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Sphingosine-1-Phosphate Modulates Vascular Permeability and Cell Recruitment in Acute Inflammation In Vivo

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ABSTRACT

The sphingosine kinase (SPK)/sphingosine-1-phosphate (S1P) pathway recently has been associated with a variety of inflammatory-based diseases. The majority of these studies have been performed in vitro. Here, we have addressed the relevance of the SPK/S1P pathway in the acute inflammatory response in vivo by using different well known preclinical animal models. The study has been performed by operating a pharmacological modulation using 1) L-cycloserine and DL-*threo*-dihydrosphingosine (DTD), S1P synthesis inhibitors or 2) 2-undecyl-thiazolidine-4-carboxylic acid (BML-241) and *N*-(2,6-dichloro-4-pyridinyl)-2-[1,3-dimethyl-4-(1-methylethyl)-1*H*-pyrazolo[3,4-*b*]pyridin-6-yl]-hydrazinecarboxamide (JTE-013), specific $S1P_2$ and $S1P_3$ receptor antagonists. After local injection of carrageenan in mouse paw S1P release significantly increases locally and decreases during the resolution phase. Expression of SPKs and $S1P_2$ and $S1P_3$ receptors is increased in inflamed tissues. Administration of L-cycloserine or DTD caused a significant anti-inflammatory effect. By using different animal models we have also demonstrated that the SPK/S1P pathway contributes to changes in vascular permeability and promotes cell recruitment. The S1P effect on cell recruitment results is receptor-mediated because both JTE-013 and BML-241 inhibited zymosan-induced cell chemotaxis without effect on vascular leakage. Conversely, changes in vascular permeability involve mainly SPK activity, because compound 48/80-induced vascular leakage was significantly inhibited by DTD. In conclusion, the SPK/S1P pathway is involved in acute inflammation and could represent a valuable therapeutic target for developing a new class of anti-inflammatory drugs.

Introduction

Sphingolipids are formed either via the metabolism of sphyngomyelin, a constituent of the plasma membrane, or by de novo synthesis (Hannun and Obeid, 2008). During the last few years, it has become clear that sphingolipids, in addition to being structural constituents of cell membranes, are sources of important signaling molecules (Bartke and Hannun, 2009; Maceyka et al., 2009). Indeed, there are different enzymatic pathways that result in the formation of various lipid mediators, which are known to have important roles in many cellular processes, including proliferation, apoptosis, and migration. In particular, the sphingolipid metabolites, ceramide and sphingosine-1-phosphate (S1P), have emerged as a new class of potent bioactive molecules implicated in a variety of pathophysiological processes (Chalfant et al., 2005).

Several studies now suggest that these sphingolipid mediators, including S1P, have an integral role in inflammation (El Alwani et al., 2006; Snider et al., 2010). In particular, S1P is involved in inflammatory-based diseases such as asthma (Ammit et al., 2001; Roviezzo et al., 2007), rheumatoid arthritis (Kitano et al., 2006) multiple sclerosis (Van Doorn et al., 2010), and inflammatory bowel disease (Duan and Nilsson, 2009). Furthermore, various sphingolipid enzymes [sphingomyelinase, sphingosine kinase (SPK), etc.] are acti-

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ABBREVIATIONS: S1P, sphingosine-1-phosphate; SPK, sphingosine kinase; CM 48/80, compound 48/80; DTD, DL-*threo*-dihydrosphingosine; L-cycl, L-cycloserine; ANOVA, analysis of variance; BML-241, 2-undecyl-thiazolidine-4-carboxylic acid; JTE-013, *N*-(2,6-dichloro-4-pyridinyl)-2-[1,3-dimethyl-4-(1-methylethyl)-1*H*-pyrazolo[3,4-*b*]pyridin-6-yl]-hydrazinecarboxamide; DMSO, dimethyl sulfoxide; MPO, myeloperoxidase; PBS, phosphate-buffered saline.

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vated by inflammatory cytokines, and their downstream lipid mediators regulate inflammatory pathway in addition to immune cell functions (Bartke and Hannun, 2009). In particular, SPKs, the enzymes responsible for the production of S1P from sphingosine, have been shown to be modulated by a plethora of stimuli, including ligands for G protein-coupled receptors (S1P, bradykinin, muscarinic receptor agonist), agonist of growth factor receptor (platelet-derived growth factor, vascular endothelial growth factor, epidermal growth fact), transforming growth factor β, tumor necrosis factor α, and interleukins (Spiegel and Milstien, 2003; Taha et al., 2006).

S1P signaling is the result of a complicated system of regulation. To date, most S1P functions have been attributed to receptor-mediated signaling. S1P exerts different cellular effects depending upon the subtype of S1P receptors (S1Pn) and the downstream G protein coupled. Other biological effects such as activation of Ras and extracellular signal-regulated kinase signaling pathways by vascular endothelial growth factor or activation of phosphatidylinositol 3-kinase/Akt have been shown to be linked to S1P intracellular action because they cannot be reproduced by exogenous addition of S1P (Chalfant and Spiegel, 2005).

Although it is clear that sphingolipids can be intimately involved in the onset and maintenance of inflammation, it is still unclear whether targeting sphingolipid action could represent a feasible anti-inflammatory therapeutic strategy. There are now several compounds that have been developed that can manipulate different component of the sphingosine pathway, e.g., S1P synthesis inhibitor or selective S1P receptors antagonists (Koide et al., 2002; Nishiuma et al., 2008; Chiba et al., 2010; Imasawa et al., 2010).

Here, we have addressed the relevance of the SPK/S1P pathway in the acute inflammatory response in vivo. We have used drugs able to interfere with 1) S1P synthesis, by pharmacological modulation with appropriate inhibitors or 2) S1P action, by using specific receptor antagonists. Our study focuses mainly on the early stage of inflammation, analyzing the SPK/S1P pathway contribution to vascular leakage or cell infiltration by using different preclinical well known models.

Materials and Methods

Materials. DL-*threo*-Dihydrosphingosine (DTD; an inhibitor of both isoforms of SPKs), L-cycloserine (L-cycl; an inhibitor of sphingolipid metabolism), and 2-undecyl-thiazolidine-4-carboxylic acid (BML-241) (an $S1P_3$ /$S1P_2$ antagonist) were purchased from Vinci Biochem (Vinci, Italy). *N*-(2,6-Dichloro-4-pyridinyl)-2-[1,3-dimethyl-4-(1-methylethyl)-1*H*-pyrazolo[3,4-*b*]pyridin-6-yl]-hydrazinecarboxamide (JTE-013) (an $S1P_2$ antagonist) was purchased from Tocris Bioscience (Bristol, UK). Anti-$S1P_1$, anti-$S1P_2$, anti-$S1P_3$, anti-$S1P_4$, anti-SPK_1, and anti SPK_2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Compound 48/80 (CM 48/80) and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Animals. Male CD-1 mice (Harlan, Milan, Italy) weighing 20 to 25 g were separated into groups ($n = 6$). All animal experiments were approved by and performed under the guidelines of the Ethical Committee for the use of animals, Barts and The London School of Medicine and Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986), and the Animal Ethical Committee of the University of Naples Federico II.

