and thus their intragranular protease composition. Upon release, these newly expressed proteases can begin to cleave pro-inflammatory cytokines such as IL-6 and IL-13, as well as SCF [139], a mast cell chemotactic factor [153-155] and the primary growth factor required for human mast cell differentiation. Furthermore, chymase can regulate blood pressure by cleaving angiotensin I to produce angiotensin II, a factor that induces vasoconstriction [139]. Engagement of $S1P_1$ on

endothelial cells can begin to enhance and preserve vascular integrity [39, 151, 156, 157], thereby helping to restore and maintain blood pressure.

The importance of S1P now extends well beyond the regulation of mast cell functions to the regulation of other immune cells and the surrounding environment. Physiologically, S1P production, degradation, and release are intricately controlled, as is regulation of its multiple cell surface receptors and mode of action (intracellular versus extracellular). Changes in S1P levels are therefore critical in modulating immune responses and these events allow for plasticity in response to this single molecule, depending on the type of cell, tissue, or surrounding environment.

4.2 SphK1 versus SphK2

Numerous S1P mediated events are relevant to the pathophysiology of allergic responses, asthma and the exacerbated anaphylactic reaction. Yet, controversies still surround the importance of SphK1 versus SphK2 in various features of mast cell-mediated responses. The differences observed (discussed previously) between SphK-deficient BMMCs or fetal-liver derived mast cells, mice with genetic deletions, and RNAi silencing may simply be due to alterations in mast cell phenotypes. Mast cell phenotype varies depending on the environment they populate or on experimental conditions employed, thus mast cells differentiated *in vitro* may not reflect the phenotype of mast cells *in vivo*. These differences may also be due to off-target effects of siRNA or possible compensatory mechanisms present in SphK-deficient mice. Use of siRNA *in vivo* or SphK-deficient mice affects multiple cell types, suggesting responses seen in these studies may not be attributed

to mast cells alone. Moreover, genetic deletion of SphKs may induce epigenetic changes, resulting in differences in mast cell phenotpyes, in mast cells or bone marrow progenitors themselves, or in cellular distribution and localization of SphKs and S1P in these cells or in the surrounding environment. Location of S1P production may be the major determinant of the resulting phenotype, its mode of action, and its pleiotropic roles in various types of immune cells. Regardless of these unresolved issues, overall these and other studies demonstrate a critical role for SphKs in mast cell-mediated allergic responses.

4.3 SphK inhibitors: new targeted anti-allergic therapies?

Current therapeutic strategies for allergic diseases are primarily targeted against mast cell mediators intending to suppress symptoms that are consequences of airway inflammation and hyperresponsiveness. Moreover, they are often of poor efficacy and associated with undesirable side effects. Complete allergen avoidance is very difficult to achieve. Desensitizing immunotherapy has been performed for many years, with doubtful efficacy in many cases and has occasionally even been hazardous. Thus, new approaches for the development of novel inhibitors of allergic diseases have great potential. Modulating the production of S1P and/or specifically targeting its receptors are attractive novel approaches for the management of mast cell-mediated allergic diseases. Promising observations in preclinical models of allergic disease provide proof of concept for the importance of SphKs and production of S1P as targets in inflammatory responses. For example, increased levels of proinflammatory cytokines in the peritoneal cavity of mice administered C5a were substantially decreased by treatment with the pan SphK inhibitor,

N,N-dimethylsphingosine (DMS) [106]. This was accompanied by suppression of C5a-induced neutropenic responses, as well as by increased vascular permeability [106]. Moreover, C5a activates SphK in human neutrophils and the SphK inhibitor DMS largely blocked C5a-stimulated calcium mobilization, chemotaxis and cytokine production [47]. However, although an *in vivo* model of bacterial lung infection revealed an accelerated progression of disease in *SphK2* but not *SphK1*-knockout mice, effector functions of SphK1- or SphK2-deficient neutrophils and their capacity to kill bacteria were normal [48].

A recent study by Lai *et al*. utilized the pan-SphK inhibitor DMS and downregulation of SphK1 expression to demonstrate that both effectively suppressed airway eosinophilia, pulmonary inflammation and secretion of T_H2 cytokines and chemokines, and markedly attenuated OVA-induced AHR in sensitized mice [127]. Serum levels of OVA-specific IgE were reduced by SphK1 siRNA, suggesting that production of S1P may regulate B-cell trafficking and IgE production. In another study, OVA inhalation caused S1P release into bronchial alveolar lavage (BAL) and expression of SphK1 around bronchial epithelial walls. Inhalation of pan-SphK inhibitors decreased S1P in BAL, accompanied by decreased eosinophil infiltration and eotaxin expression. Furthermore, bronchial hyperresponsiveness to inhaled methacholine and goblet cell hyperplasia were improved by SphK inhibitors [135].

4.4 Concluding remarks and future perspective

Although S1P has been implicated as an important component of the regulation of immune responses, there are still many questions waiting to be answered. The lack of SphK isozyme-specific inhibitors has made it challenging to assign specific functions to SphK1 and SphK2. However, the recent development and availability of specific S1P receptor agonists and antagonists, as well as our water soluble, isozyme specific SK1-I inhibitor, will likely encourage more preclinical and clinical trials to target effects mediated by S1P. In conclusion, the relevance of mast cells, S1P, SphKs and S1P receptors for the maintenance of normal physiology, or in disease states, constitutes an outstanding and intricate combination of players important for immune responses at the cellular, signaling and molecular levels. The need for specific SphK inhibitors is driving the development of new compounds by many pharmaceutical companies. Combining knowledge gained from molecular strategies and conditional gene knockouts to interfere with expression of enzymes that regulate S1P levels with pharmacological approaches will surely aid in this quest.

