S1P₄ Regulates Passive Systemic Anaphylaxis in Mice but Is Dispensable for Canonical IgE-Mediated Responses in Mast Cells

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Abstract: Mast cells are key players in the development of inflammatory allergic reactions. Cross-linking of the high-affinity receptor for IgE (FcεRI) on mast cells leads to the generation and secretion of the sphingolipid mediator, sphingosine-1-phosphate (S1P) which is able, in turn, to transactivate its receptors on mast cells. Previous reports have identified the expression of two of the five receptors for S1P on mast cells, S1P₁ and S1P₂, with functions in FcεRI-mediated chemotaxis and degranulation, respectively. Here, we show that cultured mouse mast cells also express abundant message for S1P₄. Genetic deletion of S1pr4 did not affect the differentiation of bone marrow progenitors into mast cells or the proliferation of mast cells in culture. A comprehensive characterization of IgE-mediated responses in S1P₄-deficient bone marrow-derived and peritoneal mouse mast cells indicated that this receptor is dispensable for mast cell degranulation, cytokine/chemokine production and FcεRI-mediated chemotaxis in vitro. However, interleukin-33 (IL-33)-mediated enhancement of IgE-induced degranulation was reduced in S1P₄-deficient peritoneal mast cells, revealing a potential negative regulatory role for S1P₄ in an IL-33-rich environment. Surprisingly, genetic deletion of S1pr4 resulted in exacerbation of passive systemic anaphylaxis to IgE/anti-IgE in mice, a phenotype likely related to mast cell-extrinsic influences, such as the high circulating levels of IgE in these mice which increases FcεRI expression and consequently the extent of the response to FcεRI engagement. Thus, we provide evidence that S1P₄ modulates anaphylaxis in an unexpected manner that does not involve regulation of mast cell responsiveness to IgE stimulation.

Keywords: S1P₄; S1pr4; sphingosine-1-phosphate; mast cell; anaphylaxis; chemotaxis; mediator release; degranulation; IL-33

1. Introduction

Sphingosine-1-phosphate (S1P) is a sphingolipid mediator that critically regulates multiple cellular processes including proliferation, survival, chemotaxis and immune regulation. S1P elicits these functions by binding five known G-protein coupled receptors (GPCRs), designated as S1P₁-₅, or by acting on its intracellular targets [1]. Unlike S1P₁-₃ receptors which are expressed ubiquitously, S1P₄ exhibits preferential expression in lymphoid and hematopoietic organs and cells [2]. S1P₄ has been reported to regulate neutrophil counts in circulation [3] and trafficking [4,5], dendritic cell function [6] and to modulate certain lymphocyte functions [6–9]. Global genetic deletion of S1P₄ in mice results in...
elevated serum IgE levels, enhanced T helper 2 (Th2)-and Th17-dominated immune responses and diminished Th1-responses [6]. However, the role of S1P4 in immune cells is still not well understood.

Mast cells are tissue-resident cells commonly associated with Th2 immediate hypersensitivity reactions. Mast cells recognize IgE-bound antigen (Ag) through the high-affinity receptor for IgE (FcεRI) expressed at the plasma membrane. Aggregation of FcεRI by IgE/Ag initiates signaling cascades leading to the release of both early and late mediators that cause immediate allergic reactions and contribute to chronic inflammation [10,11]. In addition to FcεRI, mast cells express other cell surface receptors that allow mast cells to respond to signals in the microenvironment that modulate FcεRI-mediated responses [12,13]. These signals and their receptors may be significant factors in the susceptibility to, or severity of, anaphylaxis in allergic individuals [14].

Elevated S1P in inflamed tissues or produced endogenously by mast cells is considered one of the factors regulating FcεRI-induced responses [15,16]. Previous studies in mast cells have indicated that following FcεRI-mediated activation, S1P is generated and induces ligand-dependent “transactivation” of S1P1 and S1P2 receptors expressed on these cells [17]. S1P1 transactivation is reported to mediate the migration of mast cells toward Ag [17,18]. S1P2 enhances FcεRI-induced degranulation, although its contributions to degranulation may depend on the type of mast cell studied and culture conditions used [17–19].

Here, we show that mouse mast cells express S1P4 receptor in addition to S1P1 and S1P2. Based on the Th2-skewed phenotype of mice lacking S1P4 and the role of S1P in regulating mast cell responses, we sought to better understand the role of S1P4 in FcεRI-mediated stimulation and allergic responses. As it will be shown, global genetic deletion of S1pr4 resulted in exacerbation of IgE-mediated systemic anaphylaxis, although S1P4 was dispensable for normal FcεRI-mediated activation in S1pr4-deficient cultured mast cells. Our data suggest that the enhanced anaphylaxis in mice lacking S1P4 is not directly linked to intrinsic alterations in mast cells, but instead may be secondary to the higher levels of IgE in vivo, which would promote membrane expression of FcεRI and thus a stronger stimulus. Our experiments also revealed an unexpected role for S1P4 in the negative regulation of innate mast cell degranulation in response to co-stimulation with IgE/Ag and IL-33 [20].

2. Results

2.1. S1P4 Is Expressed in Mast Cells

Mast cells express mRNA coding for S1P1 and S1P2, receptors known to contribute to FcεRI-mediated mast cell responses [16,17]. We found that, in addition to the expression of S1pr1 and S1pr2, mouse mast cells contained mRNA for S1P4 (but not S1P3 or S1P5) at comparable or higher relative levels than for S1P2 (Figure 1). Since S1P4 may play redundant, antagonistic or unique roles in mast cells compared to the other S1P receptors, we reasoned it was possible that deletion of this receptor could affect the expression of the other S1P receptors, which could in turn skew the functional outcome. However, the mRNA expression of the other S1P receptors in mast cells was not altered by S1pr4 deficiency (Figure S1A, open bars). As the role of S1P4 in mast cells has not been examined, we next sought to characterize the growth of mouse mast cells obtained from S1pr4-deficient mice.
presence of SCF (45.094 ± 0.862\% in S1pr4−/− mast cells (BMMC, left) and peritoneal-derived mast cells (PDMC, right). Plots represent the mean ± SE (or SD for PDMC) of data pooled from 7 independent BMMC or 2 PDMC cultures. ND: Not detected.