Sphingosine-1-Phosphate Measurement. Mice received subplantar administration of 50 μl of carrageenan (1% w/v) (Posadas et al., 2004) or vehicle (saline). Mice were sacrificed with carbon dioxide 2, 4, or 6 h after carrageenan administration. Paws were cut and centrifuged at 4000 rpm for 30 min. Exudates (supernatants) were collected with 100 μl of saline and used for S1P quantification by using a commercially available enzyme-linked immunosorbent assay kit (Echelon; Tebu-bio, Versailles, France).

Western Blot. Mice received a subplantar administration of 50 μl of carrageenan (1% w/v) or vehicle and were sacrificed after 2, 4, or 6 h. The injected paws were cut and used for Western blot analysis. In brief, mouse paws were homogenized in 10 mm HEPES pH 7.4 buffer containing saccharose (0.32 M), EDTA (100 μM), dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (1 mg/ml), and leupeptin (10 μg/ml) with a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland) (three cycles of 10 s at maximum speed). After centrifugation at 3000 rpm for 15 min, protein supernatant content was measured by Bradford reagent, and protein concentration was adjust at 30 μg. Protein samples were loaded on 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes for 45 min at 250 mA. Membranes were blocked in PBS-Tween 20 (0.1%) containing 5% nonfat milk and 0.1% bovine serum albumin for 30 min at 4°C. Membranes were washed with PBS-Tween 20 (0.1%) at 5-min intervals for 30 min and incubated with anti-$S1P_1$ (1:1000; Santa Cruz Biotechnology, Inc.) anti-$S1P_2$ (1:1000; Santa Cruz Biotechnology, Inc.), anti-$S1P_3$ (1:1000; Santa Cruz Biotechnology, Inc.), anti-$S1P_4$ (1:1000; Santa Cruz Biotechnology, Inc.), anti-SPK_1 (1:2000; Santa Cruz Biotechnology, Inc.), or anti-SPK_2 (1:2000; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Blots were washed with PBS-Tween 20 (0.1%) at 5-min intervals for 30 min and incubated with secondary antibody for 2 h at 4°C. The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Protein levels were evaluated through densitometry (how intense the stain was) versus β-actin.

Mouse Paw Edema. Mice were lightly anesthetized with enflurane and received subplantar injection of 50 μl of carrageenan (1% w/v) (Posadas et al., 2004). Paw volume was measured using a hydroplethismometer especially modified for small volumes (Ugo Basile, Comerio, Italy) immediately before the subplantar injection (basal value) and 2, 4, and 6 h thereafter.

Mice were divided into three groups ($n = 6$) and received intraperitoneal administration of 1) L-cycl, a sphingolipid metabolism regulator with inhibitory activity of serine palmitoyltransferase that catalyzes the first step of sphingolipid biosynthesis (10–100 mg/kg in 200 μl) 24 h and 30 min before the beginning of inflammation (Williams et al., 1987), 2) DTD, an inhibitor of both isoforms of SPKs (0.3–3 mg/kg), 30 min before inflammogen application (Nishiuma et al., 2008), or 3) vehicle (DMSO/saline 0.1%). To assess the efficacy of these drugs, in a separate set of experiment we evaluated S1P release after inflammatory challenge in the presence of L-cycl and DTD using the dose that was determined previously to give the maximal response.

MPO Measurement. Mice from different groups were sacrificed with CO_2 after 2, 4, and 6 h from carrageenan injection. Injected paws were cut, weighed, and homogenated in 1 ml of hexadecyltrimethyl-ammonium bromide buffer using a Polytron homogenizer (two cycles of 10 s at maximum speed). After centrifugation of homogenates at 10,000 rpm for 2 min, supernatant fractions were assayed for MPO activity using the method described by Bradley et al. (1982). In brief, samples were mixed with phosphate buffer containing 1 mM *O*-dianisidine dyhydrochloride and 0.001% hydrogen peroxide in a microtiter plate reader. Absorbance was measured at 450 nm, taking three readings at 30-s intervals. Units of MPO were calculated considering that 1 U MPO = 1 μmol H_2O_2 split, and 1 μmol H_2O_2 gives a change in absorbance of 1.13×10^{-2} nm/min.

Intravital Microscopy. Mice were divided into three groups ($n = 6$) and received intraperitoneal administration of 1) L-cycl, an inhibitor of sphingolipid metabolism (30 mg/kg in 200 μl) 24 h and 30 min before the beginning of inflammation, 2) DTD, an inhibitor of both

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isoforms of SPKs (1 mg/kg) 30 min before inflammogen application, or 3) vehicle (DMSO/saline 0.1%). Experiments began with intraperitoneal injections of 1 mg of zymosan (in 0.5 ml of sterile saline); 4 h later, when neutrophil infiltration was maximal (Gavins and Chatterjee, 2004), the vascular mesenteric bed was prepared for microscopic observation. Mice were anesthetized with diazepam (6 mg/kg s.c.) and Hypnorm (0.7 mg/kg fentanyl citrate and 20 mg/kg fluanisone i.m.). Cautery incisions were made along the abdominal region, and the mesenteric vascular bed was exteriorized and placed on a viewing Plexiglas stage (Gavins and Chatterjee, 2004). The preparation was mounted on a Zeiss Axioskop FS (Carl Zeiss GmbH, Jena, Germany) with a water immersion objective lens (magnification 40×), and an eyepiece (magnification 10×) was used to observe the microcirculation. The preparation was transilluminated with a 12-V, 100-W halogen light source. A Hitachi (Tokyo, Japan) charge-coupled device color camera (model KPC571) acquired images that were displayed on a Sony (Tokyo, Japan) Trinitron color video monitor (model PVM 1440QM) and recorded on a Sony superVHS video cassette recorder (model SVO-9500 MDP) for subsequent offline analysis. A video time-date generator (FOR.A video timer, model VTG-33; Broadcast Store, Los Angeles, CA) projected the time, date, and stopwatch function onto the monitor. Mesenteries were superfused with bicarbonate-buffered solution at 37°C (7.71 g/l NaCl, 0.25 g/l KCl, 0.14 g/l $MgSO_4$, 1.51 g/l $NaHCO_3$, and 0.22 g/l $CaCl_2$, 0.22, pH 7.4; gassed with 5% CO_2/95% N_2) at a rate of 2 ml/min. The temperature of the stage was maintained at 37°C. This procedure has no effect on rectal temperature or blood pressure (data not shown). Red blood cell velocity was measured in venules by using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, TX). Venular blood flow was calculated from the product of mean red blood cell velocity (V_{mean} = centerline velocity/1.6) and microvascular cross-sectional area, assuming a cylindrical geometry. Wall shear rate was calculated by the Newtonian definition: shear rate = 8000 × (V_{mean}/diameter). One to three randomly selected postcapillary venules (diameter between 20 and 40 μm, length at least 100 μm) were observed for each mouse; measurements were taken 5 to 10 min after exposure of the chosen vessels.