Literature Cited

Literature Cited

[1] H. Turner, J.P. Kinet, Signalling through the high-affinity IgE receptor Fc epsilonRI, Nature, 402 (1999) B24-B30.
[2] J. Rivera, A. Olivera, Src family kinases and lipid mediators in control of allergic inflammation, Immunol. Rev., 217 (2007) 255-268.
[3] J. Rivera, Molecular adapters in Fc(epsilon)RI signaling and the allergic response, Curr Opin Immunol, 14 (2002) 688-693.
[4] N. Urtz, A. Olivera, E. Bofill-Cardona, R. Csonga, A. Billich, D. Mechtcheriakova, F. Bornancin, M. Woisetschlager, J. Rivera, T. Baumruker, Early activation of sphingosine kinase in mast cells and recruitment to FcepsilonRI are mediated by its interaction with Lyn kinase, Mol. Cell Biol., 24 (2004) 8765-8777.
[5] N.N. Trivedi, G.H. Caughey, Mast cell peptidases: chameleons of innate immunity and host defense, Am J Respir Cell Mol Biol, 42 (2010) 257-267.
[6] J.R. Gordon, S.J. Galli, Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin, Nature, 346 (1990) 274-276.
[7] O.H. Choi, J.-H. Kim, J.-P. Kinet, Calcium mobilization via sphingosine kinase in signalling by the FcεRI antigen receptor, Nature, 380 (1996) 634-636.
[8] E.E. Prieschl, R. Csonga, V. Novotny, G.E. Kikuchi, T. Baumruker, The balance between sphingosine and sphingosine-1-phosphate is decisive for mast cell activation after Fc epsilon receptor I triggering, J. Exp. Med., 190 (1999) 1-8.
[9] T. Baumruker, E.E. Prieschl, The role of sphingosine kinase in the signaling initiated at the high- affinity receptor for IgE (FcepsilonRI) in mast cells, Int. Arch. Allergy Immunol., 122 (2000) 85-90.
[10] A.J. Melendez, A.K. Khaw, Dichotomy of Ca^{2+} signals triggered by different phospholipid pathways in antigen stimulation of human mast cells, J. Biol. Chem., 277 (2002) 17255-17262.
[11] P.S. Jolly, M. Bektas, A. Olivera, C. Gonzalez-Espinosa, R.L. Proia, J. Rivera, S. Milstien, S. Spiegel, Transactivation of sphingosine-1-phosphate receptors by Fc{epsilon}RI triggering is required for normal mast cell degranulation and chemotaxis, J. Exp. Med., 199 (2004) 959-970.
[12] A. Olivera, N. Urtz, K. Mizugishi, Y. Yamashita, A.M. Gilfillan, Y. Furumoto, H. Gu, R.L. Proia, T. Baumruker, J. Rivera, IgE-dependent activation of sphingosine kinases 1 and 2 and secretion of sphingosine 1-phosphate requires Fyn kinase and contributes to mast cell responses, J. Biol. Chem., 281 (2006) 2515-2525.
[13] A. Olivera, K. Mizugishi, A. Tikhonova, L. Ciaccia, S. Odom, R.L. Proia, J. Rivera, The sphingosine kinase-sphingosine-1-phosphate axis is a determinant of mast cell function and anaphylaxis, Immunity, 26 (2007) 287-297.
[14] C.A. Oskeritzian, S.E. Alvarez, N.C. Hait, M.M. Price, S. Milstien, S. Spiegel, Distinct roles of sphingosine kinases 1 and 2 in human mast-cell functions, Blood, 111 (2008) 4193-4200.
[15] S. Spiegel, S. Milstien, Sphingosine-1-phosphate: an enigmatic signalling lipid, Nat. Rev. Mol. Cell Biol., 4 (2003) 397-407.

[16] H. Rosen, E.J. Goetzl, Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network, Nat. Rev. Immunol., 5 (2005) 560-570.
[17] G.M. Strub, M. Paillard, J. Liang, L. Gomez, J.C. Allegood, N.C. Hait, M. Maceyka, M.M. Price, Q. Chen, D.C. Simpson, T. Kordula, S. Milstien, E.J. Lesnefsky, S. Spiegel, Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration, FASEB J., (2010).
[18] S.E. Alvarez, K.B. Harikumar, N.C. Hait, J. Allegood, G.M. Strub, E.Y. Kim, M. Maceyka, H. Jiang, C. Luo, T. Kordula, S. Milstien, S. Spiegel, Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2, Nature, 465 (2010) 1084-1088.
[19] N.C. Hait, J. Allegood, M. Maceyka, G.M. Strub, K.B. Harikumar, S.K. Singh, C. Luo, R. Marmorstein, T. Kordula, S. Milstien, S. Spiegel, Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate, Science, 325 (2009) 1254-1257.
[20] O. Cuvillier, G. Pirianov, B. Kleuser, P.G. Vanek, O.A. Coso, S. Gutkind, S. Spiegel, Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate, Nature, 381 (1996) 800-803.
[21] S.M. Pitson, P.A. Moretti, J.R. Zebol, H.E. Lynn, P. Xia, M.A. Vadas, B.W. Wattenberg, Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation, EMBO J., 22 (2003) 5491-5500.
[22] S.M. Pitson, P. Xia, T.M. Leclercq, P.A. Moretti, J.R. Zebol, H.E. Lynn, B.W. Wattenberg, M.A. Vadas, Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling, J. Exp. Med., 201 (2005) 49-54.
[23] N. Igarashi, T. Okada, S. Hayashi, T. Fujita, S. Jahangeer, S.I. Nakamura, Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis, J. Biol. Chem., 278 (2003) 46832–46839.
[24] H. Liu, R.E. Toman, S. Goparaju, M. Maceyka, V.E. Nava, H. Sankala, S.G. Payne, M. Bektas, I. Ishii, J. Chun, S. Milstien, S. Spiegel, Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis, J. Biol. Chem., 278 (2003) 40330-40336.
[25] N.C. Hait, A. Bellamy, S. Milstien, T. Kordula, S. Spiegel, Sphingosine kinase type 2 activation by ERK-mediated phosphorylation, J. Biol. Chem., 282 (2007) 12058-12065.
[26] K. Mizugishi, T. Yamashita, A. Olivera, G.F. Miller, S. Spiegel, R.L. Proia, Essential role for sphingosine kinases in neural and vascular development, Mol. Cell Biol., 25 (2005) 11113-11121.
[27] A. Olivera, C. Eisner, Y. Kitamura, S. Dillahunt, L. Allende, G. Tuymetova, W. Watford, F. Meylan, S.C. Diesner, L. Li, J. Schnermann, R.L. Proia, J. Rivera, Sphingosine kinase 1 and sphingosine-1-phosphate receptor 2 are vital to recovery from anaphylactic shock in mice, J. Clin. Invest., 120 (2010) 1429-1240.
[28] M.L. Allende, T. Sasaki, H. Kawai, A. Olivera, Y. Mi, G. van Echten-Deckert, R. Hajdu, M. Rosenbach, C.A. Keohane, S. Mandala, S. Spiegel, R.L. Proia, Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720, J. Biol. Chem., 279 (2004) 52487-52492.
[29] K. Venkataraman, Y.M. Lee, J. Michaud, S. Thangada, Y. Ai, H.L. Bonkovsky, N.S. Parikh, C. Habrukowich, T. Hla, Vascular Endothelium As a Contributor of Plasma Sphingosine 1-Phosphate, Circ. Res., 102 (2008) 669-676.