2.2. Maturation and Proliferation of S1pr4-Deficient Mast Cells In Vitro

Bone marrow-derived mast cells (BMMC) from S1pr4-deficient mice cultured in the presence of IL-3 and stem cell factor (SCF) differentiated with comparable kinetics to S1pr4+/+ BMMC, as evidenced by the increasing appearance over time in culture of a population of FcεRI and CD117 (KIT) double-positive cells, the characteristic cell surface markers of mast cells (Figure S1B top panel, and C). In addition, the absence of S1P4 had no significant effect on the total numbers of mast cells in culture (Figure S1B, bottom panel). Similar to BMMC, the growth and expansion of S1pr4-deficient mast cells obtained from peritoneal exudates (peritoneal-derived mast cells or PDMC), which are considered more mature than BMMC [21–23] and with functional characteristics of innate mast cells [24], was no different from S1pr4+/+ cells (Figure S1D). These data indicate that S1P4 receptor expression is not required for either the expansion of mature mast cells from the peritoneum or the in vitro differentiation/expansion of mast cells from bone marrow precursors.

2.3. Degranulation, Cytokine and Chemokine Responses in S1pr4-Deficient Mast Cells In Vitro

We next tested whether S1P4 might modulate degranulation in response to FcεRI stimulation in BMMC and PDMC cultures. Our analysis showed that S1pr4-deficient cultures bound comparable levels of IgE on the cell surface (Figure S2A–C) and showed similar degranulation to that of S1pr4+/+ cultures in response to all concentrations of Ag tested (Figure 2A). S1pr4-deficient and S1pr4+/+ cells also exhibited identical responses to pharmacological stimulation by thapsigargin, an inhibitor of Ca2+ uptake into the ER that causes increased cytosolic Ca2+ accumulation (Figure 2B). Further, degranulation in response to IgE/Ag was also similar in both groups of cultures in the presence of SCF (45.094 ± 0.862\% in S1pr4+/+ and 42.443 ± 0.804\% in S1pr4−/− stimulated with 25 ng/mL Ag + 20 ng/mL SCF), which is known to synergize with FcεRI-mediated responses [14,25].

Cultured PDMC degranulate in response to a diverse group of cationic compounds, referred to as “mast cell secretagogues” such as substance P and compound 48/80, through a class of GPCRs known as Mas-related gene (Mrg) receptors expressed on these cells [24,26,27]. Degranulation of S1pr4-deficient PDMC in response to 5 to 50 µg/mL of compound 48/80 was indistinguishable from S1pr4+/+ mast cells (27.356 ± 8.997\% in S1pr4+/+ and 20.334 ± 4.831\% in S1pr4−/− stimulated with 10 µg/mL compound 48/80). In contrast, FcεRI-induced degranulation in the presence of IL-33, a cytokine that orchestrates a variety of allergic inflammatory conditions through innate immune cells [20,28] and potentiates FcεRI mediated mast cell responses [25,29], was further potentiated in S1pr4-deficient PDMC compared to S1pr4+/+ (Figure 2C).

These effects were not observed in BMMC (15 ± 2\% in S1pr4+/+ and 17 ± 2\% in S1pr4−/− stimulated with 25 ng/mL Ag and 1 ng/mL IL-33). Overall, the data indicates that S1P4 is dispensable
for degranulation initiated through either FcεRI or the Mas-related GPCRs but diminishes the potentiating effects of IL-33 on FcεRI-mediated responses in innate mast cells.

Mast cells also generate a variety of cytokines and chemokines following activation of FcεRI as a result of enhanced gene expression, with IL-6 and TNF-α representing two of the most abundant and best characterized cytokines produced by BMMC [21,30,31]. S1pr4 deficiency did not significantly alter FcεRI-induced transcription of IL-6 and TNF-α (relative Il6 expression was 0.05609 ± 0.01661% in S1pr4+/+ and 0.0493 ± 0.01077% in S1pr4−/− stimulated with 25 ng/mL) or their release into the media (Figure 2D,E) at any of the concentrations of Ag tested. To determine whether transactivation of S1P4 might regulate the expression of other cytokines or chemokines induced by FcεRI stimulation, as has been reported for S1P2, we performed a qPCR array to examine the relative expression levels of 84 key cytokines and chemokines with critical roles in various immune responses. From those whose expression was highest and/or induced by FcεRI activation, three cytokines (IL-2, IL-5 and IL-9) and three chemokines (Ccl12, Ccl22, Ccl24) exhibited relative expression levels that were on average ≥2-fold higher in stimulated S1pr4−/− BMMC than in stimulated S1pr4+/+ controls (Table S1). The relative expression levels for several of the genes were highly variable between replicates probably due to the low abundance of these transcripts (100–1000 fold less abundant than those for IL-6) and the same was true, in general, for the fold change in expression between stimulated S1pr4−/− and S1pr4+/+ cells in separate cultures and thus, to confirm the measurements on these cytokines/chemokines, we employed droplet digital PCR (ddPCR) technology for enhanced sensitivity and reproducibility [32]. We also determined IL-6 expression by ddPCR for comparison, as a negative control, and Ccl11 since this was one of the most highly upregulated messages in both S1pr4−/− and S1pr4+/+ after stimulation and the average fold increase in S1pr4−/− compared to S1pr4+/+ cells was nearly 2 fold (Table S1). Using ddPCR we were able to accurately quantify those low and high abundance transcripts to definitively conclude that activated S1pr4−/− BMMC have indistinguishable responses to those of S1pr4+/+ cells in terms of cytokine/chemokine mRNA expression (Figure 2F,G). Only IL-2 and IL-5 showed a trend towards higher expression in stimulated S1pr4−/− although this was not statistically significant. Altogether our data demonstrates no role for S1P4 expression in the normal functioning of FcεRI-mediated responses in vitro.

