



Disodium cromoglycate inhibits asthma-like features induced by sphingosine-1-phosphate

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ABSTRACT

Compelling evidence suggests the involvement of sphingosine-1-phosphate (S1P) in the pathogenesis of asthma. The systemic administration of S1P causes asthma like features in the mouse involving mast cells. In this study we investigated whether disodium cromoglycate (DSCG), administered as a preventative treatment as in human therapy, could affect S1P effects on airways. BALB/c mice, treated with DSCG, received subcutaneous administration of S1P. Bronchi and pulmonary tissues were collected and functional, molecular and cellular studies were performed. DSCG inhibited S1P-induced airway hyper-reactivity as well as pulmonary inflammation. DSCG decreased the recruitment of solely mast cells and B cells in the lung. IgE serum levels, prostaglandin D₂, mucus production and IL-13 were also reduced when mice were pretreated with DSCG. S1P induced pulmonary expression of CD23 on T and B cells, that was reversed by DSCG. Conversely, S1P failed to upregulate CD23 in mast cell-deficient Kit^{W-sh/W-sh} mice. In conclusion we have shown that DSCG inhibits S1P-induced asthma like features in the mouse. This beneficial effect is due to a regulatory action on mast cell activity, and in turn to an inhibition of IgE-dependent T and B cells responses.

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1. Introduction

Mast cells are widely known for their harmful activity during lung allergic inflammation [1,2]. Their infiltration into airway smooth muscle cell layer characterizes allergic asthma associated with airway hyper-reactivity [3,4]. The ability of mast cells to produce a variety of bioactive products makes these cells the first-line regulators in many immune functions [5,6].

Sphingosine-1-phosphate (S1P) has been recognised as a new inflammatory mediator secreted by activated mast cells and involved in both innate and adaptive immunity [7,8]. Cross-linking of IgE for its high affinity receptor (FcεRI) on mast cells activates

sphingosine kinases (SPKs), which triggers phosphorylation of sphingosine to generate S1P. This event plays a relevant role in cell degranulation, leading to release of allergic pro-asthma mediators [9,10]. In addition, S1P can be released by mast cells to amplify their response through binding S1P receptors in a paracrine and autocrine manner [11,12].

Compelling evidence suggests an involvement of SPK/S1P pathway in the pathogenesis of chronic asthma [13]. Elevated levels of S1P in bronchoalveolar lavage (BAL) fluid were recovered from allergic asthma patients after ragweed antigen challenge [9,10]. In support, administration of S1P exacerbates antigen-induced airway inflammation in mice [15]. Furthermore, the treatment of ovalbumin (OVA)-sensitised mice with an inhibitor of SPKs ameliorates the development of bronchial smooth muscle hyper-responsiveness in a mast cell dependent manner [16,17]. Accordingly we published data demonstrating that the systemic administration of S1P induces several asthma-like features in the

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mouse involving mast cells [17–20]. Recently Oskeritzian et al. demonstrated that pretreatment with anti-S1P mAb inhibits mast cell activation *in vitro* as well as development of airway inflammation and mast cell activation *in vivo* [21].

Mast cell-derived lipid mediators and their receptors represent interesting therapeutic targets for treating allergic inflammation. However, multiple functions and complex biology are associated with these mediators. Disodium cromoglycate (DSCG) belongs to chromones that still have a niche role in the treatment of asthma and allergies [22]. DSCG, when inhaled prior to challenge, is capable to inhibit both the early and late phase response to a variety of inhaled allergens, bronchial constrictor agents and exercise [23]. The DSCG-like drugs are known as ‘mast cell stabilizers’ due to the fact that they prevent the release of mast cell histamine upon stimulation by different agonists [24]. However, many other actions of DSCG-like drugs have been also reported [25–29].

In this study we investigated whether DSCG could affect disease manifestation in a murine asthma model induced by S1P.

2. Materials and methods

2.1. Animals

Female BALB/c and mast cell-deficient Kit^{W-sh/W-sh} [30,31] mice (8weeks) were purchased from Charles River Laboratories (Milan, Italy). The animals were housed in a controlled environment and provided with standard rodent chow and water. All mice were housed with a 12 h light dark cycle and were allowed food and water *ad libitum*. All animals were allowed to acclimate for four days prior to experiments. The experiments described have been carried out in accordance with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116/92) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986). The animal studies were approved by the local ethical committee of the University of Naples Federico II on 20/07/2012 (approval number 2012/0081821).

2.1.1. Animal injections and harvest

BALB/c mice received s.c. injection of S1P (10 ng, equivalent to 0.5 µg/Kg; Enzo Life Science, Italy) in sterile saline containing BSA (0.001%) on days 0 and 7 [18,20]. Part of these mice received disodium cromoglycate (DSCG; i.p. 50 mg/Kg; Sigma Aldrich, Italy) 30 min prior to S1P administration. Vehicle mice receiving 0.1 ml of sterile saline containing BSA (0.001%) were used as control. On day 14 and 21 mice were anesthetised with i.p. ketamine/xylazine and euthanized by bleeding. Bronchial reactivity and lung function were assessed at 21 days. In another set of experiments mice were sacrificed at 14 days to take bronchoalveolar lavage and pulmonary tissues used for functional and molecular studies. Each lung was divided into two parts. One part was frozen in liquid nitrogen for 2 h before storage at –86 °C for cytokine measurements, and the other was fixed in 10% neutralized buffered formalin for histopathological and immunohistochemical detection. In another set of experiments mast cell-deficient Kit^{W-sh/W-sh} mice received s.c. injection of S1P (0.5 µg/Kg; Enzo Life Science, Italy) in sterile saline containing BSA (0.001%) on days 0 and 7. Mice were sacrificed at 14 days and lungs fixed in 10% neutralized buffered formalin for immunohistochemical analysis. Each experimental group consisted of 6 mice.

