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Title page

**Sphingosine-1-phosphate modulates vascular permeability and cell recruitment in acute inflammation in vivo**

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Running title page

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## Abstract

Sphingosine kinase (SPKs) / sphingosine-1-phosphate (S1P) pathway has been recently associated to 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 by using i) L-cycloserine and DL-threo-Dihydrosphingosine, S1P synthesis inhibitors or ii) BML-241 and JTE013, specific S1P<sub>2</sub> and S1P<sub>3</sub> receptor antagonists. Following local injection of carrageenan in mouse paw S1P release significantly increases locally and decreases during the resolution phase. Expression of SPK(s) and S1P<sub>2</sub> and S1P<sub>3</sub> receptors is increased in inflamed tissues. Administration of L-cycloserine or DL-threo-Dihydrosphingosine caused a significant antiinflammatory effect. By using different animal model we have also demonstrated that SPK/S1P pathway contributes to changes in vascular permeability and promotes cell recruitment. S1P effect on cell recruitment results receptor mediated since both JTE-013 and BML-241 inhibited zymosan induced cell chemotaxis, without effect on vascular leakage. Conversely changes in vascular permeability mainly involve SPK activity, since CM48/80 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 in order to develop a new class of anti-inflammatory drugs.

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## Introduction

Sphingolipids are formed either via the metabolism of sphingomyelin, a constituent of the plasma membrane, or by *de novo* synthesis (Hannun et al, 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 et al. 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. Particularly, 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 sphingolipids 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 Dooorn et al. 2010) and inflammatory bowel disease (Duan et al. 2009). Furthermore various sphingolipid enzymes (sphingomyelinase, sphingosine kinase etc) are activated by inflammatory cytokines and their downstream lipid mediators regulate inflammatory pathway in addition to immune cell functions (Bartke et al. 2009). In particular sphingosine kinases (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 ( PDGF, VEGF, EGF), TGF $\beta$ , TNF $\alpha$ , interleukins (Spiegel et Milstien 2003; Taha et al. 2006).

S1P signaling is the result of a complicated system of regulation. To date, most of S1P functions have been attributed to receptor-mediated signaling. S1P exerts different cellular effects depending upon the subtype of S1P receptors (S1P<sub>n</sub>) and the downstream G protein coupled. Other biological

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effects such as activation of Ras and ERK signaling pathways by VEGF or activation of PI3K/Akt have been shown to be linked to S1P intracellular action since 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 if targeting sphingolipid action could represent a feasible antiinflammatory therapeutic strategy. There are now several compounds that have been developed, which can manipulate different component of the sphingosine pathway, e.g. S1P synthesis inhibitor or selective S1P receptors antagonists (Nishiuma et al. 2008; Koide et al. 2002; Imasawa et al. 2010; Chiba 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 mainly focuses on the early stage of inflammation analyzing the SPK/S1P pathway contribute to vascular leakage or cell infiltration by using different preclinical well known models.

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## **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 BML-241 (an S1P<sub>3</sub> /S1P<sub>2</sub> antagonist ) were purchased from BIOMOL (VINCI BIOCHEM, Vinci, Italy). JTE013 (an S1P<sub>2</sub> antagonist) was purchased from Tocris Bioscience (Bristol, United Kingdom). Antibodies anti-S1P<sub>1</sub>, anti-S1P<sub>2</sub>, anti-S1P<sub>3</sub>, anti-S1P<sub>4</sub>, anti-SPK<sub>1</sub> and anti SPK<sub>2</sub> were obtained from Santa Cruz Biotechnology (Santa Cruz, California). Compound 48/80 and all other reagents were obtained from Sigma–Aldrich Chemicals (St. Louis, MO)

### **Animals**

Male CD-1 (Harlan, Italy) mice weighing 20-25 g were separated in 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) or by Animal Ethical Committer of the University of Naples Federico II.

### **Sphingosine-1-phosphate measurement**

Mice received subplantar administration of 50  $\mu$ l of carrageenan 1% w/v (Posadas et al. 2004) or vehicle (saline). Mice were sacrificed with carbon dioxide 2, 4, 6h after carrageenan administration. Paws were cut and centrifuged at 4000 r.p.m. for 30 min. Exudates (supernatants) were collected with 100  $\mu$ l of saline and were used for S1P quantification by using a commercially available ELISA kit ( Echelon, Tebu-bio).

