S1P-induced airway smooth muscle hyperresponsiveness and lung inflammation

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Summary

**Background and Purpose.** Sphingosine-1-phosphate (S1P) has been shown to be involved in the asthmatic disease as well in preclinical mouse experimental models. The aim of this study was to understand the mechanism(s) underlying S1P effects on the lung.

**Experimental Approach.** BALB/c, mast cell-deficient and Nude mice were injected with S1P subcutaneously on day 0 and 7. Functional, molecular and cellular studies were performed.

**Key Results.** S1P administration to BALB/c mice increased airway smooth muscle reactivity, mucus production, PGD$_2$, IgE, IL-4 and IL-13 release. These features were associated to a higher recruitment of mast cells to the lung. Mast cell-deficient Kit$^{W-sh/W-sh}$ mice injected with S1P did not display airway smooth muscle hyper-reactivity. However, lung inflammation and IgE production were still present. Treatment in vivo with the anti-CD23 antibody B3B4, that blocks IgE production, inhibited both S1P-induced airway smooth muscle reactivity in vitro and lung inflammation. S1P administration to Nude mice did not elicit airway smooth muscle hyper-reactivity and lung inflammation. Naïve (non-treated) mice subjected to the adoptive transfer of CD4$^+$ T cells harvested from S1P-treated mice presented all the features elicited by S1P in the lung.

**Conclusions and Implications.** S1P triggers a cascade of events that sequentially involves T cells, IgE and mast cells reproducing several asthma-like features. This model may represent a useful tool to define the role of S1P in the mechanism of action of drugs currently used as well as in order to define new therapeutic approaches in asthma-like diseases.
Abbreviations: BSA, Bovine Serum Albumin; CFSE, Carboxyfluoresceindiacetate, succinimidyl ester; DAB, Diammino-benzidinic acid; FcεRI, High affinity IgE receptor; H&E, Hematoxylin and Eosin; IgE, Immunoglobulin E; PAS, Periodic acid /Alcian blue/Schiff; PGD$_2$, Prostaglandin D$_2$; s.c., subcutaneous; S1P, Sphingosine-1-Phosphate; SPK, Sphingosine Kinase;

Introduction

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite with pleiotropic actions that mediates several biological functions of many cell types (Spiegel et al. 2003; Pyne et al. 2000; Pyne et al. 2002). S1P, produced following the activation of sphingosine kinases (SPK), exerts most of its effects binding five distinct G protein-coupled receptors designated as S1P$_{1-5}$ (Spiegel et al., 2003; Pyne et al., 2000). High levels of S1P have been found in the broncho-alveolar lavage of asthmatic patients and they directly correlated to eosinophil count (Ammit et al., 2001). Therefore, S1P has been thought to be involved in asthma (Lai et al., 2011; Ryan et al., 2008; Oskeritzian et al., 2007).

S1P aggravates antigen-induced airway inflammation in mice (Chiba et al., 2010) and increases sensitivity to methacholine (Price et al., 2012; Kume et al., 2007). Airway smooth muscle cells stimulated with S1P release high levels of RANTES and IL-6 which play a critical role in the lung (Ammit et al., 2001; Jenkins et al., 2011; Roviezzo et al., 2004). In humans it has been shown that mast cells obtained from the
airways of allergic patients produce high levels of S1P that are associated with mast cell chemotaxis, degranulation, cytokine and lipid mediator production (Rivera et al., 2008; Olivera et al., 2008). These findings have been confirmed by several pre-clinical studies showing that S1P is pivotal in IgE-mediated mast cell degranulation and secretion of pro-inflammatory cytokines. Particular relevant in this context is the finding that the ligation of FceRI, the high affinity IgE receptor, induces SPK activation and consequently the secretion of S1P by mast cells (Olivera et al., 2011).

In this context we previously demonstrated that treatment with a non-selective inhibitor of SPKs significantly inhibits ovalbumin-induced hyper-reactivity (Roviezzo et al., 2007). In addition we have also shown that subcutaneous administration of S1P by itself, in the absence of sensitization and airway challenge induces airway smooth muscle hyper-reactivity associated to an increase in lung Th2 cytokines in a time and dose dependent manner (Roviezzo et al., 2010).