[30] S.W. Paugh, S.G. Payne, S.E. Barbour, S. Milstien, S. Spiegel, The immunosuppressant FTY720 is phosphorylated by sphingosine kinase type 2, FEBS Lett., 554 (2003) 189-193.
[31] V. Brinkmann, M.D. Davis, C.E. Heise, R. Albert, S. Cottens, R. Hof, C. Bruns, E. Prieschl, T. Baumruker, P. Hiestand, C.A. Foster, M. Zollinger, K.R. Lynch, The immune modulator, FTY720, targets sphingosine 1-phosphate receptors, J. Biol. Chem., 277 (2002) 21453-21457.
[32] M. Matloubian, C.G. Lo, G. Cinamon, M.J. Lesneski, Y. Xu, V. Brinkmann, M.L. Allende, R.L. Proia, J.G. Cyster, Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1, Nature, 427 (2004) 355-360.
[33] M.L. Oo, S.H. Chang, S. Thangada, M.T. Wu, K. Rezaul, V. Blaho, S.I. Hwang, D.K. Han, T. Hla, Engagement of S1P-degradative mechanisms leads to vascular leak in mice, J Clin Invest, 121 (2011) 2290-2300.
[34] B. Zemann, B. Kinzel, M. Muller, R. Reuschel, D. Mechtcheriakova, N. Urtz, F. Bornancin, T. Baumruker, A. Billich, Sphingosine kinase type 2 is essential for lymphopenia induced by the immunomodulatory drug FTY720, Blood, 107 (2006) 1454-1458.
[35] A. Olivera, J. Rivera, Sphingolipids and the balancing of immune cell function: lessons from the mast cell, J. Immunol., 174 (2005) 1153-1158.
[36] M.J. Lee, J.R. Van Brocklyn, S. Thangada, C.H. Liu, A.R. Hand, R. Menzeleev, S. Spiegel, T. Hla, Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1, Science, 279 (1998) 1552-1555.
[37] Y. Liu, R. Wada, T. Yamashita, Y. Mi, C.X. Deng, J.P. Hobson, H.M. Rosenfeldt, V.E. Nava, S.S. Chae, M.J. Lee, C.H. Liu, T. Hla, S. Spiegel, R.L. Proia, Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation, J. Clin. Invest., 106 (2000) 951-961.
[38] B.J. McVerry, J.G. Garcia, Endothelial cell barrier regulation by sphingosine 1-phosphate, J. Cell. Biochem., 92 (2004) 1075-1085.
[39] P.A. Singleton, S.M. Dudek, E.T. Chiang, J.G. Garcia, Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by S1P1 receptor, PI3 kinase, Tiam1/Rac1, and alpha-actinin, FASEB J., 19 (2005) 1646-1656.
[40] T. Sanchez, A. Skoura, M.T. Wu, B. Casserly, E.O. Harrington, T. Hla, Induction of vascular permeability by the sphingosine-1-phosphate receptor-2 (S1P2R) and its downstream effectors ROCK and PTEN, Arterioscler. Thromb. Vasc. Biol., 27 (2007) 1312-1318.
[41] A.J. Maclennan, S.J. Benner, A. Andringa, A.H. Chaves, J.L. Rosing, R. Vesey, A.M. Karpman, S.A. Cronier, N. Lee, L.C. Erway, M.L. Miller, The S1P(2) sphingosine 1-phosphate receptor is essential for auditory and vestibular function, Hear. Res., 220 (2006) 38-48.
[42] D.R. Herr, N. Grillet, M. Schwander, R. Rivera, U. Muller, J. Chun, Sphingosine 1-phosphate (S1P) signaling is required for maintenance of hair cells mainly via activation of S1P2, J. Neurosci., 27 (2007) 1474-1478.

[43] M. Kono, I.A. Belyantseva, A. Skoura, G.I. Frolenkov, M.F. Starost, J.L. Dreier, D. Lidington, S.S. Bolz, T.B. Friedman, T. Hla, R.L. Proia, Deafness and stria vascularis defects in $S1P_2$ receptor null mice, J. Biol. Chem., 282 (2007) 10690-10696.
[44] J. Rivera, A.M. Gilfillan, Molecular regulation of mast cell activation, J. Allergy Clin. Immunol., 117 (2006) 1214-1225.
[45] S.J. Galli, M. Grimbaldeston, M. Tsai, Immunomodulatory mast cells: negative, as well as positive, regulators of immunity, Nat. Rev. Immunol., 8 (2008) 478-486.
[46] S.J. Galli, J. Kalesnikoff, M.A. Grimbaldeston, A.M. Piliponsky, C.M. Williams, M. Tsai, Mast cells as "tunable" effector and immunoregulatory cells: recent advances, Annu. Rev. Immunol., 23 (2005) 749-786.
[47] A.J. Melendez, F.B. Ibrahim, Antisense knockdown of sphingosine kinase 1 in human macrophages inhibits c5a receptor-dependent signal transduction, Ca^{2+} signals, enzyme release, cytokine production, and chemotaxis, J. Immunol., 173 (2004) 1596-1603.
[48] B. Zemann, N. Urtz, R. Reuschel, D. Mechtcheriakova, F. Bornancin, R. Badegruber, T. Baumruker, A. Billich, Normal neutrophil functions in sphingosine kinase type 1 and 2 knockout mice, Immunol. Lett., 109 (2007) 56-63.
[49] C.A. Oskeritzian, S. Milstien, S. Spiegel, Sphingosine-1-phosphate in allergic responses, asthma and anaphylaxis, Pharmacol. Ther., 115 (2007) 390-399.
[50] C.A. Oskeritzian, M.M. Price, N.C. Hait, D. Kapitonov, Y.T. Falanga, J.K. Morales, J.J. Ryan, S. Milstien, S. Spiegel, Essential roles of sphingosine-1-phosphate receptor 2 in human mast cell activation, anaphylaxis, and pulmonary edema, J. Exp. Med., 207 (2010) 465-474.
[51] N. Ancellin, C. Colmont, J. Su, Q. Li, N. Mittereder, S.S. Chae, S. Steffansson, G. Liau, T. Hla, Extracellular export of sphingosine kinase-1 enzyme: Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation, J. Biol. Chem., 277 (2002) 6667-6675.
[52] K. Venkataraman, S. Thangada, J. Michaud, M.L. Oo, Y. Ai, Y.M. Lee, M. Wu, N.S. Parikh, F. Khan, R.L. Proia, T. Hla, Extracellular export of sphingosine kinase-1a contributes to the vascular S1P gradient, Biochem. J., 397 (2006) 461-471.
[53] P. Mitra, C.A. Oskeritzian, S.G. Payne, M.A. Beaven, S. Milstien, S. Spiegel, Role of ABCC1 in export of sphingosine-1-phosphate from mast cells, Proc. Natl. Acad. Sci. U.S.A., 103 (2006) 16394-16399.
[54] Y. Yatomi, Y. Igarashi, L. Yang, N. Hisano, R. Qi, N. Asazuma, K. Satoh, Y. Ozaki, S. Kume, Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum, J. Biochem., 121 (1997) 969-973.
[55] E.V. Berdyshev, I.A. Gorshkova, N.G. JG, V. Natarajan, W.C. Hubbard, Quantitative analysis of sphingoid base-1-phosphates as bisacetylated derivatives by liquid chromatography-tandem mass spectrometry, Anal. Biochem., 339 (2005) 129-136.
[56] P. Hanel, P. Andreani, M.H. Graler, Erythrocytes store and release sphingosine 1-phosphate in blood, FASEB J., 21 (2007) 1202-1209.
[57] R. Pappu, S.R. Schwab, I. Cornelissen, J.P. Pereira, J.B. Regard, Y. Xu, E. Camerer, Y.W. Zheng, Y. Huang, J.G. Cyster, S.R. Coughlin, Promotion of lymphocyte egress into