2.1.2. Depletion of mast cells

BALB/c mice were pretreated i.p. every 12 h for four days with the mast cell degranulator compound 48/80 (CM48/80) dissolved in PBS (phosphate-buffered saline) and injected at 200 µl/cavity according to the following schemes: day 1, 0.6 mg/kg; day 2, 1.0 mg/kg; day 3, 1.2 mg/kg; day 4, 2.4 mg/kg. The administration

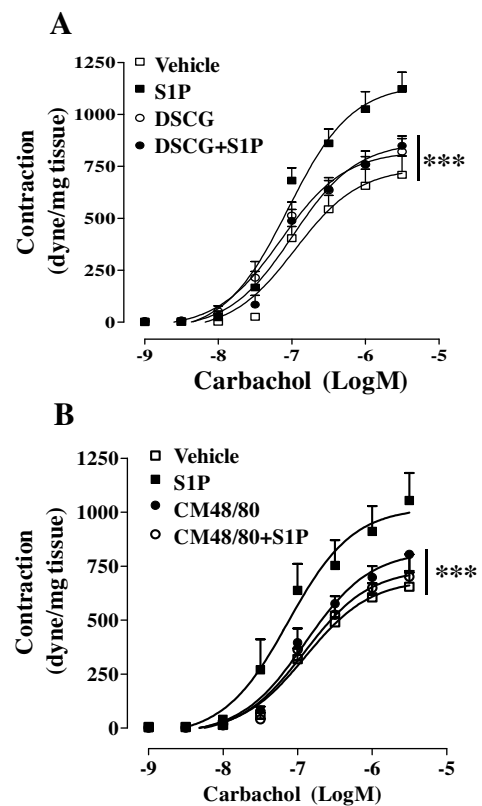


Fig. 1. DSCG inhibits S1P-induced bronchial hyper-responsiveness.

BALB/c mice received subcutaneous administration of S1P (10 ng) or vehicle (BSA 0.001% in phosphate-buffered saline) on days 0 and 7. A: DSCG (50 mg/kg) was administered i.p. 30 min before S1P or vehicle on days 0 and day 7. On day 21 bronchial reactivity to carbachol was assessed (*** P < 0.001 vs. S1P). B: The mast cell degranulator compound 48/80, dissolved in phosphate-buffered saline, was injected at 200 µl/cavity to mice i.p. every 12 h for four days, according to the following schemes: day 1, 0.6 mg/kg; day 2, 1.0 mg/kg; day 3, 1.2 mg/kg; day 4, 2.4 mg/kg. The administration of S1P was performed 24 h after the last dose of compound 48/80. On day 21, bronchial reactivity to carbachol was assessed (*** P < 0.001 vs. S1P). Data represents means $\pm$ SEM; n = 6 mice per group.

of S1P was performed 24 h after the last dose of compound 48/80 [32,33].

2.2. Airway reactivity

2.2.1. Bronchial reactivity

BALB/c mice were sacrificed and bronchial tissues were rapidly dissected and cleaned from fat and connective tissue. Rings of 1–2 mm 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). Rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 min. In each experiment, bronchial rings were challenged with carbachol (10^{-6} mol/L) until the response was reproducible. Once a reproducible response was achieved, the bronchial reactivity was assessed performing a cumulative concentration–response curve to carbachol (1×10^{-8} – 3×10^{-5} mol/L).

2.2.2. Isolated perfused mouse lung preparation

Lung function was assessed using an isolated and perfused mouse lung model [34]. Lungs were perfused in a non-circulating fashion through the pulmonary artery at constant flow of 1 ml/min resulting in a pulmonary artery pressure of 2–3 cm H₂O. The perfusion medium used was RPMI 1640 lacking phenol red (37 °C). The lungs were ventilated by negative pressure (–3 and –9 cm

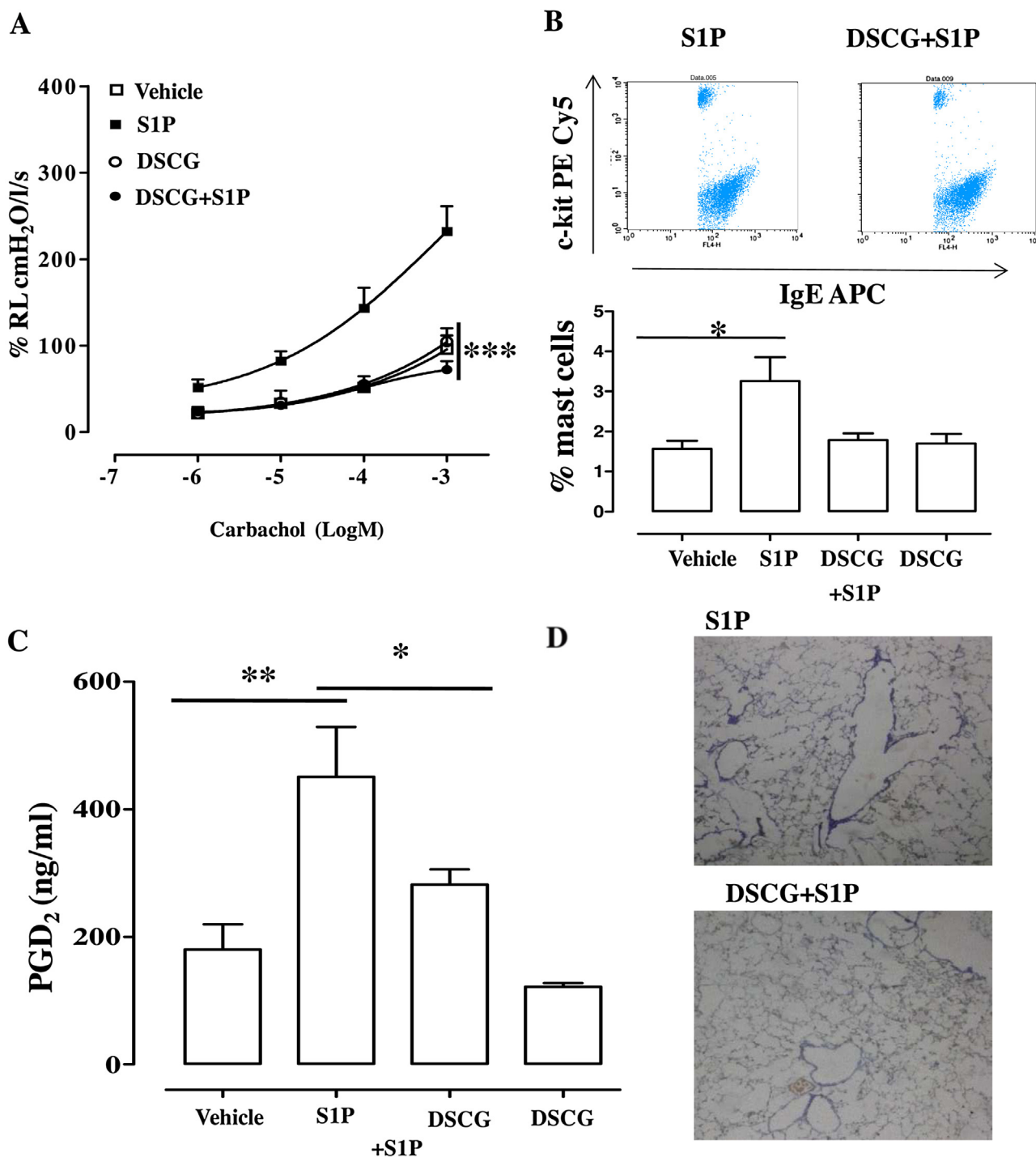


Fig. 2. DSCG reverses S1P-induced increase in airway resistances and mast cell recruitment.