The aim of this study was to understand the mechanism(s) underlying to the S1P effects on the lung. Our study has been conducted by administering S1P to BALB/c, mast cell-deficient and Nude mice and performing molecular and functional analysis.
Materials and Methods

Mice. BALB/c, mast cell-deficient Kit\textsuperscript{W-sh/W-sh} (Tono et al., 1992; Berrozpe et al., 1999), BALB/c-Nude mice were purchased by Charles River (Italy). The animals were housed in a controlled environment and provided with standard rodent chow and water. All mouse strains (20-25 g) were housed with a 12h light dark cycle and were allowed food and water \textit{ad libitum}. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986). All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals and approved by the local committee and according to ARRIVE guidelines.

S1P exposure

BALB/c, mast cell-deficient (Kit\textsuperscript{W-sh/W-sh}) or Nude mice received subcutaneous (s.c.) injection of 0.1ml of S1P (10ng; Enzo Life Science, Italy) dissolved in sterile saline containing Bovine Serum Albumin (BSA 0.001%) according to the manufacturer’s instructions. Briefly, S1P is dissolved in sterile saline containing BSA fatty acid free at the concentration of 1mg/ml. The stock solution obtained has been used to perform the serial dilution in sterile saline. Therefore when S1P is administered the final concentration of BSA is 0.001%. S1P was administered on 0 and 7 days (Figure 1A). The vehicle used in the experimental procedures contained 0.001% of BSA. Mice were sacrificed at day 7, 14 or 21. Mice were anesthetised and subjected to euthanasia.
Bronchial tissues were rapidly dissected and cleaned from fat and connective tissue. Isolated bronchi and lungs were then utilized for functional and molecular studies.

In another set of experiment mice received 30 minutes prior to S1P administration the purified rat Anti-Mouse CD23 monoclonal Ab (10µg/mouse; B3B4 clone, anti-CD23; BD Pharmingen, DBA, Italy). Each experimental group was of 6-8 mice.

**Airway responsiveness measurements.**

Mice were sacrificed and bronchial tissues were rapidly dissected and cleaned from fat and connective tissue. Rings of 1-2mm length were cut and placed in organ baths mounted to isometric force transducers (Type 7006, Ugo Basile, Comerio, Italy) and connected to a Powerlab 800 (AD Instruments, Ugo Basile, Comerio, Italy). Rings were initially stretched until a resting tension of 0.5g was reached and allowed to equilibrate for at least 30 min. In each experiment bronchial rings were challenged with carbachol (10⁻⁶mol/L) until the response was reproducible. Once a reproducible response was achieved bronchial reactivity was assessed performing a cumulative concentration-response curve to carbachol (1x10⁻⁸-3x10⁻⁵mol/L).

**Flow Cytometry Analysis.**

Lungs were isolated and digested with 1U/mL collagenase (Sigma Aldrich, Milan, Italy). Cell suspensions were passed through 70 μm cell strainers, and red blood cells were lysed. Cell suspensions were used for flow cytometric analysis of different cell subtypes (Sorrentino et al., 2008; Sorrentino et al., 2011). The composition of lung inflammatory cells was determined by flow cytometry (BD FacsCalibur Milan, Italy) using the following antibodies: CD11c-APC, CD11b-PeCy5.5, cKit-PeCy5.5 or- PE, IgE-FITC (Bioscience, San Diego, CA, USA). The different lymphocyte populations
in the proximal lymph nodes were discriminated by means of anti-CD4-Pe, anti-CD8PerCP, anti-CD45R APC antibodies (eBioscience, San Diego, CA). Appropriate isotype controls were used.

In a separate set of experiments mediastinic lymph nodes were digested (collagenase 0.5U/ml) and CD4+ T cells were isolated by using immunomagnetic beads for negative selection (Rega et al., 2013) according to the manufacturer’s instructions (EasySep, Voden, Milan, Italy). The purity of CD4+ T cells was around 90%. CD4+ T cells were then marked for carboxyfluoresceindiacetate, succinimidyl ester (CFSE; 5µM; Molecular Probes, Invitrogen) to perform proliferation assay by using CD3/CD28 stimulation (CD3/CD28 beads, Invitrogen, Milan, Italy). CFSE flow cytometry data was analyzed by means of ModFit software (BD Pharmingen).

In another set of experiments, CD4+ T cells harvested from vehicle- S1P- or S1P+anti-CD23- treated mice were adoptively transferred into naïve (non-treated mice). 1x10^6 CD4+ T cells were intravenously injected as already reported (Rega et al. 2013).