Figure 2. Cont.
Figure 2. Analysis of degranulation, cytokine and chemokine responses in primary S1P4-deficient mast cells. (A–C) Degranulation response to antigen stimulation (A, B) or to pharmacological stimulation with thapsigargin (B). Mast cells from S1pr4+/+ (solid) and S1pr4−/− mice (open) were grown in the presence of stem cell factor (SCF) and recombinant mIL-3 for 6–7 weeks (BMMC) or 14 days (PDMC) and sensitized with 100 ng/mL anti-dinitrophenyl (DNP)-IgE in cytokine-free media for 14 h. Degranulation was assessed by measuring the release of β-hexosaminidase into the media after 30 min of stimulation with the indicated concentrations of DNP (antigen; Ag) (A), 1 μM thapsigargin (B), or antigen in addition to 1 ng/mL recombinant IL-33 (C). Data represent the mean ± SE of results pooled from 4–8 independent cultures. (D,E) BMMC from S1pr4+/+ (solid bars) and S1pr4−/− mice (open bars) were sensitized overnight with 100 ng/mL anti-DNP IgE in cytokine-free media. Cells were washed, stimulated with the indicated concentrations of Ag and the amounts of IL-6 and TNF-α (E) secreted into the media measured by ELISA at 0 h post-stimulation. The limit of detection for IL-6 and TNF-α quantitation by ELISA are shown by a dotted line in panels C and D at 0.0003 ng/mL and 0.000072 ng/mL, respectively. Data is pooled from 4 independent cultures. (F,G) Validation by ddPCR of the normalized relative expression of select chemokines (F) and cytokines (G) identified as being variably upregulated in S1pr4+/+ and S1pr4−/− BMMC cultures following Ag stimulation. Relative expression of Il6 is included for comparison. Data show mean ± SE of values obtained from at least seven independent cultures of BMMC for each genotype. All comparisons between S1pr4+/+ and S1pr4−/− cells were found to be not statistically significant unless otherwise indicated. * p < 0.05.

2.4. Regulation of Mast Cell Chemotaxis by S1P4

Various S1P receptors modulate chemotaxis in a variety of cell types [33]. In mast cells, S1P1 mediates migration toward Ag while overexpression of S1P2 appears to antagonize this process [17,18]. Transwell migration of BMMC towards Ag (Figure 3A) or towards SCF (Figure 3B) were minimally affected by the absence of S1P4 expression. Addition of 100 nM S1P did enhance the number of S1pr4+/+ BMMCs exhibiting specific migration towards Ag but had no effect on S1pr4-deficient BMMC (Figure 3A). Thus, under these specific conditions, there was a trend towards reduced chemotaxis toward Ag in S1pr4-deficient BMMC. However, the data suggest that the role of S1P4 in chemotactic mast cell migration is at best marginal.
observed in S1pr4 since the number of metachromatic mast cells present in toluidine blue stained tissues is similar to that observed in S1pr4+/+ controls (Figure 4B,C).

2.5. Systemic Anaphylaxis in S1pr4−/− Mice

Mast cells grown and differentiated in the presence of IL-3 and SCF in culture may react differently to antigenic stimulation than cells undergoing activation during immune responses in vivo. To assess mast cell responses in S1pr4−/− mice in vivo, we induced an anaphylactic response using a model of passive systemic anaphylaxis (PSA). S1pr4−/− and S1pr4+/+ mice were sensitized with 3 µg of IgE to saturate IgE receptors prior to challenge with anti-IgE. Crosslinking of FccRI on mast cells in this manner results in anaphylaxis, which is manifested in mice by a drop in body temperature. S1pr4−/− mice exhibited increased hypothermia compared with S1pr4+/+ controls that was most apparent early on and was maintained throughout the course of induced anaphylaxis (Figure 4A). Histamine is a key vascular mediator released by mast cells that elicits anaphylactic symptoms in mice [34]. Previous reports indicate that anaphylactic reactions in S1pr4−/− mice are indistinguishable from those in S1pr4+/+ mice when anaphylaxis is induced by systemic administration of exogenous histamine [35], suggesting that the differences observed in absence of S1P4 in this study following administration of IgE/anti-IgE are likely not due to an overall change in sensitivity to histamine. In addition, exacerbated anaphylaxis in S1pr4−/− mice was unlikely due to a higher mast cell burden since the number of metachromatic mast cells present in toluidine blue stained tissues is similar to that observed in S1pr4+/+ controls (Figure 4B,C).
Since S1pr4−/− mice exhibit elevated levels of circulating IgE ([6] and Figure S2D) and IgE is known to regulate the amount of mast cell surface FcεRI, which in turn can determine the extent of mast cell responses [36], we measured whether the expression of FcεRI on mast cells in peritoneal exudates from S1pr4−/− mice was altered. Staining with a mAb specific for FcεRI (MAR-1) along with anti-IgE to measure signal from both occupied and unoccupied FcεRI [37] suggested that peritoneal mast cells ex vivo from unchallenged S1pr4−/− mice express higher levels of FcεRI at the plasma membrane compared to S1pr4+/+ control cells (Figure S2E,F). Since S1P4-deficient PDMC or BMMC, once removed from the influence of higher IgE levels, show no differences in FcεRI expression (Figure S2A–C) or intrinsic alterations in their responses (Figure 2), it is reasonable to surmise that the exacerbated anaphylactic responses in S1pr4−/− mice could partly be attributed to an increase in the expression levels of FcεRI due to an exposure to relatively high IgE levels.

3. Discussion

The importance and complexity of S1P signaling during allergic immune responses continues to emerge as we gain a greater understanding of how the various S1P receptors influence immune regulation. Mice deficient in S1P4 exhibit an allergy-prone phenotype [6], although little is known regarding the contribution of mast cells or other cell types to this condition. S1P generated following FcεRI activation in mast cells induces ligand-dependent transactivation of S1P1 and S1P2 which contribute to specific IgE-mediated responses [17]. In this study, we show that the S1P4 receptor is expressed in murine mast cells and that genetic deletion of S1pr4 results in increased IgE-mediated...
Previous reports have implicated S1P receptors, particularly S1P1, in the regulation of mast cell chemotaxis towards Ag [17,18]. This process is likely to be integral to allergic conditions such as bronchial asthma and allergic rhinitis where mast cell accumulation in tissues is critical for the development of disease [40]. Inhibition of S1P production by mast cells [17,19,41], inhibition of S1P transport from mast cells to the extracellular medium [18], or knockdown of S1P1 [17], results in inhibition of mast cell chemotaxis towards Ag in vitro, supporting the concept that FcεRI triggering promotes mast cells migration via S1P generation, export and transactivation of the S1P1 receptor. Given that inhibition of Gi signaling, which functions downstream of both S1P1 and S1P4 [42], effectively blocks migration of mast cells towards Ag [17] and that signaling through both S1P1 and S1P4 receptors can affect actin dynamics through activation of the small GTPases Rac and Rho, respectively [43–48], a contributory role for S1P4 on mast cell chemotaxis might be expected. Even though S1P4 activates pathways involved in cell motility, it did not appear to have a relevant role in FcεRI-induced chemotaxis. Nevertheless, there was a trend towards reduced motility to Ag, and addition of S1P as a chemoattractant together with Ag promoted migration in S1pr4+/+ but not in S1pr4−/− BMMC and thus a minor role for S1P4 in mast cell chemotaxis is possible. In general, this would be in agreement with reports that examine other immune cell types and implicate S1P4 in a contributory, albeit less prominent chemotactic role alongside S1P1 [9,49,50].