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### **Western Blot**

Mice received a subplantar administration of 50  $\mu$ l of carrageenan 1% w/v or vehicle and were sacrificed after 2, 4, 6 hours. The injected paws were cut and utilized for western blot analysis. Briefly, mouse paws were homogenized in 10 mm HEPES pH 7.4 buffer containing saccharose (0.32M), EDTA (100 $\mu$ M), dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (1 mg/ml) and leupeptin (10  $\mu$ g/ml) with a Polytron homogenizer (3 cycles of 10 seconds 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  $\mu$ g. Protein samples were loaded on 10% PAGE-SDS and transferred onto nitrocellulose membranes for 45 min at 250 mA. Membranes were blocked in PBS-Tween 20 (0.1%) containing 5% non-fat milk and 0.1% BSA 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<sub>1</sub> (1:1000 Santa Cruz) anti-S1P<sub>2</sub> (1:1000 Santa Cruz), anti-S1P<sub>3</sub> (1:1000; Santa Cruz), anti-S1P<sub>4</sub> (1:1000 Santa Cruz) anti-SPK<sub>1</sub> (1:2000; Santa Cruz) or anti-SPK<sub>2</sub> (1:2000 Santa Cruz) 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; Amersham, USA). Protein levels are evaluated through densitometry (how intense the stain is) versus  $\beta$ -actin.

### **Mouse paw edema**

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

Mice were divided in 3 groups (n=6) and received intraperitoneal administration of 1) L-cycl, a sphingolipid metabolism regulator with inhibitory activity of serine plamitoyltransferase that catalyze the first step of sphingolipid biosynthesis (10-100mg/kg in 200  $\mu$ l), 24 h and 30 min

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before beginning of inflammation (Williams et al 1987) or 2) DL-threo-Dihydrosphingosine an inhibitor of both isoforms of SPKs (DTD; 0.3-3mg/Kg), 30 min before inflammogen application (Nishiuma et al. 2008) or 3) vehicle (DMSO/saline 0.1%). In order to assess the efficacy of these drugs, in a separate set of experiment we evaluated S1P release following inflammatory challenge in presence of L-cycl and DTD using the dose that was previously determined to give the maximal response.

### **MPO measurement**

Mice from different groups were sacrificed with CO<sub>2</sub> after 2, 4 and 6 hours from carrageenan injection. Injected paws were cut, weighted and homogenated in 1 ml of HTAB (Hexadecyltrimethyl-ammonium bromide) buffer using a Polytron homogenizer (2 cycles of 10 seconds at maximum speed). After centrifugation of homogenates at 10000 rpm for 2 minutes, supernatant fractions were assayed for MPO activity using the method described by Bradley et al. (1982). Briefly, samples were mixed with phosphate buffer containing 1mM *O*-dianisidine dihydrochloride and 0.001% hydrogen peroxide in a microtiter plate reader. Absorbance was measured at 450nm taking three reading at 30 seconds intervals. Units of MPO were calculated considering that 1 U. MPO= 1 μmole H<sub>2</sub>O<sub>2</sub> split, and 1 μmole H<sub>2</sub>O<sub>2</sub> gives a change in absorbance of  $1.13 \times 10^{-2}$  nm/min.

### **Intravital microscopy**

Mice were divided in 3 groups (n=6) and received intraperitoneal administration of 1) L-cycl an inhibitor of sphingolipid metabolism (30mg/kg in 200 μl), 24 h and 30 min before beginning of inflammation or 2) DL-threo-Dihydrosphingosine, an inhibitor of both isoforms of SPKs (DTD, 1mg/Kg), 30 min before inflammogen application or 3) vehicle (DMSO/saline 0.1%). Experiments began with i.p. injections of 1 mg of zymosan (in 0.5 ml of sterile saline); 4hr later, when neutrophil infiltration is maximal (Gavins et al. 2004), the vascular mesenteric bed was prepared