**Immunohistochemistry**

Left lung lobes were fixed in OCT medium (Pella Inc., Milan, and Italy) and 7µm cryosections were cut. The degree of inflammation was scored by blinded observers by using hematoxylin and eosin (H&E) and Periodic acid /Alcian blue/Schiff staining (PAS). PAS Staining (Sigma Aldrich, Milan Italy) was performed according to the manufacturer’s instructions to detect glycoprotein. PAS+ cryosections were graded with scores 0 to 4 to describe low to severe lung inflammation as follows: 0: <5%; 1: 5 to 25%; 2: 25-50%; 3: 50-75%; 4: <75% positive staining/total lung area. Immunohistochemical detection of CD23 was performed by using anti-CD23 or rat IgG
isotype control. The diammino-benzidinic acid (DAB) system was used to detect complexes. Positive staining was quantified by means of Image J software (NIH, USA) and expressed as CD23 positive staining compared to the total area of the lung section. At least 5 sections were considered for each animal and the mean of the positive staining compared to the total area were plotted.

Measurement of serum IgE and PGD$_2$ levels.

Blood was collected from heart. Total serum IgE levels were measured by means of ELISA using matched antibody pairs (BD Pharmingen, Franklin Lakes, NJ, and USA). PGD$_2$ quantification has been performed using EIA Kit (Cayman, Ann Arbor, MI, USA)

Cytokines measurements

Pulmonary IL-4 (R&D system, UK) and IL-13 (eBioscience, CA, USA) were determined by ELISA.

Statistical analysis

Data are means ± SEM from at least 6 mice in each group. The level of statistical significance was determined by two-way analysis of variance (ANOVA) followed by Bonferroni’s test for multiple comparisons or Student’s $t$ test by using the Graph Pad Prism software, when appropriate.
Results

S1P induces airway smooth muscle hyper-reactivity and lung inflammation

Lungs harvested from mice injected with S1P (Figure 1A), displayed a progressive alteration of lung morphology. The effect was maximal at 21 days after S1P challenge (Figure 1B) and was associated to increased mucus production as determined by PAS staining (Figure 1C). Plasma levels of IgE (Figure 1D) were significantly increased, too. Flow cytometry analyses of lungs showed a significant increase in the percentage of mast cells, identified as CD11c+cKit+IgE+ cells (Figure 2A and 2B). Mast cells were stained for IgE since they covalently bind to its receptor (FcεRI) on these cells (Metzger et al. 1991). Plasma levels of PGD$_2$ were significantly increased in S1P-treated compared to vehicle treated mice (Figure 2C).

S1P-induced hyper-reactivity, but not lung inflammation, is abrogated in mast cell deficient Kit$^{W-sh/W-sh}$ mice

In mast cell-deficient Kit$^{W-sh/W-sh}$ mice S1P failed to induce bronchial hyperresponsiveness (Figure 3A). Conversely, lungs harvested from the same animals still displayed i) altered alveolar structure and ii) increased mucus production (Figure 3B and 3C) in comparison with wild type. Basal serum IgE resulted significantly reduced in Kit$^{W-sh/W-sh}$ mice when compared to wild type mice (Figure 3D). Nevertheless, S1P challenge significantly increased IgE levels in Kit$^{W-sh/W-sh}$ mice when compared to the basal levels of mast cell deficient mice (Figure 3D).
S1P induces lung inflammation and airway smooth muscle hyper-reactivity in an IgE-dependent manner

CD23 is an important regulatory receptor for IgE production and its interaction with IgE can amplify IgE-associated immune responses (Cheng et al., 2010; Morris et al., 1994; Galli et al., 2012). Immunohistochemical analysis (Figure 4A) showed that CD23 expression was significantly increased in S1P-treated mice with a maximal effect at 21 days after S1P challenge in comparison to vehicle mice (Figure 4B). Therefore, in order to investigate the role of IgE in S1P-induced airway dysfunction, we used an anti-CD23 monoclonal antibody e.g. B3B4. Administration of anti-CD23 per se did not affect S1P-induced increase in pulmonary mast cell infiltration (Figure 4C). Conversely anti-CD23 significantly reduced S1P-induced IgE increase (Figure 4D) thereby confirming the role of CD23 in S1P-induced IgE production.