blood and lymph by distinct sources of sphingosine-1-phosphate, Science, 316 (2007) 295-298.
[58] S.R. Schwab, J.P. Pereira, M. Matloubian, Y. Xu, Y. Huang, J.G. Cyster, Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients, Science, 309 (2005) 1735-1739.
[59] S.D. Ryu, H.S. Lee, H.Y. Suk, C.S. Park, O.H. Choi, Cross-linking of FcvarepsilonRI causes Ca(2+) mobilization via a sphingosine kinase pathway in a clathrin-dependent manner, Cell Calcium, (2008).
[60] H.S. Lee, C.S. Park, Y.M. Lee, H.Y. Suk, T.C. Clemons, O.H. Choi, Antigen-induced Ca(2+) mobilization in RBL-2H3 cells: Role of I(1,4,5)P(3) and S1P and necessity of I(1,4,5)P(3) production, Cell Calcium, 38 (2005) 581–592.
[61] L.B. Schwartz, Mast cells and basophils, Clin. Allergy Immunol., 16 (2002) 3-42.
[62] F.D. Finkelman, M.E. Rothenberg, E.B. Brandt, S.C. Morris, R.T. Strait, Molecular mechanisms of anaphylaxis: lessons from studies with murine models, J. Allergy Clin. Immunol., 115 (2005) 449-457.
[63] S.J. Galli, S. Nakae, M. Tsai, Mast cells in the development of adaptive immune responses, Nature Immunol., 6 (2005) 135-142.
[64] Y. Kurashima, J. Kunisawa, M. Higuchi, M. Gohda, I. Ishikawa, N. Takayama, M. Shimizu, H. Kiyono, Sphingosine 1-phosphate-mediated trafficking of pathogenic Th2 and mast cells for the control of food allergy, J. Immunol., 179 (2007) 1577-1585.
[65] J.A. Boyce, Mast cells and eicosanoid mediators: a system of reciprocal paracrine and autocrine regulation, Immunol. Rev., 217 (2007) 168-185.
[66] C.E. Brightling, P. Bradding, F.A. Symon, S.T. Holgate, A.J. Wardlaw, I.D. Pavord, Mast-cell infiltration of airway smooth muscle in asthma, N. Engl. J. Med., 346 (2002) 1699-1705.
[67] 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, R.A. Panettieri, Jr., Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma, FASEB J., 15 (2001) 1212-1214.
[68] H.M. Rosenfeldt, Y. Amrani, K.R. Watterson, K.S. Murthy, R.A. Panettieri, Jr., S. Spiegel, Sphingosine-1-phosphate stimulates contraction of human airway smooth muscle cells, FASEB J., 17 (2003) 1789-1799.
[69] P.S. Jolly, M. Bektas, K.R. Watterson, H. Sankala, S.G. Payne, S. Milstien, S. Spiegel, Expression of SphK1 impairs degranulation and motility of RBL-2H3 mast cells by desensitizing S1P receptors, Blood, 105 (2005) 4736-4742.
[70] F. Roviezzo, A. Di Lorenzo, M. Bucci, V. Brancaleone, V. Vellecco, M. De Nardo, D. Orlotti, R. De Palma, F. Rossi, B. D'Agostino, G. Cirino, Sphingosine-1-phosphate/sphingosine kinase pathway is involved in mouse airway hyper-responsiveness, Am. J. Respir. Cell. Mol. Biol., 36 (2007) 757-762.
[71] F. Roviezzo, F. Del Galdo, G. Abbate, M. Bucci, B. D'Agostino, E. Antunes, G. De Dominicis, L. Parente, F. Rossi, G. Cirino, R. De Palma, Human eosinophil chemotaxis and selective in vivo recruitment by sphingosine 1-phosphate, Proc. Natl. Acad. Sci. U.S.A., 101 (2004) 11170-11175.