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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 et al. 2004). The preparation was mounted on a Zeiss Axioskop FS with a water immersion objective lens (magnification  $\times 40$ ), and an eyepiece (magnification  $\times 10$ ) was used to observe the microcirculation. The preparation was transilluminated with a 12-V, 100-W halogen light source. A Hitachi charge-coupled device color camera (model KPC571) acquired images that were displayed on a Sony 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) projected the time, date, and stopwatch function onto the monitor. Mesenteries were superfused with bicarbonate-buffered solution at 37°C [in g/liter: NaCl, 7.71; KCl, 0.25; MgSO<sub>4</sub>, 0.14; NaHCO<sub>3</sub>, 1.51; and CaCl<sub>2</sub>, 0.22 (pH 7.4); gassed with 5% CO<sub>2</sub>/95% N<sub>2</sub>] 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 (rbc) velocity was measured in venules by using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station). Venular blood flow was calculated from the product of mean rbc velocity ( $V_{\text{mean}} = \text{centerline velocity}/1.6$ ) and microvascular cross-sectional area, assuming a cylindrical geometry. Wall shear rate was calculated by the Newtonian definition: shear rate =  $8,000 \times (V_{\text{mean}}/\text{diameter})$ . One to three randomly selected postcapillary venules (diameter between 20 and 40  $\mu\text{m}$ , length at least 100  $\mu\text{m}$ ) were observed for each mouse; measurements were taken 5–10 min after exposure of the chosen vessels.

### **Mouse air pouch**

Mice were divided in 5 groups (n=6) and received intraperitoneal administration of 1) L-cycl an inhibitor of sphingolipid metabolism (30mg/kg in 200  $\mu\text{l}$ ) 24 h and 30 min before beginning of

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inflammation or 2) DL-threo-Dihydrosphingosine an inhibitor of both isoforms of SPKs (DTD; 1mg/kg in 200  $\mu$ l), 30 min before inflammogen application or 3) an S1P<sub>3</sub> antagonist (BML-241; 0.3-3mg/kg in 200  $\mu$ l intraperitoneally) 30 min before inflammogen application or an S1P<sub>2</sub> antagonist (JTE013; 0.3-3mg/kg in 200  $\mu$ l intraperitoneally) 30 min before inflammogen application (Koide et al. 2002, Imasawa et al. 2010, Chiba et al. 2010; M Jongsma et al. 2006) 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 sterile air into the back of mice (28-30 g). Three days later 2.5 ml of sterile air was re-injected in the same cavity (Posadas et al. 2000). After another three days (six days after the first air injection), 1 ml of zymosan 1% w/v or vehicle (saline) was injected into the air pouch. After 4h from zymosan injection, when neutrophil infiltration is maximal (Posadas et al. 2000), mice were sacrificed by CO<sub>2</sub> exposure and exudate in the pouch was collected with 1 ml of saline and placed in graduated tubes and centrifuged at 125 x g for 10 minutes. The pellet was suspended in 500  $\mu$ l 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 compound 48/80 induces an acute increase in *vascular leakage* to plasma proteins (Segawa et al. 2007). Mice were divided in 5 groups (n=6) and received intraperitoneal administration of 1) L-cycl (30mg/kg in 200  $\mu$ l) 24 h and 30 min before administration of compound 48/80 or 2) DTD (1mg/kg in 200  $\mu$ l), 30 min before administration of compound 48/80 or 3) an S1P<sub>3</sub> antagonist (BML-241; 0.3-3mg/kg in 200  $\mu$ l intraperitoneally) 30 min before administration of compound 48/80 or an S1P<sub>2</sub> antagonist (JTE013; 0.3-3mg/kg in 200  $\mu$ l intraperitoneally) 30 min before administration of compound 48/80 (Koide et al. 2002, Imasawa et al. 2010, Chiba et al. 2010) or 5) vehicle (DMSO/saline 0.1%). Anesthetized mice were injected intradermally with 5  $\mu$ g of compound 48/80 dissolved in 50  $\mu$ l of physiological saline into their shaved back skin immediately after an intravenous injection of a 0.5% Evans Blue solution

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(5ml/kg) Animals were sacrificed 30 min later, and skin removed for a quantitative determination of the extravasated dye. Evans blue was neutralized by 1ml of 6M KOH at 45°C for 6h. This extract was neutralized by 1ml of 6M HCl, and then 2ml of acetone was added. The extract was clarified by filtration, its absorbance at 595nm was measured and the extracted Evans Blue content was then calculated from its calibration curve.

### **Statistical analysis**

Data are expressed as mean  $\pm$  s.e. mean. The level of statistical significance was determined by one way or two-way analysis of variance (ANOVA) followed by Bonferroni's *t*-test for multiple comparisons, using the GraphPad Prism software.