IL-4 and IL-13 are key cytokines for the initiation of Th2-mediated response and for IgE isotype class switching (Finkelman et al. 1990; Zurawski et al. 1994). As shown in Figure 4E and 4F, S1P administration induced a significant increase of both IL-4 (Figure 4E) and IL-13 (Figure 4F) in the lung. Anti-CD23 inhibited S1P-induced expression of IL-13 (Figure 4F), but not of IL-4 (Figure 4E).

However, the administration of anti-CD23 to S1P-treated mice prevented airway smooth muscle hyper-responsiveness (Figure 5A), lung damage (Figure 5B) and mucus production (Figure 5C).

T cells are involved in S1P-induced effects on lung

The major control step in IgE synthesis is the regulation of IgE class-switch recombination which is mostly T cell dependent (Geha et al. 2003; Rosenwasser et al. 2011). Our data define IgE/CD23 signaling as a key driver for the effects triggered by
S1P in the airways. In order to extend our observations on the role of CD23 and to further elucidate the role of IgE-dependent mechanisms, we evaluated T cell involvement. Nude mice injected with S1P did not develop bronchial hyper-responsiveness (Figure 6A). In addition, alteration of lung structure (Figure 6B), mucus production (Figure 6C) and mast cell infiltration (Figure 6D) in S1P-treated Nude mice were significantly lower than wild type mice. Plasma levels of IgE resulted unaltered by S1P in Nude mice compared to wild type (Figure 6E). Accordingly, we observe neither increase in CD23 pulmonary expression (Figure 6F), nor IL-4/IL-13 over-expression in the lung (data not shown).

Adoptive transfer of S1P-derived CD4+T cells into naïve mice induces airway smooth muscle hypereactivity and lung inflammation.

In order to further gain insight into the cellular mechanisms we isolated CD4+T cells. CD4+ T cells were labelled at day 0 with CFSE (parent histogram; Figure 7A) and then cultured for three days in the presence or absence of CD3/CD28 beads. CD4+T cells were isolated from the mediastinic lymph node of vehicle-(Figure 7B and 7C), S1P- (Figure 7D and 7E), IgG- (Figure 7F), anti-CD23 (Figure 7G and 7H) or S1P+anti-CD23- (Figure 7I and 7J) treated mice. CD4+ T cells harvested from S1P-treated mice (Figure 7E) displayed a basal increase in the proliferation rate as compared to vehicle-treated mice (Figure 7C), even in the absence of CD3/CD28 beads (Figure 7B and 7D; Supplementary Figure 1). We observed that CFSE+CD4+ T cells were proliferating up to seven generation when they were obtained from S1P-(Figure 7D and 7E), but not vehicle -treated mice (Figure 7B and 7C ). Proliferation rate of CD4+T cells induced by S1P was not affected by anti-CD23 treatment (Figure 7I and 7J, Supplementary Figure 1). IgG- (Figure 7F, Supplementary Figure 1) or
anti-CD23- (Figure 7G and 7H, Supplementary Figure 1) derived CD4+T cells did not show any significant increase in the proliferation rate (Supplementary Figure 1). These results imply that the CD23 role in vivo is to regulate negatively IgE production during S1P exposure without a direct significant effect on T-cell growth or differentiation. These latter data well fit with the lack of effect of anti-CD23 in regulating IL-4 expression.

To determine whether S1P could directly affect CD4+ T cells proliferation, we incubated CD4+T cells obtained from vehicle-treated mice with S1P. In these experimental conditions there was no detectable increase in proliferation index (data not shown).

Finally, adoptive transfer of CD4+T cells, harvested from the mediastinic lymph node of S1P-treated mice, into naïve mice was performed. S1P-derived CD4+ T cell adoptively transferred into naïve mice increased mast cell infiltration (Figure 8A), bronchial reactivity (Figure 8B), lung inflammation (Figure 8C), IL-4 (Figure 8D) and IL-13 (Figure 8E) release. Conversely, adoptive transfer of CD4+T cells, harvested from mice treated with anti-CD23 and S1P reversed airway smooth muscle hyper-reactivity (Figure 8B), lung inflammation (Figure 8C) and IL-13 upregulation (Figure 8E). In agreement with previous data (Figure 4) neither mast cell infiltration (Figure 8A) nor IL-4 upregulation (Figure 8D) were modified.
Discussion and conclusions

In our previous studies we showed lower bronchial reactivity and lung inflammation after the inhibition of sphingosine kinases in OVA-sensitized mice. Here we show that the subcutaneous administration of S1P in the absence of an allergen causes a progressive alteration of pulmonary parenchyma morphology, an increased mucus production, lung inflammation and higher bronchial reactivity. These morphological and functional changes were preceded by an increase in plasma levels of PGD2 and IgE and lung IL-4 and IL-13.