A: Pulmonary resistances were measured on day 21 in isolated and perfused lungs (*** $p < 0.001$ vs. S1P). B: Quantification of lung mast cells by flow cytometry identified as CD11c + c-Kit + IgE+ positive cells on day 14. C: BAL was collected (on day 14) and PGD₂ levels were determined by ELISA (** $P < 0.01$ vs. vehicle, * $P < 0.05$ vs. S1P). D: Toluidine blue staining of formalin-embedded lung sections. Data represents means $\pm$ SEM; $n = 6$ mice per group.

H₂O) with 90 breath min⁻¹ and a tidal volume of about 200 μ l. Every 5 min a hyperinflation (-20 cm H₂O) was performed. Artificial thorax chamber pressure was measured with a differential pressure transducer (Validyne DP 45-24) and airflow velocity was monitored with pneumotachograph tube connected to a differential pressure transducer (Validyne DP 45-15). The lungs respired humidified air. The arterial pressure was continuously monitored by means of pressure transducer (Isotec Healthdyne), which was connected with the cannula ending in the pulmonary

artery. All data were transmitted to a computer and analysed with Pulmodyn software (Hugo Sachs Elektronik, March Hugstetten, Germany [35]). Data were analysed through the following formula: $P = V \cdot C^{-1} + R_L \cdot dV \cdot dt^{-1}$, where P is chamber pressure, C pulmonary compliance, V tidal volume, R_L airway resistance. Successively, airway resistance value registered was corrected for the resistance of the pneumotachometer and the tracheal cannula of 0.6 cm H₂O s ml⁻¹. Lungs were perfused and ventilated for 45 min without any treatment in order to obtain baseline state. Subsequently, lungs

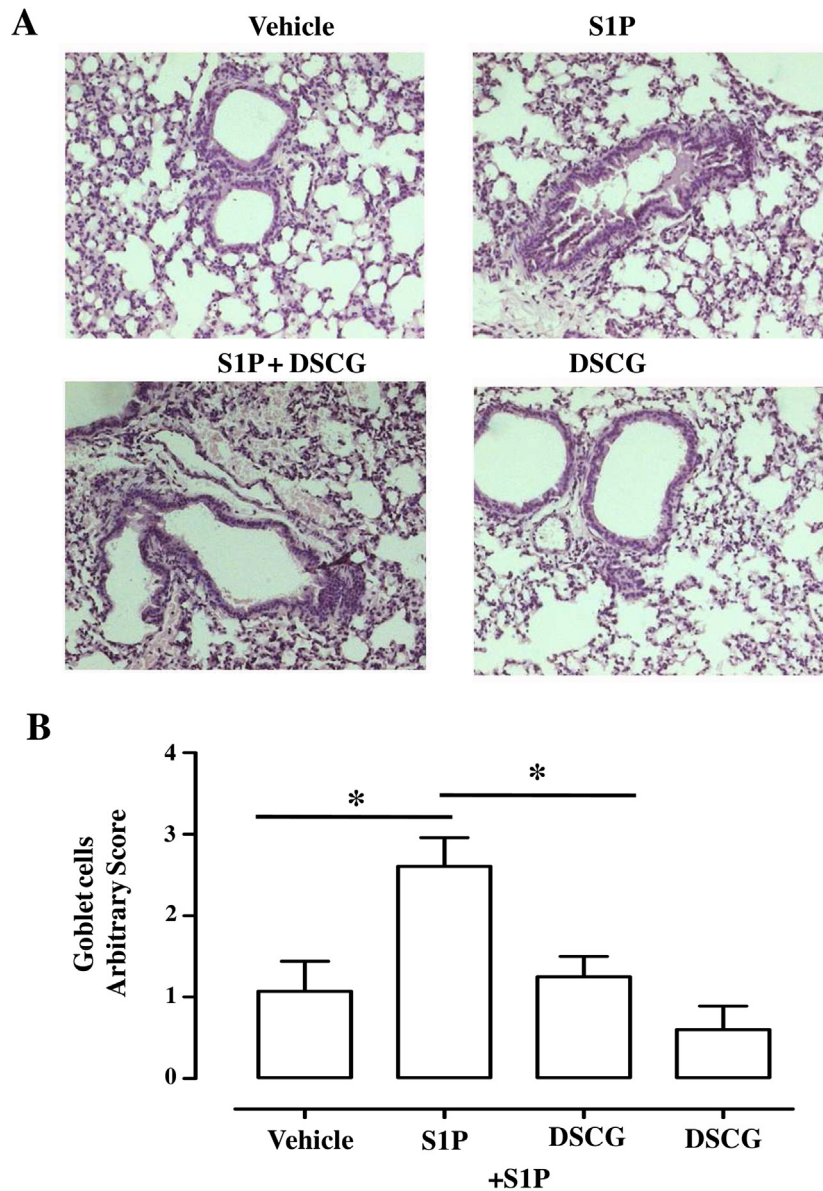


Fig. 3. DSCG inhibits S1P induced mucus production.

Lung sections were fixed and stained with periodic acid/Alcian blue/Schiff (PAS). A: Representative PAS staining for mucus in airways. B: Lung sections were photographed under light microscopy at $\times 10$ magnification. PAS+ cryosections were graded with scores 0 to 4 to describe low to severe lung inflammation (* $P < 0.05$ vs. S1P) as follows: 0: $<5\%$; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: $>75\%$ positive staining/total lung area. Lung sections were photographed under light microscopy at $\times 10$ magnification. Data are means $\pm$ SEM; $n = 6$ mice per group.

were challenged with carbachol. Dose response curve of carbachol was performed by administration of 50 μ l bolus of each dose, followed by intervals of 15 min in which lungs were perfused with buffer only.