Mouse Air Pouch. Mice were divided into five groups (n = 6) and received intraperitoneal administration of 1) L-cycl, an inhibitor of sphingolipid metabolism (30 mg/kg in 200 μl) 24 h and 30 min before the beginning of inflammation, 2) DTD, an inhibitor of both isoforms of SPKs (1 mg/kg in 200 μl) 30 min before inflammogen application, 3) an $S1P_3$ antagonist (BML-241; 0.3–3 mg/kg in 200 μl intraperitoneally) 30 min before inflammogen application, 4) an $S1P_2$ antagonist (JTE-013; 0.3–3 mg/kg in 200 μl intraperitoneally) 30 min before inflammogen application (Koide et al., 2002; Jongsma et al., 2006; Chiba et al., 2010; Imasawa et al., 2010), or 5) vehicle (DMSO/saline 0.1%). Mice were then lightly anesthetized with enflurane. Air pouches were developed by subcutaneous injection of 2.5 ml of sterile air into the back of mice (28–30 g). Three days later 2.5 ml of sterile air was reinjected into the same cavity (Posadas et al., 2000). After another 3 days (6 days after the first air injection), 1 ml of zymosan (1% w/v) or vehicle (saline) was injected into the air pouch. After 4 h from zymosan injection, when neutrophil infiltration was maximal (Posadas et al., 2000), mice were sacrificed by CO_2 exposure, and exudate in the pouch was collected with 1 ml of saline and placed in graduated tubes and centrifuged at 125g for 10 min. The pellet was suspended in 500 μl of saline, and total leukocyte count was evaluated by optical microscopy in the cell suspension diluted with Turk's solution.

Mouse Vascular Leakage. Intradermal injection of CM 48/80 induces an acute increase in vascular leakage to plasma proteins (Segawa et al., 2007). Mice were divided into five groups (n = 6) and received intraperitoneal administration of 1) L-cycl (30 mg/kg in 200 μl) 24 h and 30 min before administration of CM 48/80, 2) DTD (1 mg/kg in 200 μl) 30 min before administration of CM 48/80, 3) an $S1P_3$ antagonist (BML-241; 0.3–3 mg/kg in 200 μl intraperitoneally) 30 min before administration of CM 48/80, 4) an $S1P_2$ antagonist (JTE-013; 0.3–3 mg/kg in 200 μl intraperitoneally) 30 min before administration of CM 48/80 (Koide et al., 2002; Chiba et al., 2010; Imasawa et al., 2010), or 5) vehicle (DMSO/saline 0.1%). Anesthetized mice were injected intradermally with 5 μg of CM 48/80 dissolved in 50 μl of physiological saline into their shaved back skin immediately after an intravenous injection of a 0.5% Evans blue solution (5 ml/kg). Animals were sacrificed 30 min later, and skin was removed for a quantitative determination of the extravasated dye. Evans blue was neutralized by 1 ml of 6 M KOH at 45°C for 6 h. This extract was neutralized by 1 ml of 6 M HCl, and then 2 ml of acetone was added. The extract was clarified by filtration, its absorbance at 595 nm was measured, and the extracted Evans blue content was then calculated from its calibration curve.

Statistical Analysis. Data are expressed as mean ± S.E.M. The level of statistical significance was determined by one- or two-way analysis of variance (ANOVA) followed by Bonferroni's t test for multiple comparisons, using the Prism software (GraphPad Software Inc., San Diego, CA).

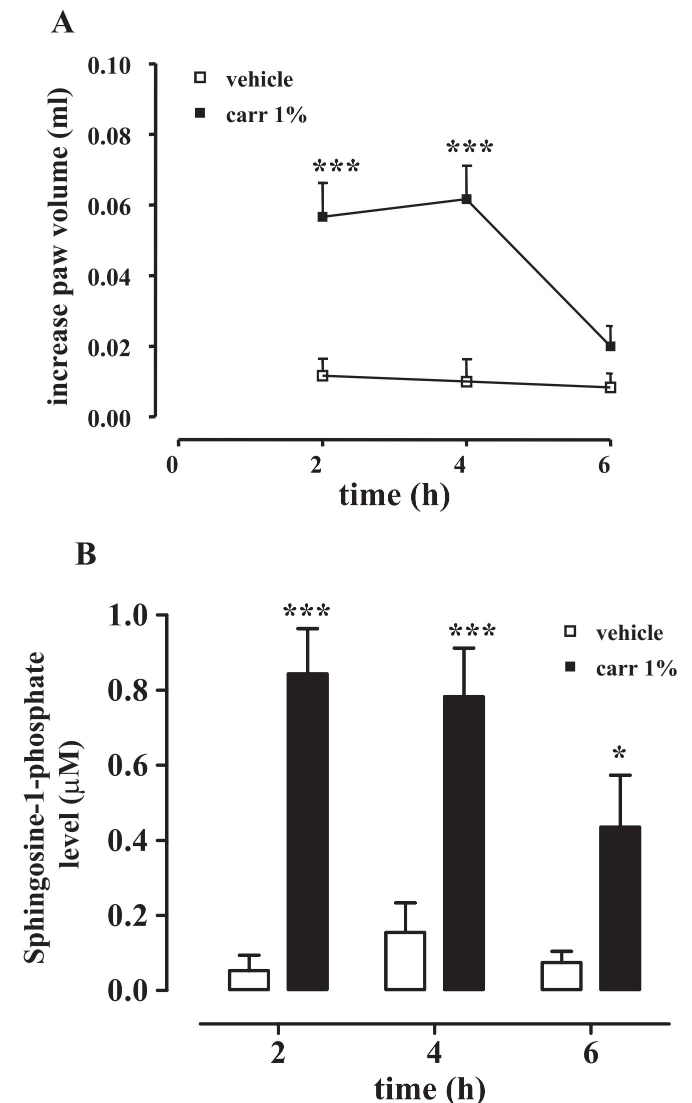

Fig. 1. S1P level is increased in inflamed tissues. A, injection of 50 μl of carrageenan (carr; 1% w/v) in the mouse paw causes an increase in paw volume that peaks at 4 h and resolves after 6 h. ***, $p < 0.001$ versus vehicle (one-way ANOVA). B, S1P levels measured in paw exudates were significantly increased at all times tested. *, $p < 0.05$; ***, $p < 0.001$ versus vehicle (two-way ANOVA).

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Results

S1P Pathway Is Up-Regulated in the Carrageenan-Injected Mouse Paw. Injection of carrageenan in mouse paw causes a time-dependent edema (Fig. 1A). S1P levels were significantly elevated in exudates harvested from mouse paws peaking at 2 h and declining at 6 h (Fig. 1B). At all time points, e.g., 2, 4, and 6 h, there was also a significant increase in $S1P_2$ (Fig. 2A) and $S1P_3$ (Fig. 2B) receptors as well as in SPK_1 (Fig. 2C) and SPK_2 (Fig. 2D). $S1P_1$ and $S1P_4$ levels were unchanged (data not shown).