A large body of evidence suggest that both IgE and mast cells are key factors for the pathophysiological changes and tissue remodelling associated with chronic allergic inflammation in asthma. Such effects include IgE-dependent regulation of mast cells, mast cell-independent IgE-mediated actions and mast cell activities that do not directly involve IgE. In order to understand the possible involvement of these mechanisms in our model we treated mast cell KO (Kit<sup>W-sh/W-sh</sup>) mice with S1P. In Kit<sup>W-sh/W-sh</sup> mice S1P did not cause airway smooth muscle hyper-reactivity. However, lung inflammation, as defined by mucus production and IgE levels, was still evident. Thus, the absence of mast cells does not affect IgE levels suggesting an alternative target for IgE in our mouse model.

CD23 is the low affinity receptor for IgE and has been implicated in a number of inflammatory conditions, and it is considered important in the regulation of IgE production. An altered expression of CD23 has been widely associated to allergic diseases (Morris et al., 1994; Cheng et al., 2010; Rosenwasser et al., 2005). Based on this notions, we found that lung CD23 expression was significantly increased in S1P-treated compared to vehicle mice. In order to define CD23’s role we treated mice with
a neutralizing antibody (B3B4 clone), extensively characterized for its high affinity for CD23 and for a reciprocal inhibitory pattern with IgE. Indeed, treatment of mice with the anti-CD23 and S1P, reduced airway smooth muscle hyper-responsiveness, pulmonary hyperplasia, IL-13 and IgE levels, but pulmonary mast cell infiltration and IL-4 levels were not altered. Therefore these data, taken together with previous evidence, imply that S1P/CD23 signalling is not directly involved in mast cell recruitment, but rather interferes with their activation, as suggested by the marked reduction in production of IgE. This hypothesis is also supported by the reduced levels of IL-13 after anti-CD23 treatment. Similarly, we did not observe any change in IL-4 release after the treatment with anti-CD23, suggesting a predominant Th2 bias in S1P-induced asthma like model in mice.

Therefore, in order to further gain insight into the cellular mechanisms, S1P was injected in Nude mice, which lack a functional adaptive immunity. In Nude mice S1P did not elicit bronchial hyper-responsiveness, pulmonary infiltration of mast cells and IgE production. These features were coupled to a lack of induction of both cytokines IL-4 and IL-13 as well as of CD23 in the lung. Thus, T cells, opposite to mast cells, trigger the functional, molecular and cellular changes elicited by S1P. Thus, both IgE-dependent pathways and Th2-like bias orchestrated S1P-induced asthmatic conditions in mice.

The idea that all these events are T cell dependent is further sustained by the finding that the CD4+ T lymphocytes, harvested from BALB/c mice exposed to S1P, showed an increased ability to proliferate in vitro. To note, CD4+ T cells harvested from S1P-treated mice were able to proliferate even in the absence of the in vitro stimulation with CD3/CD28 beads. On the other hand the proliferation rate of CD4+T cells,
obtained from mice pre-treated in vivo with the anti-CD23 prior to S1P administration, was not altered. This indicates that the major action of CD23 in vivo is to negatively regulate IgE production without a significant activity on T-cell growth or differentiation. These data fit well with the lack of effect of anti-CD23 in regulating IL-4 release, mainly involved, together with IL-13, IgE, and PGD$_2$ (Bice et al. 2014), in a Th2 skew in the lung of S1P-treated mice. That these mechanisms, triggered by S1P, require this in vivo cellular activation is strongly supported by the finding that CD4+ T cells, harvested from vehicle-treated mice, were not able to proliferate when exposed in vitro to S1P. These latter events occur in an IgE independent manner.