[72] K. Takabe, S.W. Paugh, S. Milstien, S. Spiegel, "Inside-out" signaling of sphingosine-1-phosphate: therapeutic targets, Pharmacol. Rev., 60 (2008) 181-195.
[73] S.G. Payne, C.A. Oskeritzian, R. Griffiths, P. Subramanian, S.E. Barbour, C.E. Chalfant, S. Milstien, S. Spiegel, The immunosuppressant drug FTY720 inhibits cytosolic phospholipase A2 independently of sphingosine-1-phosphate receptors., Blood, 109 (2007) 1077-1085.
[74] E. Sawicka, C. Zuany-Amorim, C. Manlius, A. Trifilieff, V. Brinkmann, D.M. Kemeny, C. Walker, Inhibition of Th1- and th2-mediated airway inflammation by the sphingosine 1-phosphate receptor agonist FTY720, J. Immunol., 171 (2003) 6206-6214.
[75] M. Idzko, H. Hammad, M. van Nimwegen, M. Kool, T. Muller, T. Soullie, M.A. Willart, D. Hijdra, H.C. Hoogsteden, B.N. Lambrecht, Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function, J. Clin. Invest., 116 (2006) 2935-2944.
[76] K. Ahn, S. Takai, R. Pawankar, A. Kuramasu, H. Ohtsu, D. Kempuraj, H. Tomita, M. Iida, K. Matsumoto, A. Akasawa, M. Miyazaki, H. Saito, Regulation of chymase production in human mast cell progenitors, J. Allergy Clin. Immunol., 106 (2000) 321-328.
[77] A.S. Kirshenbaum, J.P. Goff, S.W. Kessler, J.M. Mican, K.M. Zsebo, D.D. Metcalfe, Effect of IL-3 and stem cell factor on the appearance of human basophils and mast cells from CD34+ pluripotent progenitor cells, J. Immunol., 148 (1992) 772-777.
[78] H. Mitsui, T. Furitsu, A.M. Dvorak, A.M. Irani, L.B. Schwartz, N. Inagaki, M. Takei, K. Ishizaka, K.M. Zsebo, S. Gillis, et al., Development of human mast cells from umbilical cord blood cells by recombinant human and murine c-kit ligand, Proc. Natl. Acad. Sci. U.S.A., 90 (1993) 735-739.
[79] H. Saito, M. Ebisawa, H. Tachimoto, M. Shichijo, K. Fukagawa, K. Matsumoto, Y. Iikura, T. Awaji, G. Tsujimoto, M. Yanagida, H. Uzumaki, G. Takahashi, K. Tsuji, T. Nakahata, Selective growth of human mast cells induced by Steel factor, IL-6, and prostaglandin E2 from cord blood mononuclear cells, J. Immunol., 157 (1996) 343-350.
[80] H. Ochi, W.M. Hirani, Q. Yuan, D.S. Friend, K.F. Austen, J.A. Boyce, T helper cell type 2 cytokine-mediated comitogenic responses and CCR3 expression during differentiation of human mast cells in vitro, J. Exp. Med., 190 (1999) 267-280.
[81] H.B. Andersen, M. Holm, T.E. Hetland, C. Dahl, S. Junker, P.O. Schiotz, H.J. Hoffmann, Comparison of short term in vitro cultured human mast cells from different progenitors - Peripheral blood-derived progenitors generate highly mature and functional mast cells, J. Immunol. Methods, 336 (2008) 166-174.
[82] T.C. Moon, E. Lee, S.H. Baek, M. Murakami, I. Kudo, N.S. Kim, J.M. Lee, H.K. Min, N. Kambe, H.W. Chang, Degranulation and cytokine expression in human cord blood-derived mast cells cultured in serum-free medium with recombinant human stem cell factor, Mol. Cells, 16 (2003) 154-160.
[83] C.A. Oskeritzian, Z. Wang, J.P. Kochan, M. Grimes, Z. Du, H.W. Chang, S. Grant, L.B. Schwartz, Recombinant human (rh)IL-4-mediated apoptosis and recombinant human IL-6-mediated protection of recombinant human stem cell factor-dependent human mast cells derived from cord blood mononuclear cell progenitors, J. Immunol., 163 (1999) 5105-5115.

[84] P. Conti, D. Kempuraj, K. Kandere, M. Di Gioacchino, M. Reale, R.C. Barbacane, M.L. Castellani, U. Mortari, W. Boucher, R. Letourneau, T.C. Theoharides, Interleukin-16 network in inflammation and allergy, Allergy Asthma Proc., 23 (2002) 103-108.
[85] Y. Shimizu, T. Suga, T. Maeno, F. Aoki, H. Tsukagoshi, T. Kawata, K. Sakai, T. Narita, T. Takahashi, S. Ishikawa, Y. Morishita, T. Nakajima, F. Hara, T. Miura, M. Kurabayashi, Functional expression of high-affinity receptor for immunoglobulin E on mast cells precedes that of tryptase during differentiation from human bone marrow-derived CD34 progenitors cultured in the presence of stem cell factor and interleukin-6, Clin. Exp. Allergy, 34 (2004) 917-925.
[86] C.A. Oskeritzian, W. Zhao, A.L. Pozez, N.M. Cohen, M. Grimes, L.B. Schwartz, Neutralizing endogenous IL-6 renders mast cells of the MCT type from lung, but not the MCTC type from skin and lung, susceptible to human recombinant IL-4-induced apoptosis, J. Immunol., 172 (2004) 593-600.
[87] J. Rivera, R.L. Proia, A. Olivera, The alliance of sphingosine-1-phosphate and its receptors in immunity, Nat. Rev. Immunol., 8 (2008) 753-763.
[88] S. Bagga, K.S. Price, D.A. Lin, D.S. Friend, K.F. Austen, J.A. Boyce, Lysophosphatidic acid accelerates the development of human mast cells, Blood, 104 (2004) 4080-4087.
[89] A. Olivera, Unraveling the complexities of sphingosine-1-phosphate function: The mast cell model, Prostaglandins Other Lipid Mediat., 86 (2008) 1-11.
[90] M.C. Sullards, J.C. Allegood, S. Kelly, E. Wang, C.A. Haynes, H. Park, Y. Chen, A.H. Merrill, Jr., Structure-specific, quantitative methods for analysis of sphingolipids by liquid chromatography-tandem mass spectrometry: "inside-out" sphingolipidomics, Methods Enzymol., 432 (2007) 83-115.
[91] L.C. Edsall, S. Spiegel, Enzymatic measurement of sphingosine 1-phosphate, Anal. Biochem., 272 (1999) 80-86.
[92] M. Kambe, N. Kambe, C.A. Oskeritzian, N. Schechter, L.B. Schwartz, IL-6 attenuates apoptosis, while neither IL-6 nor IL-10 affect the numbers or protease phenotype of fetal liver-derived human mast cells, Clin. Exp. Allergy, 31 (2001) 1077-1085.
[93] A.M. Irani, T.R. Bradford, C.L. Kepley, N.M. Schechter, L.B. Schwartz, Detection of MCT and MCTC types of human mast cells by immunohistochemistry using new monoclonal anti-tryptase and anti-chymase antibodies, J Histochem Cytochem, 37 (1989) 1509-1515.
[94] C.A. Oskeritzian, W. Zhao, H.K. Min, H.Z. Xia, A. Pozez, J. Kiev, L.B. Schwartz, Surface CD88 functionally distinguishes the MCTC from the MCT type of human lung mast cell, J. Allergy Clin. Immunol., 115 (2005) 1162-1168.
[95] W. Zhao, C.A. Oskeritzian, A.L. Pozez, L.B. Schwartz, Cytokine production by skin-derived mast cells: endogenous proteases are responsible for degradation of cytokines, J. Immunol., 175 (2005) 2635-2642.
[96] H. Rosen, P. Gonzalez-Cabrera, D. Marsolais, S. Cahalan, A.S. Don, M.G. Sanna, Modulating tone: the overture of S1P receptor immunotherapeutics, Immunol Rev, 223 (2008) 221-235.