S1P Synthesis Inhibition Reduces Mouse Edema Development. The involvement of S1P pathway in mouse paw edema was confirmed by experiments of pharmacological modulation. Treatment of mice with a sphingolipid biosynthesis inhibitor, e.g., L-cycl (10–100 mg/kg), significantly reduced in a dose-dependent manner carrageenan-induced edema (Fig. 3A). The S1P level in paw exudates was also significantly reduced after L-cycl treatment (Fig. 3B). Myeloperoxidase activity was markedly reduced in mice treated with L-cycl (30 mg/kg) at all time points tested (Fig. 3C).

Intraperitoneal administration of DTD (0.3–3 mg/kg), an inhibitor of sphingosine kinases, to mice caused a significant and dose-dependent edema reduction (Fig. 4A). S1P levels in the exudates were also significantly reduced by DTD (Fig. 4B). MPO activity was significantly inhibited only at 2 h (Fig. 4C).

L-Cycloserine, but Not DTD, Inhibits Leukocyte Extravasation. Intravital microscopy allows a direct visualization of the microcirculation in inflammatory conditions. Thus, we used this experimental approach to study in vivo the effect of DTD or L-cycl on neutrophil trafficking after zymosan exposure. L-Cycl (30 mg/kg) caused a significant inhibition of neutrophil adhesion (Fig. 5A) and migration (Fig. 5B), whereas rolling (Fig. 5C) was unaffected. Conversely, DTD (1 mg/kg) did not affect any of the parameters measured (Fig. 5).

L-Cycloserine Significantly Affects Zymosan-Induced Cell Chemotaxis, Whereas DTD Significantly Inhibited Vascular Leakage. Administration of zymosan in the dorsal air pouch induces a recruitment of blood-borne leukocytes, reaching its maximum at 4 h (Posadas et al., 2000). L-Cycl (30 mg/kg) significantly inhibited the recruitment (Fig. 6A) as well as reduced MPO activity in pouch skin samples (Fig. 6B). On the other hand, DTD (1 mg/kg) was ineffective on zymosan-induced cell recruitment and MPO activity (Fig. 6B). These experiments further suggest that different signaling pathways of S1P are involved into the inflammatory response through different molecular targets.

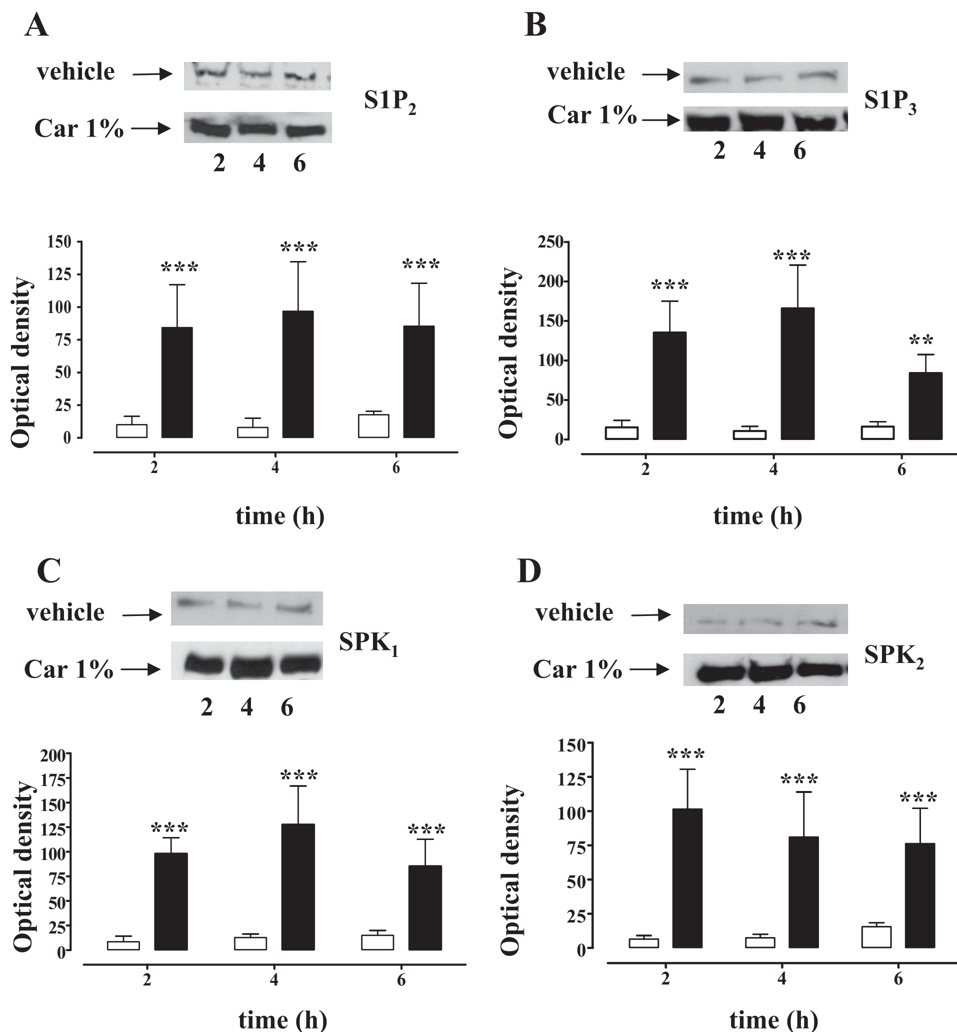

Fig. 2. Inflammation up-regulates S1P pathway. Western blot analysis for $S1P_2$ (A), $S1P_3$ (B), SPK_1 (C), or SPK_2 (D) was performed on paw tissues harvested from mice injected with 1% carrageenan (Car) after 2, 4, and 6 h. The blots are representative of three different experiments. The bar graphs were obtained by densitometric analysis performed versus β-actin. **, $p < 0.01$; ***, $p < 0.001$ versus vehicle (two-way ANOVA).