Our studies did not support a role for S1P4 receptors in regulating effector responses of Ag-stimulated PDMC or BMMC in our normal culturing conditions (in the presence of SCF and IL-3). Of note, under similar conditions, S1P2 did not affect IgE-mediated responses in PDMC or BMMC [51], although when cultured in the presence of IL-3 alone, a contribution for S1P2 in FcεRI-induced responses was manifested [51], a role which was also reported in human mast cells [17,51,52]. It is considered that BMMC cultured in the presence of IL-3 without SCF may present a more mucosal-like phenotype [53] than mast cells cultured in IL-3 and SCF. This distinction is, however, tenuous and it is unclear what culture conditions would better represent the phenotype of mast cells resident in tissues. Given the critical role of SCF in the location of mast cells within tissues and for mast cell maturation [54–56], experimental conditions that include SCF should be preferable and thus, our findings argue against a significant role for S1P4 in regulating mast cell function under homeostatic conditions. We cannot exclude, however, the possibility that signaling elicited through S1P4 in combination with other environmental cues under normal or pathological conditions [57,58] could modulate mast cell responses. In fact, our findings indicated that S1P4 negatively modulates the synergistic effect of IL-33 on IgE-mediated degranulation. These findings deserve further investigation since IL-33 is emerging as a critical player orchestrating allergic inflammation through innate immune cells, including mast cells. Increases in IL-33 in the epithelia are caused by barrier defects, microbiome alterations, irritants, allergens and other substances [28]. In addition to its effects on mast cell cytokine production [25,29], IL-33 promotes mast cell degranulation-associated responses leading to exacerbated sensitization to food or airway allergens [38,39]. Further, proteases released by mast cells during allergic reactions enhance the inflammatory potential of IL-33 by cleaving IL-33 into more active fragments [59], constituting a positive feedback loop for inflammation. As mast cells can also downregulate IL-33 actions in other models of inflammation [20], a better understanding of the mechanisms and circumstances under which S1P4 modulates IL-33 actions in mast cells may be beneficial for learning how to tamper certain allergic conditions.
We show here that mast cell-dependent, FcεRI-mediated anaphylaxis is more severe in S1pr4−/− mice, particularly at early phases of the response. Similarly enhanced anaphylactic responses to IgE/Ag in S1pr2−/− mice were attributed to an impairment in the regulation of vascular tone during anaphylaxis in these mice and thus defective histamine clearance and recovery from anaphylaxis [35,51]. Indeed, S1pr2−/− mice also have more severe anaphylaxis in response to vascular mediators such as histamine and PAF [35,51,60], which are released from mast cells and mediate vascular and temperature changes associated with IgE-induced anaphylaxis. In contrast, anaphylaxis induced by histamine administration in S1pr4−/− mice was no different than that observed in S1pr4+/+ mice [35], suggesting that there are no significant alterations in the response to vascular mediators or in the recovery from anaphylaxis and implicating instead enhanced mast cell responses. The increased severity of anaphylaxis in S1pr4−/− mice was unexpected given that cultured mast cells lacking S1P4 had no significant intrinsic alterations in their IgE-mediated responses in vitro. The heightened anaphylactic responses might be in part attributed to elevated levels of circulating IgE in these mice ([6] and Figure S2D), since IgE levels are known to regulate cell surface FcεRI expression and mast cell responsiveness [36]. In support of this notion, peritoneal mast cells from S1pr4−/− mice expressed higher FcεRI levels than S1pr2+/+ controls ex vivo, unlike cultured S1pr4−/− mast cells in vitro that lack chronic exposure to IgE. However, we cannot exclude the possibility that complex and dynamic cues in the tissues of S1pr4−/− mice mold the phenotype of mast cells in a manner that cannot be recapitulated in vitro. There is also precedence for the notion that S1P4 may be indirectly influencing FcεRI-mediated mast cell degranulation in vivo through its documented function in other cell types [6,47,61].

In summary, our studies demonstrate that S1P4 is expressed in mast cells, but mast cell-intrinsic expression is dispensable for most IgE-mediated responses in vitro. We, however, unveil a modulatory role for S1P4 in the exacerbation of innate type mast cell degranulation by IL-33. This observation may be of importance and requires further study, especially in the context of allergic inflammation where IL-33 is key. S1P receptor expression and signaling is clearly important for allergic mast cell-mediated responses in vivo and studies employing mice that harbor conditional or tissue-specific S1pr4 deletion will be critical for further dissecting the role of this molecule and its biology in the context of complex immunologic responses.

4. Materials and Methods

4.1. Mice

Mice were maintained and used in accordance with NIH guidelines and animal study proposals approved by the NIAID (LAD2E; 1/12/2017) and NIDDK (K007-GDB-15; 17/2/2015) animal care and use committee. S1pr4−/− mice and S1pr4+/+ littermate mice were obtained from crossing heterozygous mating pairs (strain B6.129P2-S1pr4tm1Dgen/J) from the Jacksons Laboratory (Bar Harbor, Maine). Mice had been backcrossed to C57/BL6 at least 7 times and maintained at NIH vivaria. Genotyping was performed using the following primers: (5′-GGC CTA CGT GGT CAA CGT GCT G-3′), (5′-CCG TAG AGG CTC AGG ATA GCC AC-3′) and (5′-GAC GAG TTC TTC TGA GGG GAT CGA TC-3′) which distinguished a 379 bp fragment in the WT from a 605 bp fragment in samples where S1pr4 was deleted.

4.2. Mast Cell Cultures

Mouse bone marrow-derived mast cells (BMMC) were differentiated from the marrow of tibias and femurs of S1pr4+/+ and S1pr4−/− littermate mice and cultured for at least 6 weeks in RPMI 1640 supplemented with 10% FBS, 1M HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 4 mM L-glutamine, 1 mM sodium pyruvate, 50 µM 2-meraptoethanol, 20 ng/mL IL-3, 20 ng/mL stem cell factor (SCF) and non-essential amino acids, as described [22]. Under these conditions, the proportion of mast cells in culture increases overtime and by 4-6 weeks >98% of the cells are mast cells. Mast cell expand particularly after 20 days in culture, when already >85% of the cells are mast cells.
The purity of mast cells in the cultures was monitored by assessing the percentage of cells expressing the receptor for SCF, CD117 (Kit) and the IgE receptor, FcεRI, by flow cytometry. Functional studies were conducted on cultures containing >95% double-positive mast cells as described [62]. The total number of mast cells was calculated as: (Absolute total cell count in the culture X percentage of mast cells (FceRI+/CD117+))/100 for each time point.