[97] C. Dahl, H. Saito, H.V. Nielsen, P.O. Schiotz, The establishment of a combined serum-free and serum-supplemented culture method of obtaining functional cord blood-derived human mast cells, J. Immunol. Methods, 262 (2002) 137-143.
[98] M. Yamaguchi, H. Azuma, M. Fujihara, H. Hamada, H. Ikeda, Generation of a considerable number of functional mast cells with a high basal level of FcepsilonRI expression from cord blood CD34+ cells by co-culturing them with bone marrow stromal cell line under serum-free conditions, Scand. J .Immunol., 65 (2007) 581-588.
[99] M.A. Beaven, D.L. Aiken, E. Woldemussie, A.H. Soll, Changes in histamine synthetic activity, histamine content and responsiveness to compound 48/80 with maturation of rat peritoneal mast cells, J. Pharmacol. Exp. Ther., 224 (1983) 620-626.
[100] M.A. Lowman, P.H. Rees, R.C. Benyon, M.K. Church, Human mast cell heterogeneity: histamine release from mast cells dispersed from skin, lung, adenoids, tonsils, and colon in response to IgE-dependent and nonimmunologic stimuli, J. Allergy Clin. Immunol., 81 (1988) 590-597.
[101] S.S. Craig, N.M. Schechter, L.B. Schwartz, Ultrastructural analysis of human T and TC mast cells identified by immunoelectron microscopy, Lab. Invest., 58 (1988) 682-691.
[102] C. Hornuss, R. Hammermann, M. Fuhrmann, U.R. Juergens, K. Racke, Human and rat alveolar macrophages express multiple EDG receptors, Eur J Pharmacol, 429 (2001) 303-308.
[103] M. Fueller, A. Wang de, G. Tigyi, W. Siess, Activation of human monocytic cells by lysophosphatidic acid and sphingosine-1-phosphate, Cell Signal, 15 (2003) 367-375.
[104] C.Q. Duong, S.M. Bared, A. Abu-Khader, C. Buechler, A. Schmitz, G. Schmitz, Expression of the lysophospholipid receptor family and investigation of lysophospholipid-mediated responses in human macrophages, Biochim. Biophys. Acta, 1682 (2004) 112-119.
[105] C.C. Chen, M.A. Grimbaldeston, M. Tsai, I.L. Weissman, S.J. Galli, Identification of mast cell progenitors in adult mice, Proc. Natl. Acad. Sci. U.S.A., 102 (2005) 11408-11413.
[106] L.P. Vlasenko, A.J. Melendez, A critical role for sphingosine kinase in anaphylatoxin-induced neutropenia, peritonitis, and cytokine production in vivo, J. Immunol., 174 (2005) 6456-6461.
[107] J. Mallen-St Clair, C.T. Pham, S.A. Villalta, G.H. Caughey, P.J. Wolters, Mast cell dipeptidyl peptidase I mediates survival from sepsis, J. Clin. Invest., 113 (2004) 628-634.
[108] M. Metz, A.M. Piliponsky, C.C. Chen, V. Lammel, M. Abrink, G. Pejler, M. Tsai, S.J. Galli, Mast cells can enhance resistance to snake and honeybee venoms, Science, 313 (2006) 526-530.
[109] Z. Orinska, M. Maurer, F. Mirghomizadeh, E. Bulanova, M. Metz, N. Nashkevich, F. Schiemann, J. Schulmistrat, V. Budagian, J. Giron-Michel, E. Brandt, R. Paus, S. Bulfone-Paus, IL-15 constrains mast cell-dependent antibacterial defenses by suppressing chymase activities, Nat. Med., 13 (2007) 927-934.
[110] M. Maurer, J. Wedemeyer, M. Metz, A.M. Piliponsky, K. Weller, D. Chatterjea, D.E. Clouthier, M.M. Yanagisawa, M. Tsai, S.J. Galli, Mast cells promote homeostasis by limiting endothelin-1-induced toxicity, Nature, 432 (2004) 512-516.

[111] G.H. Caughey, Mast cell tryptases and chymases in inflammation and host defense, Immunol. Rev., 217 (2007) 141-154.
[112] A.S. Kirshenbaum, E. Swindle, M. Kulka, Y. Wu, D.D. Metcalfe, Effect of lipopolysaccharide (LPS) and peptidoglycan (PGN) on human mast cell numbers, cytokine production, and protease composition, BMC Immunol, 9 (2008) 45-58.
[113] H. Nishio, S. Takai, M. Miyazaki, H. Horiuchi, M. Osawa, K. Uemura, K. Yoshida, M. Mukaida, Y. Ueno, K. Suzuki, Usefulness of serum mast cell-specific chymase levels for postmortem diagnosis of anaphylaxis, Int. J. Legal Med., 119 (2005) 331-334.
[114] F.E. Simons, A.J. Frew, I.J. Ansotegui, B.S. Bochner, D.B. Golden, F.D. Finkelman, D.Y. Leung, J. Lotvall, G. Marone, D.D. Metcalfe, U. Muller, L.J. Rosenwasser, H.A. Sampson, L.B. Schwartz, M. van Hage, A.F. Walls, Risk assessment in anaphylaxis: current and future approaches, J. Allergy Clin. Immunol., 120 (2007) S2-24.
[115] H.Y. Kim, R.H. DeKruyff, D.T. Umetsu, The many paths to asthma: phenotype shaped by innate and adaptive immunity, Nat Immunol, 11 (2010) 577-584.
[116] S. Spiegel, S. Milstien, The outs and the ins of sphingosine-1-phosphate in immunity, Nat Rev Immunol, 11 (2011) 403-415.
[117] Y. Chiba, K. Suzuki, E. Kurihara, M. Uechi, H. Sakai, M. Misawa, Sphingosine-1-phosphate aggravates antigen-induced airway inflammation in mice, Open Respir Med J, 4 (2010) 82-85.
[118] P. Puneet, C.T. Yap, L. Wong, Y. Lam, D.R. Koh, S. Moochhala, J. Pfeilschifter, A. Huwiler, A.J. Melendez, SphK1 regulates proinflammatory responses associated with endotoxin and polymicrobial sepsis, Science, 328 (2010) 1290-1294.
[119] P.N. Pushparaj, J. Manikandan, H.K. Tay, C. H'Ng S, S.D. Kumar, J. Pfeilschifter, A. Huwiler, A.J. Melendez, Sphingosine kinase1 is pivotal for Fc{epsilon}RI-mediated mast cell signaling and functional responses in vitro and in vivo, J. Immunol., 183 (2009) 221-227.
[120] S.W. Paugh, B.S. Paugh, M. Rahmani, D. Kapitonov, J.A. Almenara, T. Kordula, S. Milstien, J.K. Adams, R.E. Zipkin, S. Grant, S. Spiegel, A selective sphingosine kinase 1 inhibitor integrates multiple molecular therapeutic targets in human leukemia, Blood, 112 (2008) 1382-1391.
[121] A. Olivera, J. Rivera, An emerging role for the lipid mediator sphingosine-1-phosphate in mast cell effector function and allergic disease, Adv Exp Med Biol, 716 (2011) 123-142.
[122] C.M. Williams, S.J. Galli, Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice, J. Exp. Med., 192 (2000) 455-462.
[123] M. Yu, M. Tsai, S.Y. Tam, C. Jones, J. Zehnder, S.J. Galli, Mast cells can promote the development of multiple features of chronic asthma in mice, J. Clin. Invest., 116 (2006) 1633-1641.
[124] E.M. Campbell, I.F. Charo, S.L. Kunkel, R.M. Strieter, L. Boring, J. Gosling, N.W. Lukacs, Monocyte chemoattractant protein-1 mediates cockroach allergen-induced bronchial hyperreactivity in normal but not CCR2-/- mice: the role of mast cells, J Immunol, 163 (1999) 2160-2167.