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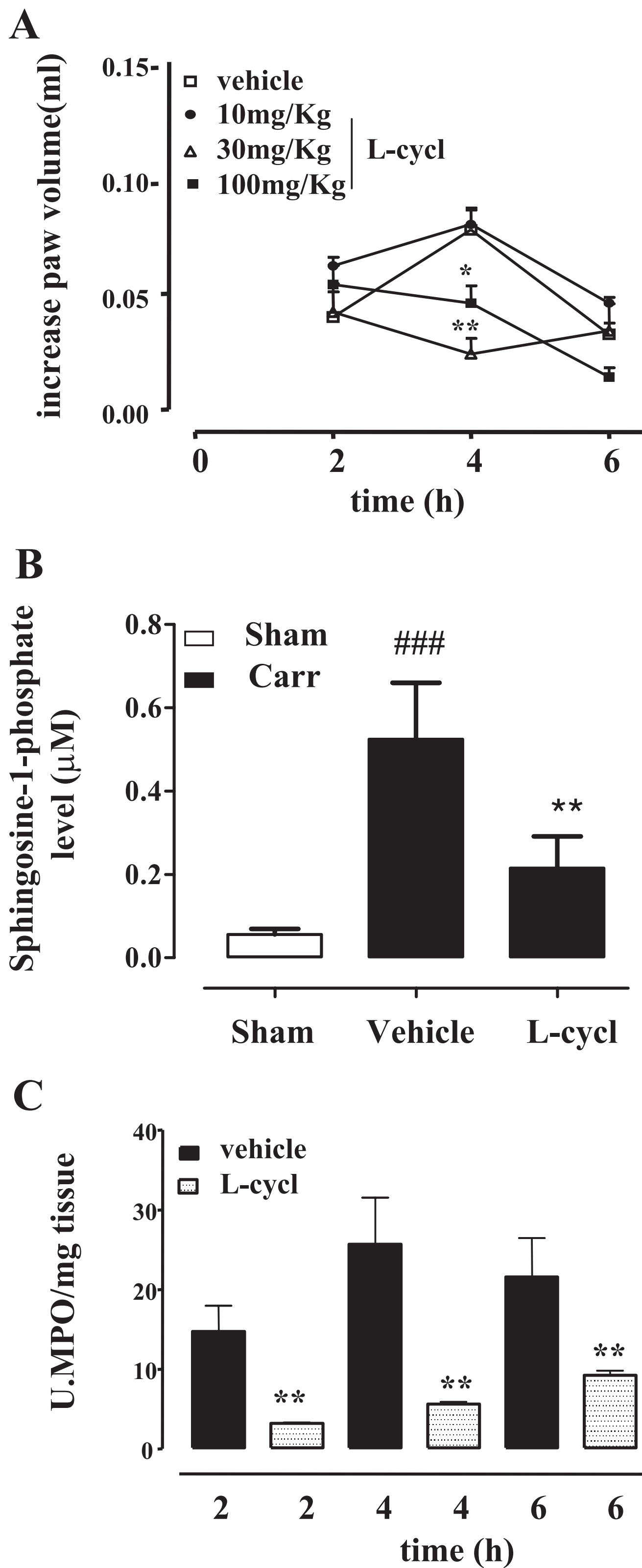

Fig. 3. Effect of L-cycl on carrageenan (Carr)-induced mouse paw edema. A, L-Cycl (10–100 mg/kg), administered to mice 24 h and 30 min before carrageenan subplantar injection, significantly reduced paw edema development. *, $p < 0.05$; **, $p < 0.01$ versus vehicle (two-way ANOVA). B, exudates harvested from mice treated with L-cycl (30 mg/kg) displayed a significant reduction in S1P levels. ###, $p < 0.001$ versus sham; **, $p < 0.01$ versus vehicle (one-way ANOVA). C, MPO levels were significantly reduced by treatment with L-cycl (30 mg/kg). **, $p < 0.01$ versus vehicle (two-way ANOVA).

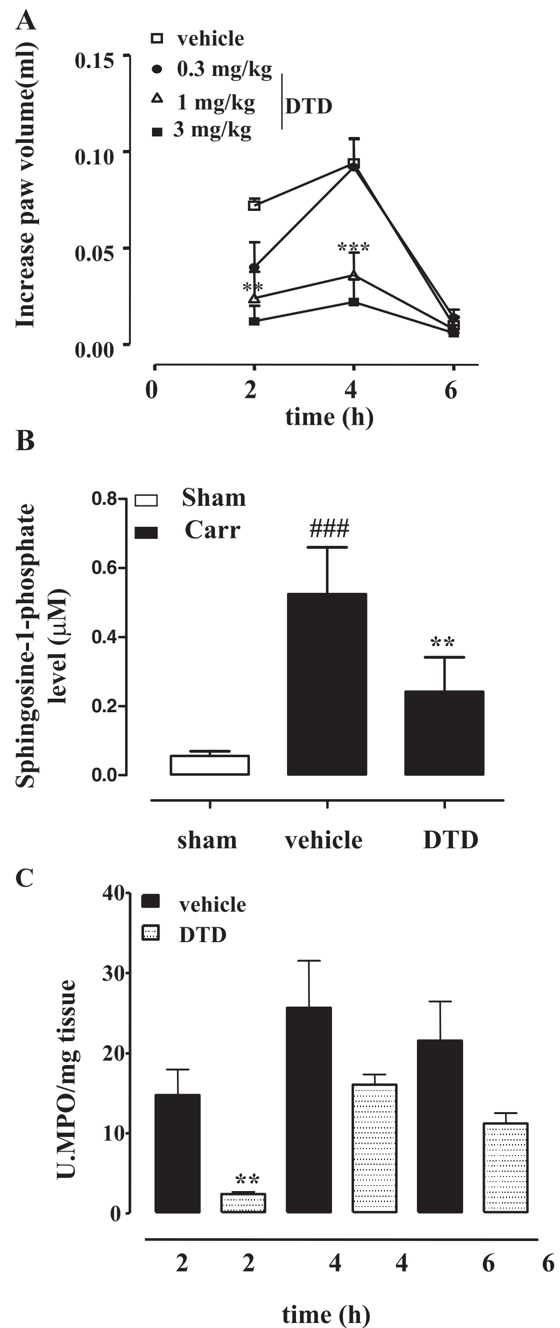

Fig. 4. Effect of DTD on carrageenan (Carr)-induced mouse paw edema. A, DTD (0.3–3 mg/kg) administered to mice 30 min before carrageenan subplantar injection significantly inhibited paw edema development. **, $p < 0.01$; ***, $p < 0.001$ versus vehicle (two-way ANOVA). B, S1P levels were significantly inhibited in exudates obtained from mice treated with DTD (1 mg/kg). ###, $p < 0.001$ versus sham; **, $p < 0.01$ versus vehicle (one-way ANOVA). C, DTD (1 mg/kg) significantly reduced MPO levels in paw tissue at 2 h. **, $p < 0.01$ versus vehicle (two-way ANOVA).

Intradermal injection of CM 48/80 (5 μg) induced an acute increase in vascular leakage (Fig. 6C). DTD (1 mg/kg) pretreatment significantly inhibited Evans blue extravasation induced by CM 48/80 (Fig. 6C). Conversely L-cycl (30 mg/kg) did not modify vascular leakage (Fig. 6C).