2.3. Flow cytometry

Lungs were isolated and digested with 1 U/mL collagenase (Sigma Aldrich, Milan, Italy). Cell suspensions were passed through 70 μ m cell strainers, and red blood cells were lysed. Cell viability of lung cell dispersion was checked by means of propidium iodide (data not shown). We had less than 5% of non-viable cells. Cell suspensions were used for flow cytometric analysis of different cell subtypes. The composition of lung inflammatory cells was determined by flow cytometry (BD FACS Calibur, Milan, Italy) using the following antibodies: CD11c-APC, CD11b-PeCy5.5, cKit-PeCy5.5 or

PE, IgE-FITC, CD3-PeCy5.5, anti-CD4-FITC, anti-CD8-APC, B220-PE, CD19-PeCy5.5, CD23-PE (eBioscience, San Diego, CA). Appropriate isotype controls were used.

2.4. Immunohistochemistry

Lung lobes were formalin-embedded and 7 μ m sections were obtained. The degree of inflammation was scored by blinded observers by using hematoxylin and eosin (H&E) and periodic acid/Alcian blue/Schiff (PAS) staining. PAS Staining (Sigma Aldrich, Milan Italy) was performed according to the manufacturer's instructions. PAS+ cryosections were graded with scores 0 to 4 to describe low to severe lung inflammation as follows: 0: $<5\%$; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: $>75\%$ positive staining/total lung area. Lung sections were photographed under light microscopy at $\times 10$ magnification. CD23 expression was performed by using

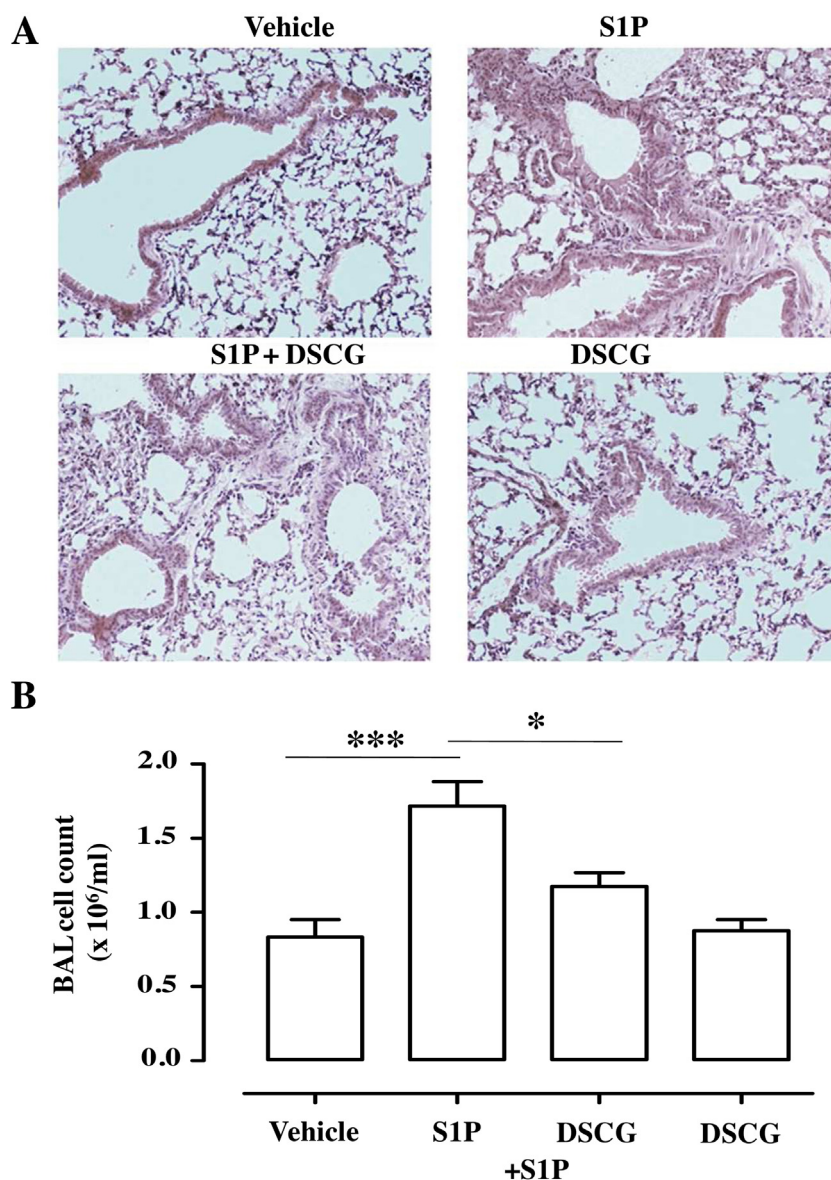


Fig. 4. DSCG inhibits S1P-induced pulmonary inflammation.

A: Lung sections were fixed and stained with H&E and photographed under light microscopy at $\times 10$ magnification. B: Lung inflammatory cell accumulation was quantified in bronchoalveolar lavage fluid (BAL; * $P < 0.05$ vs. S1P; *** $P < 0.001$ vs. S1P). Data are means $\pm$ SEM; $n = 6$ mice per group.

anti-CD23 (eBioscience, San Diego, CA) or rat IgG isotype control. The diaminobenzidine 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 with the total area of the lung section. At least five sections were considered for each animal and the mean of the positive staining compared with the total area was plotted.

2.5. Assessment of inflammatory cells in bronchoalveolar lavage (BAL) and PGD₂ levels

BAL was collected (14 days) through a 20-gauge angiocath by instilling 0.5 ml of sterile PBS into the mouse lung and repeated three times. BAL was centrifuged 1200g for 10 min at 4 °C. The total cell numbers were counted using a hemocytometer. PGD₂ quantification has been performed by using EIA Kit (Cayman, Ann Arbor, MI, USA).

2.6. Measurement of serum IgE and cytokine expression in the lung

Mice were sacrificed at 14 days. Blood was collected by cardiac puncture. Total serum IgE levels were measured by using ELISA kit assay (BD Pharmingen, Franklin Lakes, NJ, USA). Lungs were isolated and digested with 1 U/mL collagenase (Sigma Aldrich, Milan, Italy). The homogenate was centrifuged (4 °C, 6000g, 10 min). Total protein concentration was measured by the Bradford method with bovine serum albumin (BSA) as standard. The levels of IL-4 (R&D System, UK) and IL-13 (eBioscience, CA, USA) were measured with commercially available enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. The cytokine levels has been calculated as pg/mg of protein.