Peritoneal mast cells (PDMC) obtained from the peritoneal lavage of these mice were expanded in culture for 2 to 3 weeks in the same culture media as BMMC [22,63,64].

4.3. Degranulation Assays

Degranulation was assessed by a colorimetric detection of the granule marker, β-hexosaminidase, as described [65]. Briefly, mast cells were sensitized with 100 ng/mL anti-DNP IgE (clone H1-DNPe-26.82) [66] overnight in cytokine-free medium. Cells (3 × 10^4 PDMC or 5 × 10^4 BMMC) were plated in 96-well 340 µL V-bottom polypropylene (Corning, New York, NY, USA) plates in 100 µL of HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na2HPO4·7H2O, 5.6 mM Glucose, 1.8 mM CaCl2·2H2O, 1.3 mM MgSO4·7H2O). Cells were stimulated for 30 min with the indicated concentrations of 2,4-Dinitrophenyl-Human Serum Albumin (DNP-HSA) (Sigma, St. Louis, MO, USA), compound 48/80 trihydrochloride (Abcam, Cambridge, MA, USA) and/or recombinant mouse mature IL-33 (Ser 109 through Ile 266, Accession #AK075849) (eBioscience, Waltham, MA, USA). Cells were then centrifuged and supernatants separated from the cell pellets. Fifty µL of the supernatants and cell pellets lysed in 200 µL of 0.1% Triton X were transferred to 96-well plates to determine β-hexosaminidase activity. Degranulation was expressed as the percentage of β-hexosaminidase activity released into the media compared to total cellular β-hexosaminidase activity.

4.4. Flow Cytometry

Cells were resuspended at 10^7 cells/mL in PBS + aqua live/dead stain (Thermo Fisher, Waltham, MA, USA) according to manufacturer’s instructions. Cells were then washed and resuspended in FACS buffer (PBS + 2% FCS + 0.05% sodium azide) and a total of 10^6 cells (10^7 cells/mL) were incubated with anti-CD16/CD32 (clone 2.4G2—BD Pharmingen, San Jose, CA, USA), then stained with an optimal amount of antibody conjugate; anti-CD117-APC (clone ACK2—eBioscience), anti-IgE-FITC (1:100) (clone R35-72—BD Bioscience), anti-FcεRI-FITC (1:200) or -PE (1:1000) (clone MAR-1—eBioscience). Data acquisition was performed on a LSR II flow cytometer (BD Biosciences, Sparks, MD, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

4.5. Measurement of Cytokine Release

Mast cells (10^6/mL) were sensitized with anti-DNP IgE (100 ng/mL) overnight in culture media. Cells were washed ×3 and 10^6 cells plated in triplicate to 48-well plates in a volume of 1 mL of cytokine-free RPMI/well and stimulated with the indicated concentrations of DNP-HSA. After 4 h, supernatants were collected and IL-6 and TNF-α secretion was measured by ELISA (R & D systems, Minneapolis, MN, USA) as described [62].

4.6. RT-PCR and Gene Expression Analysis

Mast cells were sensitized and challenged with 25 ng/mL DNP-HSA (or media alone for unstimulated controls) as for cytokine release described above for 2 or 4 h, as indicated. Total RNA from 10^6 mast cells was isolated using the RNAeasy plus mini-kit (Qiagen, Germantown, MD, USA) according to the manufacturer’s instructions with inclusion of the QIAshredder step. RNA quantity and purity were determined using the NanoDrop ND-2000 (Nanodrop Technologies, Wilmington, DE, USA).

For qPCR analysis, 1 µg of total RNA was reverse-transcribed using the SuperScript III first-strand synthesis system with random hexamer primers (ThermoFisher, Waltham, MA, USA). cDNA and corresponding reactions in samples without reverse transcription were assessed, in triplicate,
by real-time PCR using the CFX96 Sequence Detection System (BioRad, Hercules, CA, USA). Gene-specific cDNA was amplified using Taqman gene expression probes (Table S3). The threshold cycle method, i.e., $\Delta C_T$, was used to quantify the relative abundance of each cDNA, using corresponding Gapdh (glyceraldehyde-3-phosphate dehydrogenase) levels for normalization [67]. The $C_T$ values in control samples without reverse-transcription did not exceed background levels.

For RT$^2$ gene expression array analysis, cDNA was generated from total RNA (0.5 µg) using the RT$^2$ First Strand Kit (Qiagen, Germantown, MD, USA) mixed with RT$^2$ SYBR Green qPCR Master Mix (Qiagen, Germantown, MD, USA) and aliquoted onto mouse cytokine & chemokine PCR-array plates (PAMM-150ZD-12—Qiagen). All steps were done according to the manufacturer’s protocol for the BioRad CFX96 Sequence Detection System. Data normalization was based on correcting all $C_T$ values for the average $C_T$ values of several consistently expressed housekeeping genes (HKGs) present on the array. Data was analyzed online using the manufacturer’s website (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

For droplet digital PCR (ddPCR) analysis, cDNA was generated from total RNA (160 ng) using the iScript Advanced cDNA synthesis kit for RT–qPCR (BioRad, Hercules, CA, USA). The PrimePCR ddPCR gene expression probe assay was carried out according to the manufacturer’s suggested protocol using Gapdh for high expressing transcripts or Hprt for low-expressing transcripts as reference probes (Table S2). Droplets for each sample were generated using a BioRad Droplet generator and transferred to a 96-well PCR plate. PCR reactions in the droplets were performed utilizing a 2-step thermocycling protocol [95 °C × 10 min; 40 cycles × [(94 °C × 30 s, 60 °C × 60 s); 98 °C × 10 min, ramp rate set at 2.5 °C/s]] in a BioRad C1000 Touch thermocyler and the number of transcripts in each well determined using a QX100 Droplet Reader. Data was analyzed using QuantaSoft analysis software (BioRad, Hercules, CA, USA).