[125] S. Klemm, J. Gutermuth, L. Hultner, T. Sparwasser, H. Behrendt, C. Peschel, T.W. Mak, T. Jakob, J. Ruland, The Bcl10-Malt1 complex segregates Fc epsilon RI-mediated nuclear factor kappa B activation and cytokine production from mast cell degranulation, J. Exp. Med., 203 (2006) 337-347.
[126] G. Gomez, C. Gonzalez-Espinosa, S. Odom, G. Baez, M.E. Cid, J.J. Ryan, J. Rivera, Impaired FcepsilonRI-dependent gene expression and defective eicosanoid and cytokine production as a consequence of Fyn deficiency in mast cells, J. Immunol., 175 (2005) 7602-7610.
[127] W.Q. Lai, H.H. Goh, Z. Bao, W.S. Wong, A.J. Melendez, B.P. Leung, The role of sphingosine kinase in a murine model of allergic asthma, J. Immunol., 180 (2008) 4323-4329.
[128] R.V. Haberberger, C. Tabeling, S. Runciman, B. Gutbier, P. Konig, M. Andratsch, H. Schutte, N. Suttorp, I. Gibbins, M. Witzenrath, Role of sphingosine kinase 1 in allergen-induced pulmonary vascular remodeling and hyperresponsiveness, J Allergy Clin Immunol, 124 (2009) 933-941 e931-939.
[129] Y. Chiba, H. Takeuchi, H. Sakai, M. Misawa, SKI-II, an inhibitor of sphingosine kinase, ameliorates antigen-induced bronchial smooth muscle hyperresponsiveness, but not airway inflammation, in mice, J Pharmacol Sci, 114 (2010) 304-310.
[130] K. Takeda, E. Hamelmann, A. Joetham, L.D. Shultz, G.L. Larsen, C.G. Irvin, E.W. Gelfand, Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice, J. Exp. Med., 186 (1997) 449-454.
[131] S. Nakae, C. Lunderius, L.H. Ho, B. Schafer, M. Tsai, S.J. Galli, TNF can contribute to multiple features of ovalbumin-induced allergic inflammation of the airways in mice, J Allergy Clin Immunol, 119 (2007) 680-686.
[132] M. Yu, M.R. Eckart, A.A. Morgan, K. Mukai, A.J. Butte, M. Tsai, S.J. Galli, Identification of an IFN-gamma/mast cell axis in a mouse model of chronic asthma, J Clin Invest, (2011).
[133] J. Kalesnikoff, S.J. Galli, New developments in mast cell biology, Nat. Immunol., 9 (2008) 1215-1223.
[134] S. Klemm, J. Ruland, Inflammatory signal transduction from the Fc epsilon RI to NF-kappa B, Immunobiology, 211 (2006) 815-820.
[135] T. Nishiuma, Y. Nishimura, T. Okada, E. Kuramoto, Y. Kotani, S. Jahangeer, S. Nakamura, Inhalation of sphingosine kinase inhibitor attenuates airway inflammation in asthmatic mouse model, Am. J. Physiol. Lung Cell. Mol. Physiol., 294 (2008) L1085-L1093.
[136] F. Roviezzo, B. D'Agostino, V. Brancaleone, L. De Gruttola, M. Bucci, G. De Dominicis, D. Orlotti, E. D'Aiuto, R. De Palma, F. Rossi, R. Sorrentino, G. Cirino, Systemic administration of sphingosine-1-phosphate increases bronchial hyperresponsiveness in the mouse, Am. J. Respir. Cell Mol. Biol., 42 (2010) 572-577.
[137] J.M. Brewer, M. Conacher, C.A. Hunter, M. Mohrs, F. Brombacher, J. Alexander, Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling, J Immunol, 163 (1999) 6448-6454.