2.7. Statistical analyses

Data are means $\pm$ SEM from at least 6 mice in each group. The level of statistical significance was determined by using GraphPad

Prism software. Two-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons or Student's *t*-test were carried out when appropriate. *P* value <0.05 was considered statistically relevant.

3. Results

3.1. DSCG inhibits S1P-induced airway hyper-responsiveness

BALB/c mice received subcutaneous administration of S1P on days 0 and 7. S1P caused a significant increase in bronchial reactivity to carbachol (Fig. 1A). Intraperitoneal administration of DSCG (50 mg/kg) on days 0 and 7 brought down S1P-induced bronchial hyper-reactivity (Fig. 1A). It has been widely demonstrated the key role of S1P signaling in mast cell dependent allergic inflammation and airway hyper-responsiveness. Accordingly we have already showed that S1P-induced increase in airway reactivity was associated to pulmonary mast cells recruitment in the lung [18]. In order to further correlate the active role of mast cells to S1P-induced airway hyper-reactivity, we performed additional experiments depleting mast cells by using CM48/80. The results obtained showed that treatment of BALB/c mice with CM48/80, similarly to DSCG, abrogated S1P-induced hyper-reactivity (Fig. 1B).

To confirm that the changes observed in bronchial responsiveness were coupled to similar effects on the lung function, pulmonary resistances (R_L) were measured in anesthetized, tracheotomized and ventilated mice using a whole-body plethysmography. R_L measurements demonstrated a significant increase in the response to carbachol in S1P-treated mice, that was abrogated by DSCG (Fig. 2A). This beneficial effect on lung function well correlated with a significant reduction in S1P-induced mast cell recruitment as evidenced by flow cytometry analysis (Fig. 2B).

In order to further correlate DSCG effect on S1P-induced airway hyper-reactivity to mast cell activity, we measured pulmonary expression of PGD_2 , a major cyclooxygenase product generated by activated mast cells during an allergic response. As expected, S1P induced an increased PGD_2 biosynthesis, that was significantly inhibited by pretreatment with DSCG (Fig. 2C). Similarly, we observed a significant reduction of S1P-induced mast cell degranulation following DSCG treatment (Fig. 2D). Conversely, DSCG administration to vehicle treated mice did not induce any effect on all parameters measured (Figs. 1 and 2). Therefore, a correlation between beneficial effects of DSCG on airway reactivity and mast cell activity exists in S1P-treated mice.

3.2. DSCG inhibits S1P-induced allergic inflammation

Lungs, harvested from S1P-treated mice, displayed an altered bronchial structure with a marked hyperplasia (Fig. 3A) and an increased mucus production as determined by PAS staining (Fig. 3B). Treatment with DSCG prior to S1P challenge significantly reduced goblet cells staining (Fig. 3A and B). In order to assess whether these effects were sustained by an inflammatory reaction, cell infiltration was analysed. The data obtained showed a progressive S1P-induced cell infiltration that was partly reduced when the mice were pretreated with DSCG (Fig. 4A and B).

S1P, as we already demonstrated, promotes a Th2-associated inflammation, characterized by a significant increase in IgE plasma levels. Interestingly, DSCG treatment significantly reduced serum IgE levels after S1P challenge (Fig. 5A). Evaluation of cytokine production in mouse lungs after S1P challenge showed that S1P promotes the production of "classical" Th2-type cytokines, mainly IL-4 and IL-13. However, treatment with DSCG did not show any significant effect on IL-4 release in S1P-treated mice (Fig. 5B), while

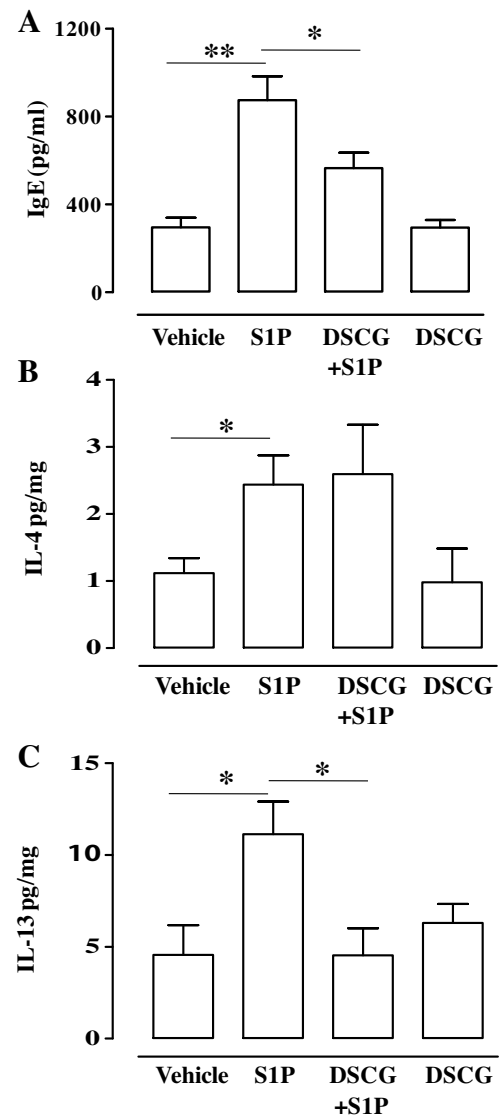


Fig. 5. DSCG decreases IgE and IL-13, but not IL-4 levels.

A: Serum levels of total IgE were determined by a specific ELISA (**P* < 0.05; ***P* < 0.01 vs. S1P). B and C: Lungs were isolated and digested with 1 U/mL collagenase. Expression of IL-4 and IL-13 have been determined by ELISA (**P* < 0.05 vs. S1P). Data are means ± SEM; *n* = 6 mice per group

a significant reduction was observed in pulmonary IL-13 release (Fig. 5C).