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## **Results**

### **S1P pathway is upregulated in the carrageenan-injected mouse paw**

Injection of carrageenan in mouse paw causes a time dependent edema (Fig 1 panel A). S1P levels were significantly elevated in exudates harvested from mouse paws peaking at 2h and declining at 6h point (Fig1 panel B). At all time points, e.g. 2, 4,6h, there was also a significant increase in S1P<sub>2</sub> (Fig 2 panel A) and S1P<sub>3</sub> (Fig 2 panel B) receptors as well as in SPK<sub>1</sub> (Fig 2 panel C) and SPK<sub>2</sub> (Fig 2 panel D). S1P<sub>1</sub>, S1P<sub>4</sub> 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 (10-100mg/Kg) significantly reduced in a dose dependent manner carrageenan-induced edema (Figure 3 panel A). S1P level in paw exudates was also significantly reduced following L-cycl treatment (Fig 3 panel B). Myeloperoxidase activity was markedly reduced in mice treated with L-cycl (30mg/kg) at all time-points tested (Figure 3 panel C).

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

### **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 following zymosan exposure. L-cycl (30m/Kg) caused a significant inhibition of neutrophil adhesion (Figure 5 panel A) and migration (Figure 5 panel B), while

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rolling (Figure 5 panel C) was unaffected. Conversely, DTD (1mg/kg) did not affect any of the parameters measured (Figure 5).

### **L-cycloserine significantly affects zymosan-induced cell chemotaxis, while DTD significantly inhibited vascular leakage**

Administration of zymosan in dorsal air pouch induces a recruitment of blood-borne leukocytes reaching its maximum at 4h (Posadas et al. 2000). L-cycl (30mg/kg) significantly inhibited the recruitment (Figure 6 panel A) as well as reduced MPO activity in pouch skin samples (Figure 6 panel B). On the other hand, DTD (1mg/Kg) was ineffective on zymosan induced cell recruitment and MPO activity (Figure 6 panel B). These experiments further suggest that different signaling pathway of S1P are involved into the inflammatory response through different molecular targets.

Intradermal injection of compound 48/80 (5 µg) induces an acute increase in vascular leakage (Figure 6 panel C). DTD (1mg/Kg) pretreatment significantly inhibited Blue Evans extravasation induced by compound 48/80 (figure 6 panel C). Conversely L-cycl (30 mg/Kg) did not modify vascular leakage (Figure 6 panel C).

### **S1P<sub>2</sub> and S1P<sub>3</sub> antagonists block cell recruitment**

It is known that S1P chemotactic proprieties are receptor mediated and in particular a key role is played by S1P<sub>2</sub> and S1P<sub>3</sub>. The specific antagonists JTE-013 (blocking S1P<sub>2</sub>;1mg/Kg) and BML-241 (blocking S1P<sub>2</sub> and S1P<sub>3</sub>;1mg/Kg) inhibited in a dose-dependent manner the cell recruitment provoked by zymosan (Figure 7 panel A). Nor JTE013 (1mg/Kg) or BML-241 (1mg/Kg) inhibited Blue Evans extravasation in mouse skin induced by compound 48/80 (Figure 7 panel B).

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## Discussion

The aim of this paper was to investigate on the role played in inflammation by S1P pathway *in vivo* in pre-clinical relevant models. S1P is rapidly released during acute inflammation at the site of injury and it is actively involved in the onset of the inflammatory response. Indeed S1P level significantly increases locally following the inflammatory challenge, and declines when inflammation resolves. At the same time in the paw there is a significant upregulation of S1P<sub>2</sub> and S1P<sub>3</sub> receptors as well as of 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(s) inhibitor or L-cycloserine, a sphingolipid metabolism inhibitor, prior to carrageenan administration significantly inhibited the edema. DTD mainly modulates vascular permeability, while L-cycloserine significantly reduces cell recruitment. In fact, DTD abrogates the first phase of edema that mainly relies on changes in vascular permeability (Posadas et al. 2000), while 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 infiltration 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. the 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, while DTD did not significantly affect both cell adhesion and emigration.

S1P has chemotactic activity (Thangada et al. 2010, Harvey t al. 2010, Ishii e al. 2009, Hashimoto et al. 2008, Roviezzo et al. 2004). In order to clarify if this effect on adhesion and migration was mediated by chemotactic action of S1P on inflammatory cells, we used the mouse air pouch, a pre-clinical experimental model that allows evaluating chemotaxis *in vivo*.