4.7. Chemotaxis

$S1pr4^{+/+}$ and $S1pr4^{-/-}$ BMMC were incubated overnight in serum-free, cytokine-free RPMI supplemented with 0.04% fatty acid-free BSA (FAF-RPMI) with or without 100 ng/mL anti-DNP IgE (clone H1-DNPε-26.82) [66]. Cells were then washed twice and suspended in 100 µL FAF-RPMI in the top wells of a 5 µm pore size Transwell plate (Costar, Tewksbury MA, USA) in triplicate for 30 min, with 600 µL FAF-RPMI in the lower chamber. The cells in the upper wells were then transferred to a test well containing 600 µL FAF-RPMI in the top wells of a 5 µm pore size Transwell plate (Costar, Tewksbury MA, USA) in triplicate for 30 min, with 600 µL FAF-RPMI in the lower chamber. The cells in the upper wells were then transferred to a test well containing 600 µL FAF-RPMI in the lower chamber. Cells and media in the lower chamber were then spun down, resuspended with 15 µL PBS and counted using a LUNA-FL cell counter (Logos Biosystems, Annandale, VA, USA). Total cells counts were calculated and averaged from 3 technical replicates for each experimental condition.

4.8. Passive Systemic Anaphylaxis

$S1pr4^{+/+}$ or $S1pr4^{-/-}$ mice were sensitized (i.v.) with 3 µg DNP-specific IgE (clone H1-DNPε-26.82) [66] (0.2 mL volume) and challenged 24 h later by i.v. injection of 9 µg purified monoclonal rat anti-mouse IgE (BD Biosciences). IPTT Implantable electronic transponders (BioMedic Data Systems, Inc.—Seaford, DE, USA) were inserted under the dorsal skin fold immediately prior to the systemic administration of IgE. All injections were conducted on anesthetized mice (2% isoflurane, 98% oxygen mix for 2 to 3 min) in a closed chamber. Basal body temperature was determined prior to induction of anaphylaxis with anti-IgE and changes in temperature were measured using a DAS-8007 wireless reader system (BioMedic Data Systems, Inc., Seaford, DE, USA) at the indicated time intervals for a total period of 1 h.

4.9. Statistical Analysis

Statistical analysis comparing two groups was performed using a two-tailed unpaired t-test or two-way ANOVA if indicated (prism version 7.0; GraphPad Software, Inc., San Diego, CA, USA). Differences were considered significant when $p < 0.05$. 
Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/5/1279/s1.

Author Contributions: Joseph M. Kulinski designed and performed the experiments, analyzed and interpreted the data and wrote the manuscript; Richard L. Proia advised on the study and provided experimental mice; Elisabeth M. Larson performed and analyzed experiments; Dean D. Metcalfe supervised the study and contributed to drafting of the manuscript. Ana Olivera supervised the study, designed and interpreted data and wrote the manuscript.

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Abbreviations

- GPCR G Protein-coupled receptor
- S1P Sphingosine-1-phosphate
- S1P1–5 Sphingosine-1-phosphate receptors 1-5
- S1PR Sphingosine-1-phosphate receptor
- FcεRI High affinity IgE receptor, Fc Epsilon receptor I
- Ag Antigen
- SCF Stem cell factor
- BMMC Bone marrow-derived mast cells
- PDMC Peritoneum-derived mast cells
- WT Wild type
- qPCR Quantitative real-time PCR
- FAF BSA Fatty acid-free bovine serum albumin
- ddPCR Droplet digital PCR
- PSA Passive systemic anaphylaxis
- mAb Monoclonal antibody

References

1. Strub, G.M.; Maceyka, M.; Hait, N.C.; Milstien, S.; Spiegel, S. Extracellular and intracellular actions of sphingosine-1-phosphate. *Adv. Exp. Med. Biol.* 2010, 688, 141–155. [PubMed]
2. Graler, M.H.; Bernhardt, G.; Lipp, M. EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. *Genomics* 1998, 53, 164–169. [CrossRef] [PubMed]
3. Group, C.C.H.W. Meta-analysis of rare and common exome chip variants indentifies S1PR4 and other loci influencing blood cell traits. *Nat. Genet.* 2016, 8, 10.
4. Allende, M.L.; Bektas, M.; Lee, B.G.; Bonifacino, E.; Kang, J.; Tuymetova, G.; Chen, W.; Saba, J.D.; Proia, R.L. Sphingosine-1-phosphate lyase deficiency produces a pro-inflammatoryatory response while impairing neutrophil trafficking. *J. Biol. Chem.* 2011, 286, 7348–7358. [CrossRef] [PubMed]
5. Gorlino, C.V.; Ranocchia, R.P.; Harman, M.F.; Garcia, I.A.; Crespo, M.I.; Moron, G.; Maletto, B.A.; Pistoresi-Palencia, M.C. Neutrophils exhibit differential requirements for homing molecules in their lymphatic and blood trafficking into draining lymph nodes. *J. Immunol.* 2014, 193, 1966–1974. [CrossRef] [PubMed]
6. Schulze, T.; Golfer, S.; Tabeling, C.; Rabel, K.; Graler, M.H.; Wittenrath, M.; Lipp, M. Sphingosine-1-phosphate receptor 4 (S1P4) deficiency profoundly affects dendritic cell function and TH17-cell differentiation in a murine model. *FASEB J.* 2011, 25, 4024–4036. [CrossRef] [PubMed]
7. Wang, W.; Graeler, M.H.; Goetzl, E.J. Type 4 sphingosine 1-phosphate G protein-coupled receptor (SIP4) transduces S1P effects on T cell proliferation and cytokine secretion without signaling migration. *FASEB J.* 2005, 19, 1731–1733. [CrossRef] [PubMed]
8. Sic, H.; Kraus, H.; Madl, J.; Flittner, K.A.; von Munchow, A.L.; Pieper, K.; Rizzi, M.; Kienzler, A.K.; Ayata, K.; Rauer, S.; et al. Sphingosine-1-phosphate receptors control B-cell migration through signaling components associated with primary immunodeficiencies, chronic lymphocytic leukemia, and multiple sclerosis. *J. Allergy Clin. Immunol.* 2014, 134, 420–428. [CrossRef] [PubMed]

9. Kleinwort, A.; Luhrs, F.; Heidecke, C.D.; Lipp, M.; Schulze, T. S1P Signalling Differentially Affects Migration of Peritoneal B Cell Populations In Vitro and Influences the Production of Intestinal IgA In Vivo. *Int. J. Mol. Sci.* 2018, 19, 391. [CrossRef] [PubMed]

10. Gilfillan, A.M.; Peavy, R.D.; Metcalfe, D.D. Amplification mechanisms for the enhancement of antigen-mediated mast cell activation. *Immunol. Res.* 2009, 43, 15–24. [CrossRef] [PubMed]