3.3. DSCG reverses S1P-induced CD23 up-regulation in the lung

Mast cells account for the airway responses to antigen challenge. However, in this context a critical role is attributed to IgE dependent systemic mast cell activation [36]. CD23 is an important regulatory receptor for IgE production and its interaction with IgE amplifies IgE-associated immune responses [37] and we have already demonstrated its involvement in our experimental model [18]. On the other hand, IL-4 is the most potent inducer of CD23 expression. Here, we observed that S1P up-regulated CD23 level in the lung, as demonstrated by immunohistochemistry, and this effect was reversed by DSCG treatment (Fig. 6A and B). In order to assess the role of mast cells in CD23 up-regulation we followed a similar approach by using mast cell-deficient Kit^{W-sh/W-sh} mice. We have already demonstrated that systemic administration of S1P in the C57/Bl6 mice, the background of mast cell-deficient mice, induces

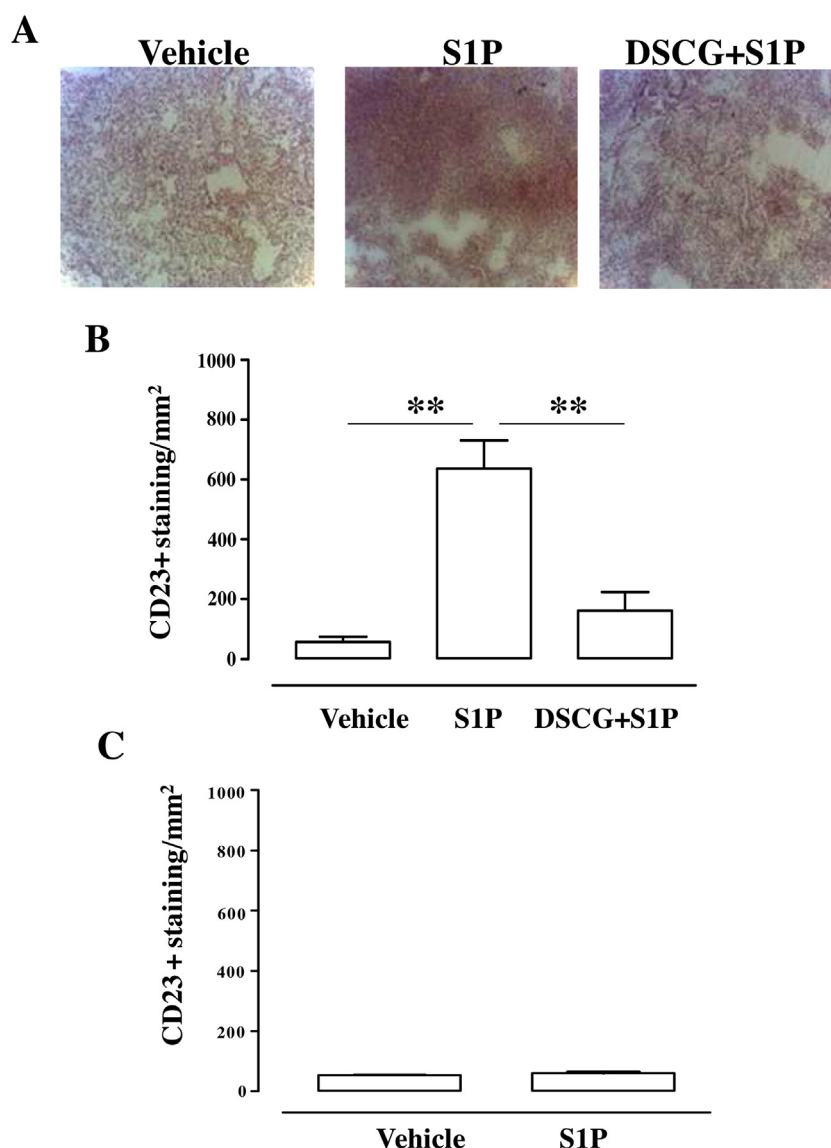


Fig. 6. S1P increases CD23 expression in the lung.

A: Immunohistochemical detection of CD23 was performed on lung sections. **B:** Positive staining was quantified and expressed as CD23 positive staining compared with the total area of the lung section. At least five sections were considered for each animal and the mean of the positive staining compared with the total area was plotted (** $P < 0.01$ vs. S1P). **C:** Pulmonary immunohistochemical detection of CD23 in mast cell-deficient ($\text{Kit}^{\text{W-sh/W-sh}}$) mice receiving s.c. administration of S1P (10 ng) or vehicle (BSA 0.001%) on days 0 and 7. Data are means $\pm$ SEM; $n = 6$ mice per group.

asthma like features too. As shown in Fig. 6C, lungs harvested from $\text{Kit}^{\text{W-sh/W-sh}}$ mice did not display any increase in CD23 expression following exposure to S1P. This data well fits with published evidence demonstrating that administration of anti-CD23 *per se* did not affect S1P-induced increase in pulmonary mast cell infiltration. Conversely, anti-CD23 significantly reduced S1P-associated IgE increase and, in turn, airway hyper-reactivity and lung inflammation when give *in vivo* [18]. Thus, collectively this data suggests that DSCG interferes with S1P/CD23 signaling.

3.4. DSCG reduces B cells recruitment and CD23 expression in the lung

In order to further gain insight into the molecular mechanisms underlying the beneficial action of DSCG on S1P-induced effects on lung, we performed flow cytometry analysis on whole lung at 14 days. Following S1P challenge, we did not observe any effect on either CD4⁺ (Fig. 7A) or CD8⁺ T cells (Fig. 7B) recruitment to the

lung; nonetheless, S1P administration increased influx of B cells (Fig. 7C). DSCG treatment reduced the recruitment of B cells to the lung after S1P exposure (Fig. 7C) accordingly with the observed reduced IgE levels.

Although a similar number of T cells was observed in the lungs harvested from all mice treated with vehicle or S1P or S1P plus DSCG, treatment of S1P-exposed mice with DSCG significantly reduced the expression of CD23 on both CD4⁺ (Fig. 7D) and CD8⁺ T cells (Fig. 7E). Similarly, DSCG reduced CD23 expression on B cells in the lung of S1P-exposed mice (Fig. 7F). Quadrant flow cytometry graphs have been reported as Supplemental Fig. 1.