[138] Y. Kono, T. Nishiuma, T. Okada, K. Kobayashi, Y. Funada, Y. Kotani, S. Jahangeer, S. Nakamura, Y. Nishimura, Sphingosine kinase 1 regulates mucin production via ERK phosphorylation, Pulm Pharmacol Ther, 23 (2010) 36-42.
[139] K.M. Heutinck, I.J. ten Berge, C.E. Hack, J. Hamann, A.T. Rowshani, Serine proteases of the human immune system in health and disease, Mol Immunol, 47 (2010) 1943-1955.
[140] M. Bektas, M.L. Allende, B.G. Lee, W. Chen, M.J. Amar, A.T. Remaley, J.D. Saba, R.L. Proia, Sphingosine 1-phosphate lyase deficiency disrupts lipid homeostasis in liver, J Biol Chem, 285 (2010) 10880-10889.
[141] Y.A. Hannun, C. Luberto, K.M. Argraves, Enzymes of sphingolipid metabolism: from modular to integrative signaling, Biochemistry, 40 (2001) 4893-4903.
[142] L.G. Ledgerwood, G. Lal, N. Zhang, A. Garin, S.J. Esses, F. Ginhoux, M. Merad, H. Peche, S.A. Lira, Y. Ding, Y. Yang, X. He, E.H. Schuchman, M.L. Allende, J.C. Ochando, J.S. Bromberg, The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral tissue T lymphocytes into afferent lymphatics, Nat Immunol, 9 (2008) 42-53.
[143] M. Kitano, T. Hla, M. Sekiguchi, Y. Kawahito, R. Yoshimura, K. Miyazawa, T. Iwasaki, H. Sano, Sphingosine 1-phosphate/sphingosine 1-phosphate receptor 1 signaling in rheumatoid synovium: Regulation of synovial proliferation and inflammatory gene expression, Arthritis Rheum., 54 (2006) 742-753.
[144] W.Q. Lai, A.W. Irwan, H.H. Goh, H.S. Howe, D.T. Yu, R. Valle-Onate, I.B. McInnes, A.J. Melendez, B.P. Leung, Anti-inflammatory effects of sphingosine kinase modulation in inflammatory arthritis, J. Immunol., 181 (2008) 8010-8017.
[145] C.E. Brightling, A.J. Ammit, D. Kaur, J.L. Black, A.J. Wardlaw, J.M. Hughes, P. Bradding, The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle, Am J Respir Crit Care Med, 171 (2005) 1103-1108.
[146] A. Zanini, A. Chetta, M. Saetta, S. Baraldo, R. D'Ippolito, A. Castagnaro, M. Neri, D. Olivieri, Chymase-positive mast cells play a role in the vascular component of airway remodeling in asthma, J Allergy Clin Immunol, 120 (2007) 329-333.
[147] H.H. Mwamtemi, K. Koike, T. Kinoshita, S. Ito, S. Ishida, Y. Nakazawa, Y. Kurokawa, K. Shinozaki, K. Sakashita, K. Takeuchi, M. Shiohara, T. Kamijo, Y. Yasui, A. Ishiguro, Y. Kawano, K. Kitano, H. Miyazaki, T. Kato, S. Sakuma, A. Komiyama, An increase in circulating mast cell colony-forming cells in asthma, J Immunol, 166 (2001) 4672-4677.
[148] P. Jolly, H. Rosenfeldt, S. Milstien, S. Spiegel, The roles of sphingosine-1-phosphate in asthma, Mol. Immunol., 38 (2002) 1239-1245.
[149] M. Idzko, E. Panther, S. Corinti, A. Morelli, D. Ferrari, Y. Herouy, S. Dichmann, M. Mockenhaupt, P. Gebicke-Haerter, F. Di Virgilio, G. Girolomoni, J. Norgauer, Sphingosine 1-phosphate induces chemotaxis of immature and modulates cytokine-release in mature human dendritic cells for emergence of Th2 immune responses, FASEB J., 16 (2002) 625-627.

[150] T. Walzer, L. Chiossone, J. Chaix, A. Calver, C. Carozzo, L. Garrigue-Antar, Y. Jacques, M. Baratin, E. Tomasello, E. Vivier, Natural killer cell trafficking in vivo requires a dedicated sphingosine 1-phosphate receptor, Nat. Immunol., 8 (2007) 1337-1344.
[151] J.F. Lee, S. Gordon, R. Estrada, L. Wang, D.L. Siow, B.W. Wattenberg, D. Lominadze, M.J. Lee, Balance of S1P1 and S1P2 signaling regulates peripheral microvascular permeability in rat cremaster muscle vasculature, Am. J. Physiol. Heart Circ. Physiol., 296 (2009) H33-H42.
[152] J.N. Lorenz, L.J. Arend, R. Robitz, R.J. Paul, A.J. MacLennan, Vascular dysfunction in S1P2 sphingosine 1-phosphate receptor knockout mice, Am J Physiol Regul Integr Comp Physiol, 292 (2007) R440-446.
[153] B. Huang, Z. Lei, G.M. Zhang, D. Li, C. Song, B. Li, Y. Liu, Y. Yuan, J. Unkeless, H. Xiong, Z.H. Feng, SCF-mediated mast cell infiltration and activation exacerbate the inflammation and immunosuppression in tumor microenvironment, Blood, 112 (2008) 1269-1279.
[154] C.J. Meininger, H. Yano, R. Rottapel, A. Bernstein, K.M. Zsebo, B.R. Zetter, The c-kit receptor ligand functions as a mast cell chemoattractant, Blood, 79 (1992) 958-963.
[155] G. Nilsson, J.H. Butterfield, K. Nilsson, A. Siegbahn, Stem cell factor is a chemotactic factor for human mast cells, J Immunol, 153 (1994) 3717-3723.
[156] M.J. Lee, S. Thangada, K.P. Claffey, N. Ancellin, C.H. Liu, M. Kluk, M. Volpi, R.I. Sha'afi, T. Hla, Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate, Cell, 99 (1999) 301-312.
[157] E. Camerer, J.B. Regard, I. Cornelissen, Y. Srinivasan, D.N. Duong, D. Palmer, T.H. Pham, J.S. Wong, R. Pappu, S.R. Coughlin, Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice, J. Clin. Invest., 119 (2009) 1871-1879.

APPENDIX A

Publications

1. **Price MM**, Oskeritzian CA, Falanga YT, Harikumar KB, Allegood JC, Alvarez SE, Conrad DH, Ryan JJ, Milstien S and Spiegel S. A specific sphingosine kinase 1 inhibitor attenuates airway hyperresponsiveness and inflammation in a mast cell-dependent mouse model of allergic asthma. *In preparation.*

2. Gomez L, Paillard M, **Price MM**, Chen Q, Teixeira G, Spiegel S, and Lesnefsky J. A novel role for sphingosine kinase 2 in mPTP mediated cell survival during cardioprotection. *In preparation.*

3. Strub, GM, Paillard M, Liang J, Gomez L, Allegood JC, Hait NC, Maceyka M, **Price MM**, Chen Q, Simpson DC, Kordula T, Milstien S, Lesnefsky EJ, Spiegel S. Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. *FASEB J. 2011 Feb;25(2):600-12.*

4. Oskeritzian CA, **Price MM**, Hait NC, Kapitonov D, Falanga Y, Morales JK, Ryan JJ, Milstien S and Spiegel S. Essential role of sphingosine-1-phosphate receptor 2 in human mast cell activation and anaphylaxis. *J Ex Med 2010 Mar 15;207(3):465-74.*

5. **Price MM**, Kapitonov D, Allegood J, Milstien S, Oskeritzian CA, Spiegel S. Sphingosine-1-phosphate induces development of functionally mature chymase-expressing human mast cells from hematopoietic progenitors. *FASEB J. 2009 Oct;23(10):3506-15.*

6. **Price MM**, Oskeritzian CA, Milstien S, Spiegel S. Sphingosine-1-phosphate synthesis and functions in mast cells. *Future Lipidol. 2008 Dec 1;3(6):665-674*.

7. Oskeritzian CA, Alvarez SE, Hait NC, **Price MM**, Milstien S, Spiegel S. Distinct roles of sphingosine kinases 1 and 2 in human mast-cell functions. *Blood. 2008 Apr 15;111(8):4193-200.*

VITA

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