11. Blank, U.; Rivera, J. The ins and outs of IgE-dependent mast-cell exocytosis. *Trends Immunol.* 2004, 25, 266–273. [CrossRef] [PubMed]

12. Tsai, M.; Grimbaldeston, M.; Galli, S.J. Mast cells and immunoregulation/immunomodulation. *Adv. Exp. Med. Biol.* 2011, 716, 186–211. [PubMed]

13. Galli, S.J.; Tsai, M. Mast cells: Versatile regulators of inflammation, tissue remodeling, host defense and homeostasis. *J. Dermatol. Sci.* 2008, 49, 7–19. [CrossRef] [PubMed]

14. Gilfillan, A.M.; Beaven, M.A. Regulation of mast cell responses in health and disease. *Crit. Rev. Immunol.* 2011, 31, 475–529. [CrossRef] [PubMed]

15. Olivera, A.; Allende, M.L.; Proia, R.L. Shaping the landscape: Metabolic regulation of S1P gradients. *Biochim. Biophys. Acta* 2013, 1831, 193–202. [CrossRef] [PubMed]

16. Kulinski, J.M.; Munoz-Cano, R.; Olivera, A. Sphingosine-1-phosphate and other lipid mediators generated by mast cells as critical players in allergy and mast cell function. *Eur. J. Pharmacol.* 2015, 778, 56–67. [CrossRef] [PubMed]

17. Jolly, P.S.; Bektas, M.; Olivera, A.; Gonzalez-Espinosa, C.; Proia, R.L.; Rivera, J.; Milstien, S.; Spiegel, S. Transactivation of sphingosine-1-phosphate receptors by FcεRI triggering is required for normal mast cell degranulation and chemotaxis. *J. Exp. Med.* 2004, 199, 959–970. [CrossRef] [PubMed]

18. Mitra, P.; Oskeritzian, C.A.; Payne, S.G.; Beaven, M.A.; Milstien, S.; Spiegel, S. Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. *Proc. Natl. Acad. Sci. USA* 2006, 103, 16394–16399. [CrossRef] [PubMed]

19. Olivera, A.; Urtz, N.; Mizugishi, K.; Yamashita, Y.; Gilfillan, A.M.; Furumoto, Y.; Gu, H.; Proia, R.L.; Baumruker, T.; Rivera, J. 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.* 2006, 281, 2515–2525. [CrossRef] [PubMed]

20. Olivera, A.; Beaven, M.A.; Metcalfe, D.D. Mast Cells Signal their Importance in Health and Disease. *J. Allergy Clin. Immunol.* 2018. [CrossRef] [PubMed]

21. Metcalfe, D.; Baram, D.; Mekori, Y.A. Mast cells. *Physiol. Rev.* 1997, 77, 1033–1079. [CrossRef] [PubMed]

22. Jensen, B.M.; Swindle, E.J.; Iwaki, S.; Gilfillan, A.M. Generation, isolation, and maintenance of rodent mast cells and mast cell lines. *Curr. Protoc. Immunol.* 2006. [CrossRef]

23. Vukman KV, M.M.; Maurer, M.; O’Neill, SM. Isolation and Culture of Peritoneal Cell-derived Mast Cells. *Bio Protoc.* 2014, 4, e1052. [CrossRef]

24. Kashem, S.W.; Subramanian, H.; Collington, S.J.; Magotti, P.; Lambris, J.D.; Ali, H.G. protein coupled receptor specificity for C3a and compound 48/80-induced degranulation in human mast cells: Roles of Mas-related genes MrgX1 and MrgX2. *Eur. J. Pharmacol.* 2011, 668, 299–304. [CrossRef] [PubMed]

25. Bandara, G.; Beaven, M.A.; Olivera, A.; Gilfillan, A.M.; Metcalfe, D.D. Activated mast cells synthesize and release soluble ST2-a decoy receptor for IL-33. *Eur. J. Immunol.* 2015, 45, 3034–3044. [CrossRef] [PubMed]

26. McNeil, B.D.; Pandir, P.; Meeker, S.; Han, L.; Undem, B.J.; Kulka, M.; Dong, X. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature* 2015, 519, 237–241. [CrossRef] [PubMed]

27. Mousli, M.; Hugli, T.E.; Landry, Y.; Bronner, C. Peptidergic pathway in human skin and rat peritoneal mast cell activation. *Immunopharmacology* 1994, 27, 1–11. [CrossRef]

28. Liew, F.Y.; Girard, J.P.; Turnquist, H.R. Interleukin-33 in health and disease. *Nat. Rev. Immunol.* 2016, 16, 676–689. [CrossRef] [PubMed]

29. Joulia, R.; L’Faqhi, F.E.; Valitutti, S.; Espinosa, E. IL-33 fine tunes mast cell degranulation and chemokine production at the single-cell level. *J. Allergy Clin. Immunol.* 2017, 140, 497–509. [CrossRef] [PubMed]
44. Lee, M.J.; Thangada, S.; Paik, J.H.; Sapkota, G.P.; Ancellin, N.; Chae, S.S.; Wu, M.; Morales-Ruiz, M.; Oskeritzian, C.A.; Alvarez, S.E.; Hait, N.C.; Price, M.M.; Milstien, S.; Spiegel, S. Distinct roles of sphingosine-1-phosphate (S1P) in migratory response via a novel signaling pathway involving S1P4 transactivation upstream of Rho-kinase-2 activation. *Cell. Signaling Attenuates ILT 7 Internalization To Limit IFN-β* 2016, 196, 693–704. [CrossRef] [PubMed]

45. Van Brocklyn, J.R.; Graler, M.H.; Bernhardt, G.; Hobson, J.P.; Lipp, M.; Spiegel, S. Sphingosine-1-phosphate (S1P) is a ligand for the G protein-coupled receptor EDG-1, required for endothelial cell chemotaxis. *Mol. Cell.* 2001, 8, 693–704. [CrossRef]

46. Graler, M.H.; Grosse, R.; Kusch, A.; Kremmer, E.; Gudermann, T.; Lipp, M. The sphingosine-1-phosphate receptor S1P4 regulates cell shape and motility via coupling to Gi and G12/13. *J. Cell. Biochem.* 2003, 89, 507–519. [CrossRef] [PubMed]

47. Dillmann, C.; Ringel, C.; Ringleb, J.; Mora, J.; Olesch, C.; Fink, A.F.; Roberts, E.; Brune, B.; Weigert, A. S1P4 Signaling Attenuates ILT7 Internalization To Limit IFN-α Production by Human Plasmacytoid Dendritic Cells. *J. Immunol.* 2016, 196, 1579–1590. [CrossRef] [PubMed]