4. Discussion

Sphingolipids and their altered metabolism have emerged as potential key contributors to the pathogenesis of asthma [38]. The balance between sphingosine and its phosphorylated form represents a rheostat for the induction of allergic responsiveness, during

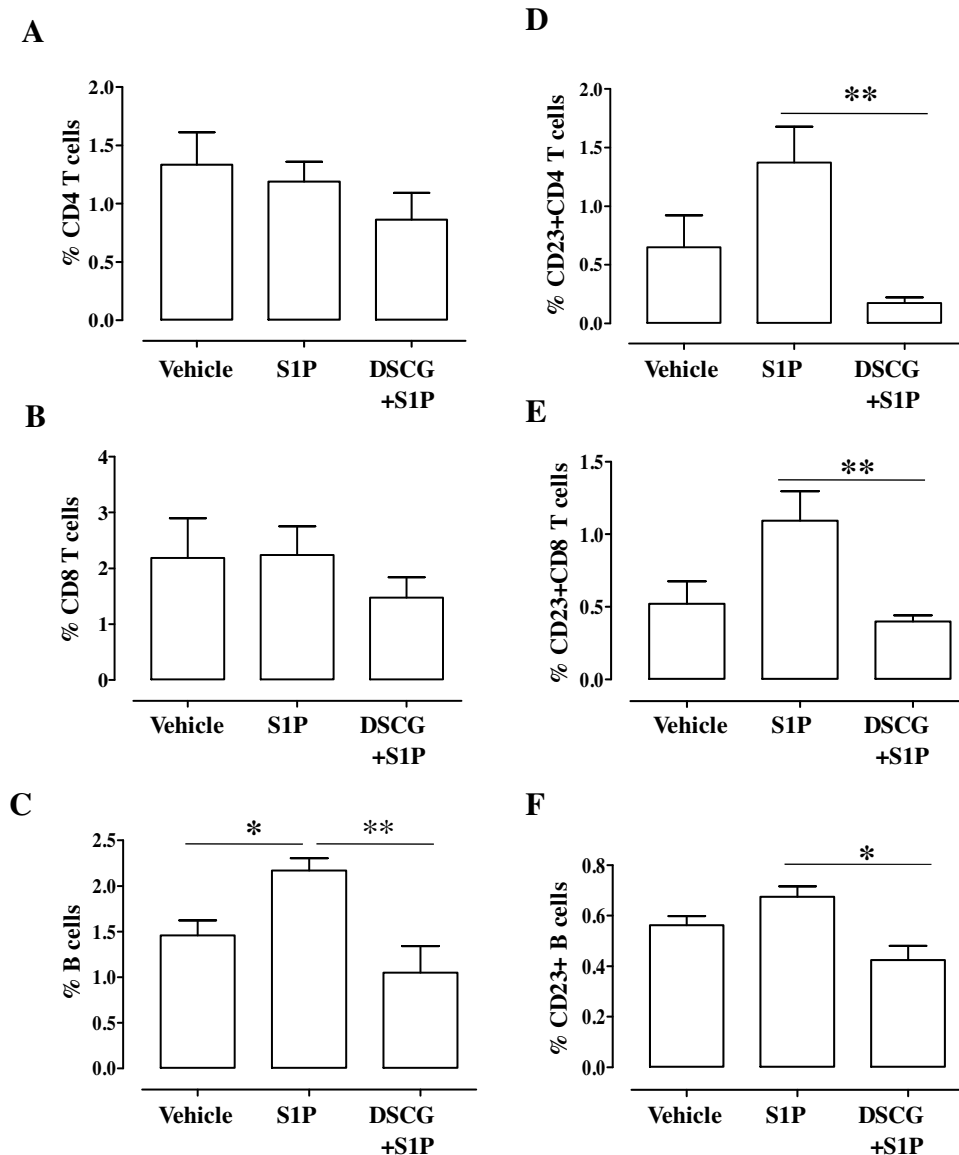


Fig. 7. DSCG reduces S1P-induced CD23 expression on CD4+ T, CD8+ T and B lymphocytes.

Flow cytometry analysis was performed on enzymatically digested lungs harvested from vehicle-, S1P-, or S1P + DSCG-treated mice. Cell suspensions were stained for CD3-PeCy5.5, CD4-FITC, CD8-APC to identify CD4+ (A) and CD8+ T cells (B). B cells were identified as B220-FITC, CD19-PeCy5.5 (C). The expression of CD23 was evaluated by using an anti-CD23-PE. CD23 expression was reduced on CD4+ T cells (D), CD8+ T cells (E) and B cells (F) recruited to the lung of S1P + DSCG treated vs. S1P-treated mice (* $P < 0.05$; ** $P < 0.01$ vs. S1P). Data are means $\pm$ SEM; $n = 6$ mice per group.

which mast cells play a pivotal role. Preclinical studies suggest that targeting S1P generation may be a productive mean of treating inflammatory diseases that involve mast cells [8]. Here, we demonstrate the beneficial effects of DSCG on asthma like features induced by S1P in the mouse.

DSCG has been shown not to be equally effective in all asthma phenotypes. This finding has been ascribed to species- or tissue-dependent differences in the responsiveness of mast cell populations [39]. Therefore mast cell heterogeneity is a crucial point to consider. Indeed, influenced by signals in their environment, they acquire their characteristic granularity by storing a variety of substances, including proteases, proteoglycans and vascular mediators in intracellular vesicles [40,41]. On the other hand S1P is widely recognised as a critical mediator inducing degranulation and chemokine/cytokine release from both human and rodent mast cells [13]. Genetic deletion or silencing of SPKs result in pronounced deficiencies in both immediate and delayed mast cell responses [7].

Our previous paper has established that systemic administration of S1P induces a significant increase in airway reactivity coupled to a pulmonary inflammation in the mouse. Here we show that DSCG treatment prevented both S1P-induced airway hyper-responsiveness and lung inflammation. Similar results were obtained when mice were depleted of mast cells with CM48/80. The involvement of mast cells in DSCG beneficial action was further supported by the modulation of the pulmonary levels of PGD₂, a major cyclooxygenase product generated by activated mast cells during an allergic response [42]. Indeed, S1P-induced increase of pulmonary PGD₂ was reversed by DSCG.

Since contradictory data are present in literature about the ability of cromones to affect mast cells in mice [29,43], we performed additional experiments. In particular we evaluated DSCG ability to affect S1P-induced mast cell recruitment and degranulation in our experimental conditions. The results obtained confirmed the efficacy of DSCG to modulate mast cell activity in the mouse when challenged with S1P.

We have previously demonstrated that mast cells are critical player of a cascade of events that sequentially involves T-cells, B cells and IgE, leading to the asthma-like symptoms in S1P-treated mice. Considering the contradictory data on DSCG's effectiveness and its selectivity on mast cells, we went on, further investigating on the involvement of S1P pathway in the cellular mechanisms underlying DSCG beneficial action. The data obtained demonstrate that DSCG did not interfere with Th2 bias. Indeed, DSCG reversed S1P effect on IgE, IL-13 and PGD₂, but did not affect IL-4 release as well as pulmonary T cell infiltration.