$S1P_2$ and $S1P_3$ Antagonists Block Cell Recruitment. It is known that S1P chemotactic proprieties are receptor-mediated, and, in particular, a key role is played by $S1P_2$ and $S1P_3$. The specific antagonists JTE-013 (blocking $S1P_2$; 1

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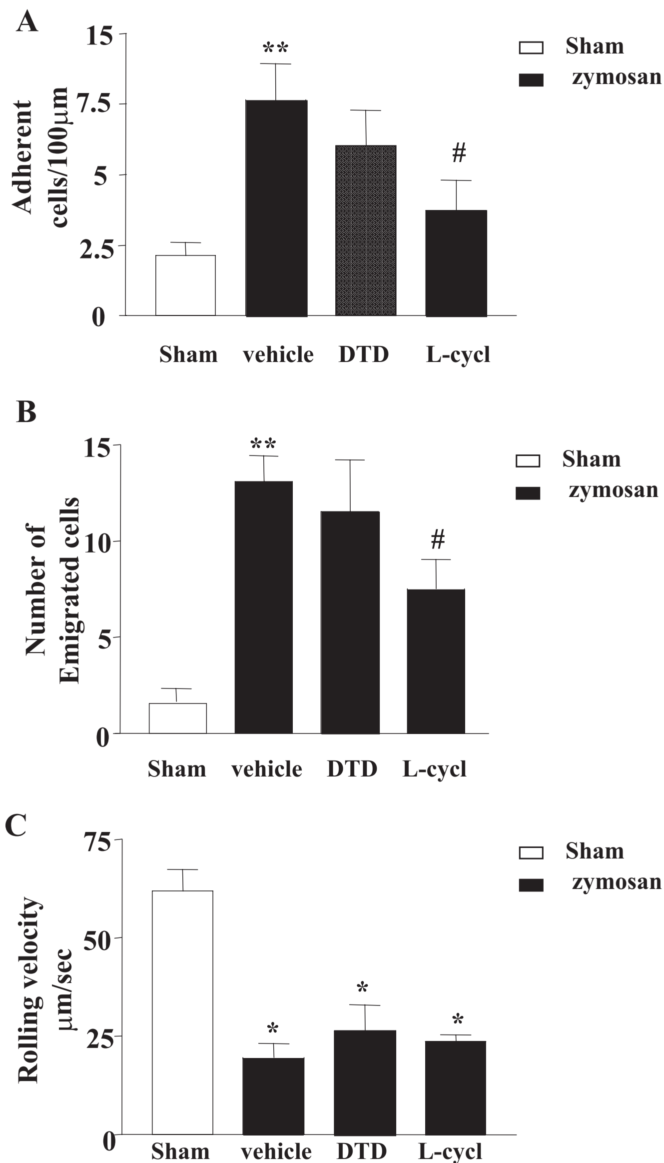

Fig. 5. Effect of L-cycl or DTD on leukocyte extravasation. L-Cycl (30 mg/kg) significantly reduced zymosan-induced adhesion (A; **, $p < 0.01$ versus sham; #, $p < 0.05$ versus vehicle) and numbers of migrated cells (B; **, $p < 0.01$ versus sham; #, $p < 0.05$ versus vehicle), whereas it did not modify rolling velocity (C; *, $p < 0.05$ versus sham). DTD (1 mg/kg) did not affect any of the parameters measured. Data were analyzed with one-way ANOVA.

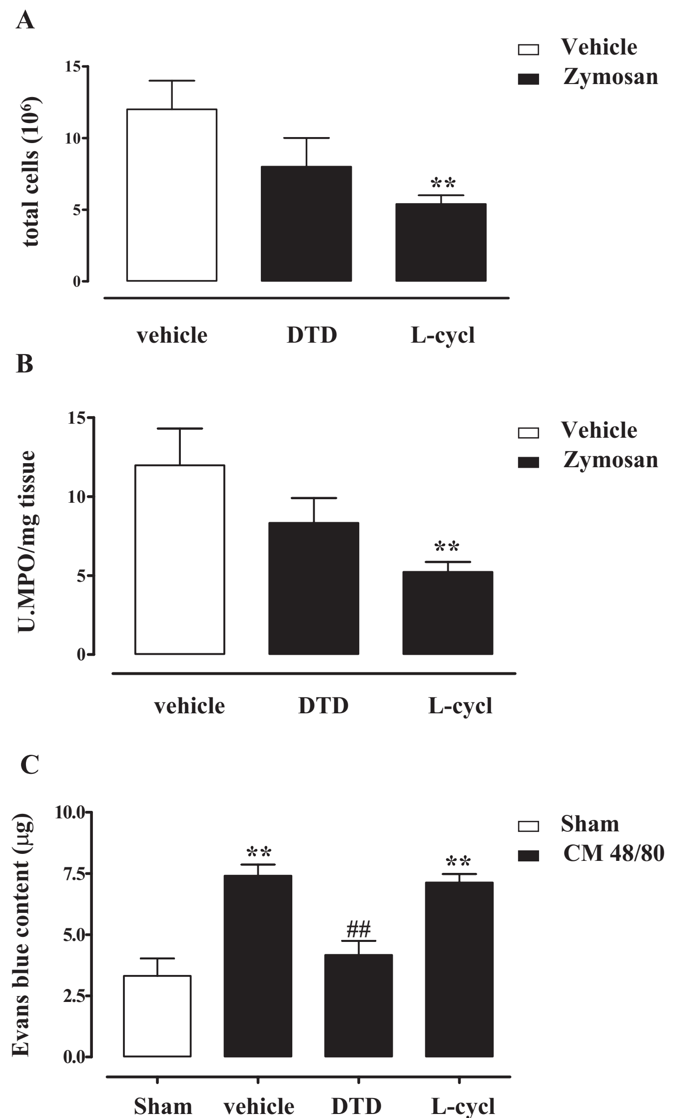

Fig. 6. Effect of L-cycl or DTD on cell infiltration and vascular permeability. A, L-cycl, but not DTD, significantly inhibited cell migration into the pouch induced by zymosan. **, $p < 0.01$ vs. vehicle. B, L-cycl, but not DTD, significantly inhibited MPO levels in skin harvested from mice pouches. **, $p < 0.01$ vs. vehicle. C, DTD, but not L-cycl, significantly inhibited CM 48/80-induced extravasations as determined by Evans blue determination. **, $p < 0.01$ versus sham; ##, $p < 0.01$ versus vehicle. Data were analyzed with one-way ANOVA.

mg/kg) and BML-241 (blocking $S1P_2$ and $S1P_3$; 1 mg/kg) inhibited in a dose-dependent manner the cell recruitment provoked by zymosan (Fig. 7A). JTE-013 (1 mg/kg) or BML-241 (1 mg/kg) did not inhibit Evans blue extravasation in mouse skin induced by CM 48/80 (Fig. 7B).

Discussion

The aim of this article was to investigate the role played in inflammation by S1P pathway in vivo in preclinical relevant models. S1P is rapidly released during acute inflammation at the site of injury and is actively involved in the onset of the inflammatory response. Indeed, S1P level significantly increases locally after inflammatory challenge and declines when inflammation resolves. At the same time in the paw there is a significant up-regulation of $S1P_2$ and $S1P_3$ receptors and both sphingosine kinases. This pattern of expression implies that the S1P pathway is involved in the onset and development of an early inflammatory reaction. This hypothesis is supported by the finding that treatment of mice with DTD, a sphingosine kinase inhibitor, or L-cycloserine, a sphingolipid metabolism inhibitor, before carrageenan administration significantly inhibited the edema. DTD modulates mainly vascular permeability, whereas L-cycloserine significantly reduces cell recruitment. In fact, DTD abrogates the first phase of edema that relies mainly on changes in vascular permeability (Posadas et al., 2000), whereas L-cycloserine markedly inhibited MPO activity, which is an index of neutrophil infiltration.