To further confirm that S1P induces a modulation of the adaptive response, we did a confirmatory experiment by performing adoptive transfer experiments. S1P-derived CD4+ T cell adoptively transferred into naïve mice increased mast cell infiltration, bronchial reactivity, pulmonary inflammation, IL-4 and IL-13 release. As expected adoptive transfer of CD4+ T cells derived from S1P-treated mice receiving anti-CD23 into naïve mice did not promote any effect on the lung. In particular we did not observe any increase in airway smooth muscle reactivity and the lack of an inflammatory response. Conversely IL-4 over-expression was still present. These data together with the studies performed in mast cell KO and Nude mice confirm that CD4+ T cells are the main cells involved in S1P-induced effects. In addition this implies an obligatory role for CD23/IgE signalling to trigger IgE-mediated immune responses in order to observe functional changes in the lung. However, the role of innate immune cells in this context still remains to be elucidated.

In conclusion systemic administration of S1P triggers a cascade of events that sequentially involves T cells, IgE and mast cells which leads to the asthma-like...
symptoms in mice. Therefore the model herein described and characterized may represent a useful tool to define the role of S1P in the mechanism of action of drugs currently used as well as in order to define new therapeutic approaches.

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**List of Author Contributions**

Fiorentina Roviezzo and Giuseppe Cirino conceived and designed experiments and wrote the manuscript; Luana De Gruttola and Antonio Bertolino performed functional experiments of bronchial reactivity and measurement of cytokines; Aldo Pinto and Michela Terlizzi performed experiments of adoptive transfer. Rosalinda Sorrentino performed flow cytometry analysis and immunohistochemistry; Angela Ianaro, M Napolitano and G. Castello performed proliferation experiments; Bruno D’Agostino and Raffaella Sorrentino analysed data.

**Statement of conflicts of interest**

None
References


Figures and Legends

Figure 1: Systemic S1P administration induces lung inflammation. A. Mice received subcutaneous administration of S1P (10ng) or vehicle (BSA 0.001%) at day 0 and 7. B. Lung sections were fixed and stained with Hematoxylin and Eosin. Lung sections were photographed under light microscopy at x10 magnification. C. Periodic acid /Alcian blue/Schiff staining (PAS) was performed to detect glycoprotein (*p<0.05 vs vehicle). D. Sera were collected and levels of total IgE were determined by using specific ELISA (**p<0.01 vs vehicle). Data are means ± SEM n= 6 mice in each group.

Figure 2: S1P increases mast cell infiltration in the lung. A. Mast cells were identified as IgE+cKit+ cells by flow cytometry as shown in the representative dot plot. B. Mast cells infiltration was quantified after 7, 14 and 21 days following S1P administration. C. Sera were collected and PGD$_2$ levels were determined by using specific ELISA.*p<0.05, **p<0.01 vs. vehicle. Data are means ± SEM n= 6 mice in each group.

Figure 3: Mast cells are essential for the development of S1P-induced bronchial hyper-reactivity, but not for lung inflammation. Mast cell-deficient Kit$^{W-sh/W-sh}$ or wild type mice received subcutaneous administration of S1P (10ng) or vehicle (BSA 0.001%) at day 0 and 7. Mice were sacrificed on day 21. A. Assessment of bronchial reactivity to carbachol (*** p<0.001 vs. vehicle). B. Lung sections were fixed and stained with periodic acid /Alcian blue/Schiff (PAS; *p<0.05). Lung sections were photographed under light microscopy at x10 magnification. C. PAS staining was quantified as described in method section. D. Sera were collected and levels of IgE
were determined by ELISA (*p<0.05; **p<0.01). Data are means ± SEM n=6 mice in each group.

**Figure 4: S1P enhances pulmonary CD23 (FcRII) expression.** A. Immunohistochemical detection of CD23 was performed on lung sections harvested from mice challenged with vehicle or S1P by using anti-CD23 monoclonal Ab (B3B4, anti-CD23) or rat IgG isotype control as shown in the representative lung section staining. Lung sections were photographed under light microscopy at x10 magnification. B. CD23 quantification was performed at 7, 14 and 21 days after S1P challenge (*p<0.05 vs. vehicle). In another set of experiments, anti-CD23 (B3B4; 10µg/mice) was administered intraperitoneally 30 minutes prior to S1P or vehicle at day 0 and day 7. On day 21 mice were sacrificed. C. Pulmonary mast cells were identified as IgE+cKit+ cells by flow cytometry (**p<0.01). D. Sera were collected to determine IgE levels by ELISA (**p<0.01); E, F. Pulmonary expression of IL-4 (*p<0.05) and IL-13 (**p<0.01) were determined by ELISA. Data are means ± SEM n=6 mice in each group.