We have previously shown a predominant Th2 bias in S1P-induced asthma like model in mice with an obligatory role for CD23/IgE signaling to trigger immune responses [18]. Considering the efficacy of DSCG in the control of all S1P-induced asthma like features, we also evaluated its effect on CD23 signaling. DSCG treatment reversed S1P induced pulmonary CD23 upregulation. Again we wondered about the role of mast cells and for this purpose we used mast cell-deficient Kit^{W-sh/W-sh} mice. Therefore, we administered S1P to these mice and we found that, in this case, S1P failed to modulate CD23 levels in lungs. The involvement of CD23 signaling in the mechanism of action of DSCG has been already proposed in the relevant literature also in humans [44]. In particular, Holen et al. showed that DSCG delivers an inhibitory signal to PBMC harvested from allergic patients expressing CD23. Accordingly, we found that DSCG reduced expression of CD23 on CD4+ and CD8+ T cells without altering the T lymphocytes recruitment to the lung. Since changes in CD23 expression alter T cells-dependent signaling [45], our data implies that DSCG could regulate T cell phenotype by reducing CD23 expression. In support of this hypothesis, we observed that, following treatment with DSCG, there is a reduction in B cell pulmonary infiltration paralleled by a reduced expression of CD23. This chain of events could explain the attenuated propagation of pulmonary inflammation, as demonstrated by reduced levels of IgE, PGD₂ and IL-13.

5. Conclusions

Asthma is one of the most common chronic inflammatory disorder of the airways. One of the barriers to successful management is the heterogeneity of asthma that can be subdivided into a number of different phenotypes [46]. Our study demonstrate that DSCG inhibits all asthma like features induced by sphingosine-1-phosphate. This beneficial effect is due to a regulatory action on mast cell activity, and, in turn, to an inhibition of IgE-dependent T and B cells responses. Since extensive species- or tissue-dependent differences in the responsiveness of mast cell populations to the inhibitory effects of DSCG have been widely reported, it is feasible that the efficacy of DSCG could be linked to asthmatic phenotypes, e.g. where this pathway plays a major role.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgment

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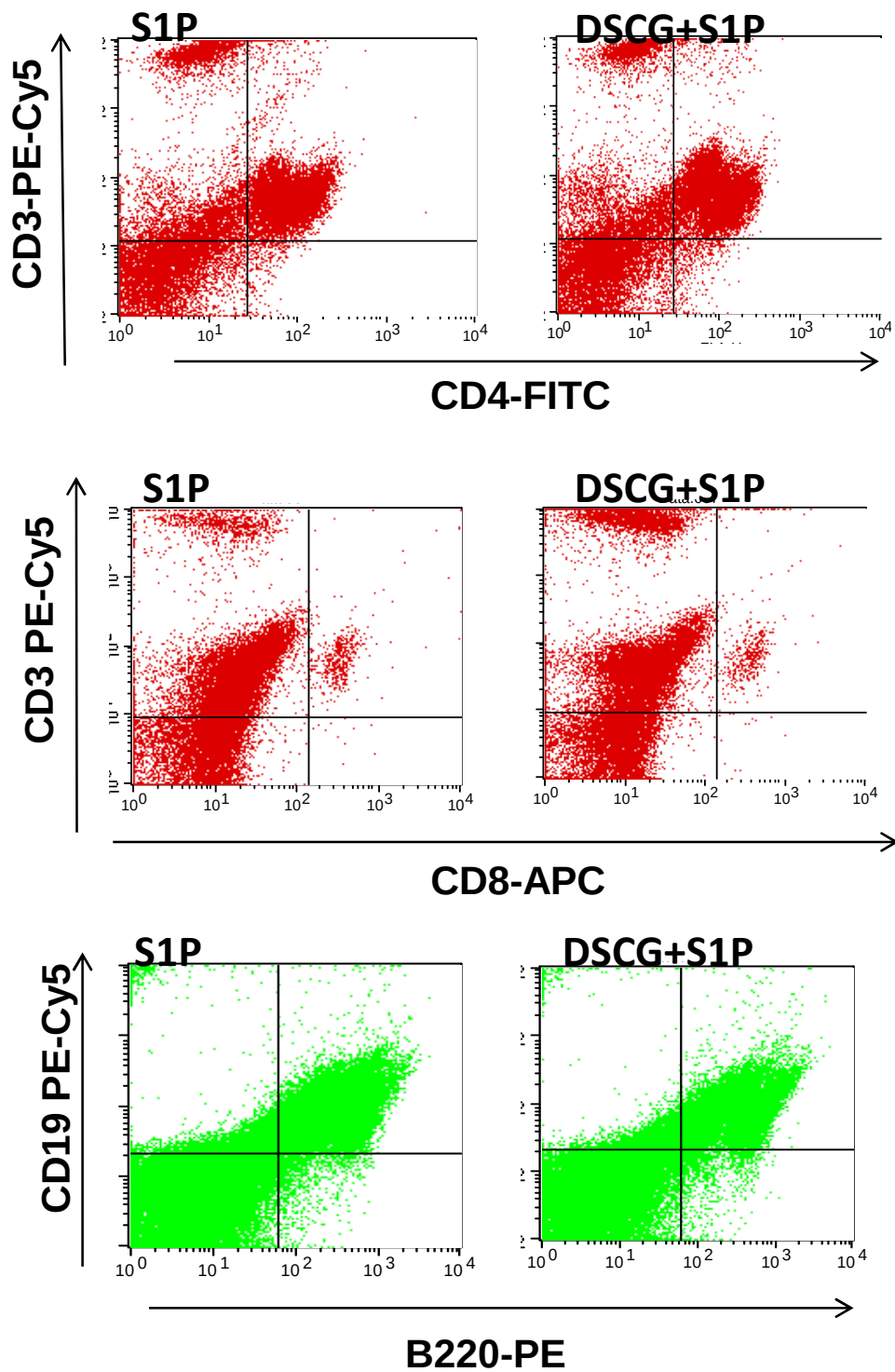
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phrs.2016.09.014>.

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Supplemental figure 1.

Representative flow cytometry quadrants of enzymatically digested lungs harvested from S1P- or S1P+DSCG-treated mice. Cell suspensions were stained for CD3-PeCy5.5, CD4-FITC, CD8-APC to identify CD4⁺ and CD8⁺ T cells . B cells were identified as B220-FITC, CD19-PeCy5.5