48. Cencetti, F.; Bernacchioni, C.; Tonelli, F.; Roberts, E.; Donati, C.; Bruni, P. TGFB1 evokes myoblast apoptotic response via a novel signaling pathway involving S1P4 transactivation upstream of Rho-kinase-2 activation. *FASEB J.* 2013, 27, 4532–4546. [CrossRef] [PubMed]

49. Matsuyuki, H.; Maeda, Y.; Yano, K.; Sugahara, K.; Chiba, K.; Kohno, T.; Igarashi, Y. Involvement of sphingosine-1-phosphate (S1P) receptor type 1 and type 4 in migratory response of mouse T cells toward S1P. *Cell Mol. Immunol.* 2006, 3, 429–437. [PubMed]
50. Olesch, C.; Ringel, C.; Brune, B.; Weigert, A. Beyond Immune Cell Migration: The Emerging Role of the Sphingosine-1-phosphate Receptor S1PR4 as a Modulator of Innate Immune Cell Activation. *Mediators Inflamm*. 2017, 2017, 6059203. [CrossRef] [PubMed]

51. Olivera, A.; Dillahunt, S.E.; Rivera, J. Interrogation of sphingosine-1-phosphate receptor 2 function in vivo reveals a prominent role in the recovery from IgE and IgG-mediated anaphylaxis with minimal effect on its onset. *Immunol. Lett.* 2013, 150, 89–96. [CrossRef] [PubMed]

52. Oskeritzian, C.A.; Price, M.M.; Halt, N.C.; Kapitonov, D.; Falanga, Y.T.; Morales, J.K.; Ryan, J.J.; Milstien, S.; Spiegel, S. Essential roles of sphingosine-1-phosphate receptor 2 in human mast cell activation, anaphylaxis, and pulmonary edema. *J. Exp. Med.* 2010, 207, 465–474. [CrossRef] [PubMed]

53. Gurish, M.F.; Austen, K.F. Developmental origin and functional specialization of mast cell subsets. *Immunity* 2012, 37, 25–33. [CrossRef] [PubMed]

54. Kuehn, H.S.; Radinger, M.; Brown, J.M.; Ali, K.; Vanhaesebroeck, B.; Beaven, M.A.; Metcalfe, D.D.; Gilfillan, A.M. Btk-dependent Rac activation and actin rearrangement following FcepsilonRI aggregation promotes enhanced chemotactic responses of mast cells. *J. Cell Sci.* 2010, 123, 2576–2585. [CrossRef] [PubMed]

55. Lennartsson, J.; Jelacic, T.; Linnekin, D.; Shivakrupa, R. Normal and oncogenic forms of the receptor tyrosine kinase kit. *Stem Cells* 2005, 23, 16–43. [CrossRef] [PubMed]

56. Linnekin, D. Early signaling pathways activated by e-Kit in hematopoietic cells. *Int. J. Biochem. Cell Biol.* 1999, 31, 1053–1074. [CrossRef]

57. Moon, T.C.; St Laurent, C.D.; Morris, K.E.; Marcet, C.; Yoshimura, T.; Sekar, Y.; Befus, A.D. Advances in mast cell biology: New understanding of heterogeneity and function. *Mucosal. Immunol.* 2010, 3, 111–128. [CrossRef] [PubMed]

58. Crivellato, E.; Ribatti, D. The mast cell: An evolutionary perspective. *Biol. Rev. Camb. Philos. Soc.* 2010, 85, 347–360. [CrossRef] [PubMed]

59. Lefrancais, E.; Duval, A.; Mirey, E.; Roga, S.; Espinosa, E.; Cayrol, C.; Girard, J.P. Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proc. Natl. Acad. Sci. USA* 2014, 111, 15502–15507. [CrossRef] [PubMed]

60. Cui, H.; Okamoto, Y.; Yoshioka, K.; Du, W.; Takuwa, N.; Zhang, W.; Asano, M.; Shibamoto, T.; Takuwa, Y. Sphingosine-1-phosphate receptor 2 protects against anaphylactic shock through suppression of endothelial nitric oxide synthase in mice. *J. Allergy Clin. Immunol.* 2013, 132, 1205–1214. [CrossRef] [PubMed]

61. Sekar, D.; Hahn, C.; Brune, B.; Roberts, E.; Weigert, A. Apoptotic tumor cells induce IL-27 release from human DCs to activate Treg cells that express CD69 and attenuate cytotoxicity. *Eur. J. Immunol.* 2012, 42, 1585–1598. [CrossRef] [PubMed]

62. Kuehn, H.S.; Radinger, M.; Gilfillan, A.M. Measuring mast cell mediator release. *Curr. Protoc. Immunol.* 2010. [CrossRef]

63. Olivera, A.; Mizugishi, K.; Tikhonova, A.; Ciaccia, L.; Odom, S.; Proia, R.L.; Rivera, J. The sphingosine kinase-sphingosine-1-phosphate axis is a determinant of mast cell function and anaphylaxis. *Immunity* 2007, 26, 287–297. [CrossRef] [PubMed]

64. Charles, N.; Watford, W.T.; Ramos, H.L.; Hellman, L.; Oettgen, H.C.; Gomez, G.; Ryan, J.J.; O’Shea, J.J.; Rivera, J. Lyn kinase controls basophil GATA-3 transcription factor expression and induction of Th2 cell differentiation. *Immunity* 2009, 30, 533–543. [CrossRef] [PubMed]

65. Ozawa, K.; Szallasi, Z.; Kazanietz, M.G.; Blumberg, P.M.; Mischak, H.; Mushinski, J.F.; Beaven, M.A. Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent isoforms of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells. Reconstitution of secretory responses with Ca<sup>2+</sup> and purified isoforms in washed permeabilized cells. *J. Biol. Chem.* 1993, 268, 1749–1756. [PubMed]

66. Liu, F.T.; Bohn, J.W.; Ferry, E.L.; Yamamoto, H.; Molinario, C.A.; Sherman, L.A.; Klinman, N.R.; Katz, D.H. Monoclonal dinitrophenyl-specific murine IgE antibody: Preparation, isolation, and characterization. *J. Immunol.* 1980, 124, 2728–2737. [PubMed]

67. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>−<sub>ΔΔCt</sub></sup> Method. *Methods* 2001, 25, 402–408. [CrossRef] [PubMed]

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