**Figure 5: Anti-CD23 (B3B4) abrogates S1P mediated effects on lung.** Anti-CD23 (B3B4; 10µg/mice) was administered intraperitoneally 30 minutes prior to S1P at day 0 and 7. Mice were sacrificed on day 21. A. Bronchial response to carbachol was evaluated (***p<0.001 vs. S1P). B. Representative staining of lung sections with Hematoxylin and Eosin. C. Quantification of PAS staining performed as described in method section (*p<0.05). Lung sections were photographed under light microscopy at x10 magnification. Data are means ± SEM n=6 mice in each group.
Figure 6: **T cell plays a key role in S1P-mediated effects on lung.** Nude athymic mice or wild type mice received subcutaneous administration of S1P (10ng) or vehicle (BSA 0.001%) at day 0 and 7. Mice were sacrificed on day 21. A. Assessment of bronchial response to carbachol (**p<0.001**). B. Representative staining of lung sections with Hematoxylin and Eosin. C. Quantification of PAS staining performed as described in method section (*p<0.05, ** p<0.01). D. Pulmonary mast cells quantification by flow cytometry (** p<0.01). E. Sera IgE levels determination by ELISA(** p<0.01). F. Immunohistochemical detection of CD23 (*p<0.05) on lung sections by using anti-CD23 monoclonal Ab (B3B4). Data are means ± SEM n= 6 mice in each group.

Figure 7: **Lymphocytes harvested from S1P-treated BALB/c mice have an increased ability to proliferate.** CD4+T cells were isolated from the mediastinic lymph node of vehicle-, S1P-, IgG- or S1P+anti-CD23- treated mice. CD4+ T cells were labelled at day 0 with CFSE (parent histogram-A) and then cultured for three days in the presence (C, E, F H, J) or absence (B, D, G, I) of CD3/CD28 beads. Histograms in the panel from B to J represent the proliferation of CD4+ T cells isolated from the lymph node of vehicle-(B and C), S1P- (D and E), IgG- (F), anti-CD23- (G and H) and S1P+anti-CD23- (I and J) treated mice. Experiments were performed in three differential experimental times. The histograms reported are representative. Data was analyzed by means of ModFlt3 program (BD FACSCalibur).

Figure 8: **Adoptive transfer of CD4+ cell from S1P treated mice into naïve (non-treated) mice mimics S1P-induced effects in BALB/c mice.** Adoptive transfer of CD4+ T cells derived from vehicle- , S1P- or anti-CD23+S1P-treated mice into naïve
BALB/c mice was performed. Mice were sacrificed at 3 and 7 days following adoptive transfer. **A.** Mast cells identification as CD11c+cKit+ IgE+cells by flow cytometry. **B.** Assessment of bronchial response to carbachol. **C.** PAS staining of lung sections harvested from mice after 7 days of adoptive transfer. **D.** Pulmonary levels of IL-4 (**p<0.01**). **E** Pulmonary levels of IL-13 (**p<0.001**). Data are means ± SEM n=6 mice in each group.
Figure A shows a timeline indicating the administration of S1P (s.c. 10ng) on Day 0 and Day 7, followed by S1P (s.c. 10ng) on Day 14. Figure B presents lung sections stained with PAS for different treatment groups: Vehicle, S1P 7 days, S1P 14 days, and S1P 21 days. Figure C compares PAS-positive staining between the vehicle and treated groups at different time points (vehicle, 7, 14, 21 days). Figure D illustrates the IgE levels (ng/mL) for the vehicle and treated groups at 7, 14, and 21 days.
Figure 3:

(A) Graph showing the effect of carbachol on contraction (dyne/ng) with different groups: vehicle/Kit^W-sh/W-sh, SIP/Kit^W-sh/W-sh, vehicle/wild type, and SIP/wild type. The graph includes error bars indicating standard deviation.

(B) Images of tissue samples with stains indicating the effects of vehicle and SIP on tissue appearance.

(C) Bar graph showing PAS positive staining with vehicle and SIP treatments. The bars are labeled as vehicle and SIP, with a legend indicating wild type and Kit^W-sh/W-sh.

(D) Bar graph showing IgE levels with vehicle and SIP treatments. The bars are labeled as vehicle and SIP, with a legend indicating wild type and Kit^W-sh/W-sh.

bph_13033_f3
A. Isotype Ctr vs vehicle

B. CD23 positive staining mm²

C. 

D. 

E. 

F. 

bph_13033_f4
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