MPO evaluation does not give dynamic information on cellular trafficking but it is an indirect measure of cell infil-

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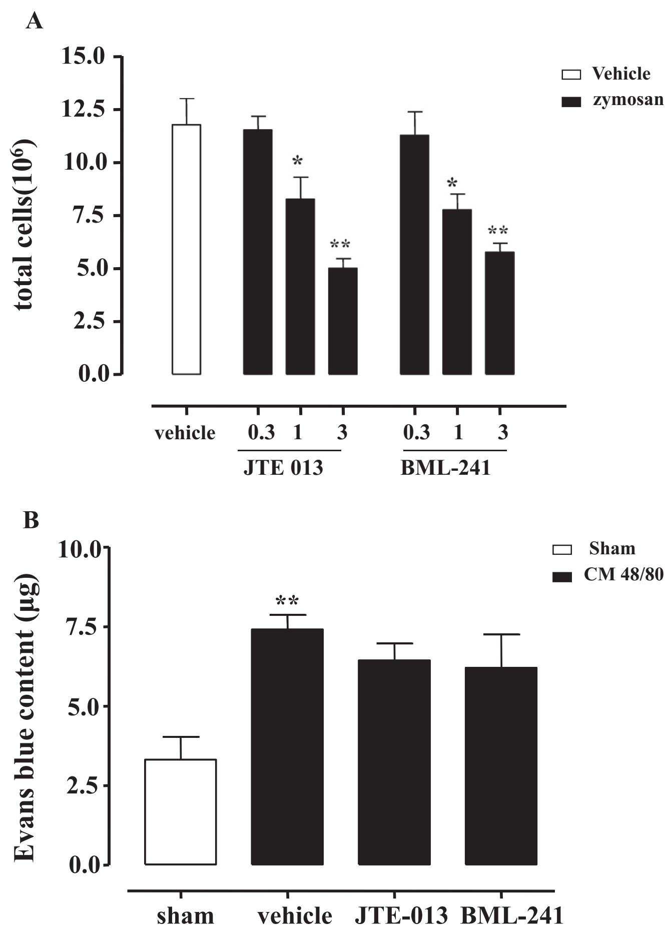

Fig. 7. Effect of antagonists of $S1P_2$ or $S1P_3$ on cell infiltration and vascular permeability. A, JTE-013 (0.3–3 mg/kg) and BML-241 (0.3–3 mg/kg) reduced in a dose-dependent manner cell accumulation in mouse air pouch. *, $p < 0.05$; **, $p < 0.01$ versus vehicle (two-way ANOVA). B, JTE-013 (1 mg/kg) and BML-241 (1 mg/kg) did not significantly inhibit CM 48/80-induced extravasations as determined by Evans blue determination. **, $p < 0.01$ versus sham (one-way ANOVA).

tration performed, in our experimental conditions, when the response peaks. To further clarify the relevance of S1P signaling in acute inflammation we used a dynamic assay, e.g., intravital microscopy. Intravital microscopy allows the identification of specific steps operating when a leukocytes leaves the bloodstream and moves to the inflamed tissue. L-Cycloserine significantly reduced cell adhesion and emigration, whereas DTD did not significantly affect both cell adhesion and emigration.

S1P has chemotactic activity (Roviezzo et al., 2004; Hashimoto et al., 2008; Ishii et al., 2009; Harvey et al. 2010; Thangada et al., 2010). To clarify whether this effect on adhesion and migration was mediated by chemotactic action of S1P on inflammatory cells, we used the mouse air pouch, a preclinical experimental model that allows evaluating chemotaxis in vivo.

L-Cycloserine produced a marked inhibition of the cell recruitment elicited by zymosan, whereas DTD was ineffective. Thus, the sphingosine kinase pathway seems not to be essential for neutrophil recruitment, as confirmed by the finding that mice nullified for both sphingosine kinases displayed normal neutrophil functions (Michaud et al., 2006).

These preliminary data confirm a key role for S1P in acute inflammation but suggest different molecular mechanisms underlying the anti-inflammatory action of L-cycloserine and DTD. L-Cycloserine and DTD are widely used as S1P synthesis inhibitors, interfering at different levels in sphingolipid metabolism. The sphingolipid metabolic pathway has an intricate network of reactions, e.g., S1P can be synthesized through de novo pathway or through the hydrolysis of complex lipids, especially sphyngomielin (Bartke and Hannun, 2009). Furthermore, the levels of the various S1P precursors display great differences among different type of cells. These observations lead us to speculate that L-cycloserine and DTD could modulate different cellular sources of S1P and, in turn, different downstream signaling.

To gain further insight into the role played by sphingosine we used available S1P receptor antagonists. It is known that the chemotactic action of S1P relies on receptor interaction (Roviezzo et al., 2004; Hashimoto et al., 2008; Ishii et al., 2009; Harvey et al., 2010; Thangada et al., 2010). $S1P_2$ and $S1P_3$ antagonists significantly inhibited in vivo zymosan-induced neutrophil accumulation. Thus, the S1P pathway is involved in cell recruitment in acute inflammation through a receptor-mediated mechanism. Our data also suggest that the sphingosine kinase does not play a major role in cell recruitment. However, its inhibition exerted a degree of anti-inflammatory effect in mouse paw edema. This apparent discrepancy could be explained by the specific role played by SPK in vascular permeability. Indeed, DTD significantly inhibited vascular extravastion in the skin assay, an animal model specifically developed to evaluate vascular permeability. This hypothesis is further corroborated by the fact that sphingosine kinases are widely expressed on endothelium, and endothelial cells are a key source of plasma S1P (Venkataraman et al., 2008).

In conclusion, our data demonstrate that S1P is an early inflammatory mediator. The main events in acute inflammation in which the S1P pathway is involved are changes in vascular permeability and cell recruitment. S1P exerts its effect on cell recruitment by increasing leukocyte trafficking through receptor-mediated mechanisms. On the other hand, changes in vascular permeability are mediated mainly by SPK activation. Therefore, selective antagonists of $S1P_2$ or $S1P_3$ receptors as well as SPK inhibitors could represent a feasible alternative therapeutic strategy to be developed for modulating inflammatory-based diseases.

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Authorship Contributions

Participated in research design: Roviezzo, Bucci, Sorrentino, Perretti, and Cirino.

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Wrote or contributed to the writing of the manuscript: Perretti and Cirino.

References

Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP, Kane SA, Peters SP, Penn RB, Spiegel S, et al. (2001) Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J* **15:**1212–1214.

Bartke N and Hannun YA (2009) Bioactive sphingolipids: metabolism and function. *J Lipid Res* **50:**S91–S96.

Bradley PP, Priebat DA, Christensen RD, and Rothstein G (1982) Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* **78:**206–209.

Chalfant CE and Spiegel S (2005) Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling. *J Cell Sci* **118:**4605–4612.

Chiba Y, Suzuki K, Uechi M, Kurihara E, Goto K, Sakai H, and Misawa M (2010) Downregulation of sphingosine-1-phosphate receptors in bronchial smooth muscle of mouse experimental asthma. *Pharmacol Res* **62:**357–363.

Duan RD and Nilsson A (2009) Metabolism of sphingolipids in the gut and its relation to inflammation and cancer development. *Prog Lipid Res* **48:**62–72.

El Alwani M, Wu BX, Obeid LM, and Hannun YA (2006) Bioactive sphingolipids in the modulation of the inflammatory response. *Pharmacol Ther* **112:**171–183.

Gavins FN and Chatterjee BE (2004) Intravital microscopy for the study of mouse microcirculation in anti-inflammatory drug research: focus on the mesentery and cremaster preparations. *J Pharmacol Toxicol Methods* **49:**1–14.

Hannun YA and Obeid LM (2008) Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol* **9:**139–150.

Harvey KA, Welch Z, Sliva D, and Siddiqui RA (2010) Role of Rho kinase in sphingosine 1-phosphate-mediated endothelial and smooth muscle cell migration and differentiation. *Mol Cell Biochem* **342:**7–19.

Hashimoto M, Wang X, Mao L, Kobayashi T, Kawasaki S, Mori N, Toews ML, Kim HJ, Cerutis DR, Liu X, et al. (2008) Sphingosine 1-phosphate potentiates human lung fibroblast chemotaxis through the S1P2 receptor. *Am J Respir Cell Mol Biol* **39:**356–363.

Imasawa T, Koike K, Ishii I, Chun J, and Yatomi Y (2010) Blockade of sphingosine 1-phosphate receptor 2 signaling attenuates streptozotocin-induced apoptosis of pancreatic β-cells. *Biochem Biophys Res Commun* **392:**207–211.

Ishii M, Egen JG, Klauschen F, Meier-Schellersheim M, Saeki Y, Vacher J, Proia RL, and Germain RN (2009) Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. *Nature* **458:**524–528.

Jongsma M, Hendriks-Balk MC, Michel MC, Peters SL, and Alewijnse AE (2006) BML-241 fails to display selective antagonism at the sphingosine-1-phosphate receptor, S1P(3). *Br J Pharmacol* **149:**277–282.

Kitano M, Hla T, Sekiguchi M, Kawahito Y, Yoshimura R, Miyazawa K, Iwasaki T, Sano H, Saba JD, and Tam YY (2006) Sphingosine 1-phosphate/sphingosine 1-phosphate receptor 1 signaling in rheumatoid synovium: regulation of synovial proliferation and inflammatory gene expression. *Arthritis Rheum* **54:**742–753.

Koide Y, Hasegawa T, Takahashi A, Endo A, Mochizuki N, Nakagawa M, and Nishida A (2002) Development of novel EDG3 antagonists using a 3D database search and their structure-activity relationships. *J Med Chem* **45:**4629–4638.

Maceyka M, Milstien S, and Spiegel S (2009) Sphingosine-1-phosphate: the Swiss army knife of sphingolipid signaling. *J Lipid Res* **50:**S272–S276.

Michaud J, Kohno M, Proia RL, and Hla T (2006) Normal acute and chronic inflammatory responses in sphingosine kinase 1 knockout mice. *FEBS Lett* **580:**4607–4612.

Nishiuma T, Nishimura Y, Okada T, Kuramoto E, Kotani Y, Jahangeer S, and Nakamura S (2008) Inhalation of sphingosine kinase inhibitor attenuates airway inflammation in asthmatic mouse model. *Am J Physiol Lung Cell Mol Physiol* **294:**L1085–L1093.

Posadas I, Bucci M, Roviezzo F, Rossi A, Parente L, Sautebin L, and Cirino G (2004) Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression. *Br J Pharmacol* **142:**331–338.

Posadas I, Terencio MC, Guillén I, Ferrándiz ML, Coloma J, Payá M, and Alcaraz MJ (2000) Co-regulation between cyclo-oxygenase-2 and inducible nitric oxide synthase expression in the time-course of murine inflammation. *Naunyn Schmiedebergs Arch Pharmacol* **361:**98–106.

Roviezzo F, Del Galdo F, Abbate G, Bucci M, D'Agostino B, Antunes E, De Dominicis G, Parente L, Rossi F, Cirino G, et al. (2004) Human eosinophil chemotaxis and selective in vivo recruitment by sphingosine-1-phosphate. *Proc Natl Acad Sci USA* **101:**11170–11175.

Roviezzo F, Di Lorenzo A, Bucci M, Brancaleone V, Vellecco V, De Nardo M, Orlotti D, De Palma R, Rossi F, D'Agostino B, et al. (2007) Sphingosine-1-phosphate/sphingosine kinase pathway is involved in mouse airway hyperresponsiveness. *Am J Respir Cell Mol Biol* **36:**757–762.

Segawa S, Takata Y, Kaneda H, and Watari J (2007) Effects of a hop water extract on the compound 48/80-stimulated vascular permeability in ICR mice and histamine release from OVA-sensitized BALB/c mice. *Biosci Biotechnol Biochem* **71:**1577–1581.

Snider AJ, Orr Gandy KA, and Obeid LM (2010) Sphingosine kinase: role in regulation of bioactive sphingolipid mediators in inflammation. *Biochimie* **92:**707–715.

Spiegel S and Milstien S (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* **4:**397–407.

Taha TA, Hannun YA, and Obeid LM (2006) Sphingosine kinase: biochemical and cellular regulation and role in disease. *J Biochem Mol Biol* **39:**113–131.

Thangada S, Khanna KM, Blaho VA, Oo ML, Im DS, Guo C, Lefrancois L, and Hla T (2010) Cell-surface residence of sphingosine 1-phosphate receptor 1 on lymphocytes determines lymphocyte egress kinetics. *J Exp Med* **207:**1475–1483.

Van Doorn R, Van Horssen J, Verzijl D, Witte M, Ronken E, Van Het Hof B, Lakeman K, Dijkstra CD, Van Der Valk P, Reijerkerk A, et al. (2010) Sphingosine 1-phosphate receptor 1 and 3 are up-regulated in multiple sclerosis lesions. *Glia* **58:**1465–1476.

Venkataraman K, Lee YM, Michaud J, Thangada S, Ai Y, Bonkovsky HL, Parikh NS, Habrukowich C, and Hla T (2008) Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. *Circ Res* **102:**669–676.

Williams RD, Sgoutas DS, Zaatari GS, and Santoianni RA (1987) Inhibition of serine palmitoyltransferase activity in rabbit aorta by L-cycloserine. *J Lipid Res* **28:**1478–1481.

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