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L-cycloserine produced a marked inhibition of the cell recruitment elicited by zymosan, while DTD was ineffective. Thus, sphingosine kinase pathway appears not to be essential for neutrophil recruitment, as confirmed by the finding that mice nullified for both sphingosine kinases display 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 antiinflammatory action of L-cycloserine and DTD. L-cycloserine and DTD are widely used as S1P synthesis inhibitors interfering at different level 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 sphingomyelin (Bartke et al. 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 source 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. (Thangada et al. 2010, Harvey et al. 2010, Ishii et al. 2009, Hashimoto et al. 2008, Roviezzo et al. 2004). S1P<sub>2</sub> and S1P<sub>3</sub> antagonists significantly inhibited in vivo zymosan-induced neutrophil accumulation. Thus, S1P pathway is involved in cell recruitment in acute inflammation through a receptor-mediated mechanism. Our data also suggest that 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 on vascular permeability. Indeed, DTD significantly inhibited vascular extravastion in the skin assay, an animal model specifically developed in order to evaluate vascular permeability. This hypothesis is further corroborated by the fact that sphingosine kinase(s) are widely expressed on endothelium and endothelial cells are a key source of plasma S1P (Venkataraman K. et al. 2008)

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In conclusion our data demonstrate that S1P is an early inflammatory mediator. The main events in acute inflammation in which S1P pathway is involved are changes in vascular permeability and cell recruitment. S1P exerts its effect on cell recruitment by increasing leukocytes trafficking through a receptor mediated mechanisms. On the other hand, changes in vascular permeability are mainly mediated by SPKs activation. Therefore selective antagonist of S1P<sub>2</sub> or S1P<sub>3</sub> receptors as well as SPKs inhibitors could represent a feasible alternative therapeutic strategy to be developed to modulate inflammatory based diseases.



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**Authorship contributions**

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Performed data analysis: **Valentina Vellecco and Bruno D'Agostino;**

Participated in research design and contributed to the writing of the manuscript: **Mauro Perretti and Giuseppe Cirino;**

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## Legends

### **Figure 1. S1P level is increased in inflamed tissues**

Panel A. Injection of 50  $\mu$ l of carrageenan 1% w/v in the mouse paw causes an increase in paw volume that peaks at 4 hour and resolves after 6 hour (\*\*\*  $p < 0.001$  vs vehicle; one way ANOVA analysis). Panel B. S1P levels measured in paw exudates were significantly increased at all time tested (\*  $p < 0.05$  \*\*\* $p < 0.001$  vs vehicle; two way ANOVA analysis).

### **Figure 2. Inflammation upregulates S1P pathway.**

Western blot analysis for S1P<sub>2</sub> (panel A), S1P<sub>3</sub> (panel B), SPK<sub>1</sub> (panel C) or SPK<sub>2</sub> (panel D) was performed on paw tissues harvested from mice injected with carrageenan 1% after 2, 4 and 6 hours. The blots are representative of three different experiments. The bar graph is obtained by densitometric analysis performed versus  $\beta$ -actin (\*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  vs. vehicle; two way ANOVA analysis).

### **Figure 3. Effect of L-cycl on carrageenan-induced mouse paw edema.**

Panel A. L-cycl (10-100mg/kg), administered to mice 24h and 30 minutes before carrageenan subplantar injection, significantly reduced paw edema development (\*  $p < 0.05$ ; \*\*  $p < 0.01$  vs. vehicle; two way analysis). Panel B. Exudates harvested from mice treated with L-cycl (30mg/Kg) displayed a significant reduction in S1P levels (###  $p < 0.001$  vs sham \*\*  $p < 0.01$  vs. vehicle; one way ANOVA analysis). Panel C. MPO levels were significantly reduced by treatment with L-cycl (30mg/Kg) (\*\*  $p < 0.01$  vs. vehicle; two way ANOVA analysis).

### **Figure 4. Effect of DTD on carrageenan-induced mouse paw edema.**

Panel A. DTD (0.3-3mg/Kg) administered to mice 30 minutes before carrageenan subplantar injection significantly inhibited paw edema development (\*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  vs. vehicle; two



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way ANOVA analysis). Panel B. S1P levels were significantly inhibited in exudates obtained from mice treated with DTD (1mg/Kg) (### p<0.001 vs sham, \*\* p<0.01 vs. vehicle; one way ANOVA analysis). Panel C. DTD (1mg/Kg) significantly reduced MPO levels in paw tissue at 2h (\*\* p<0.01 vs. vehicle; two way ANOVA analysis).

**Figure 5. Effect of L-cycl or DTD on leukocyte extravasation.**

L-cycl (30 mg/Kg) significantly reduced zymosan-induced adhesion (panel A; \*\* p< 0.01 vs. sham # p< 0.05 vs. vehicle) and number of migrated cell (panel B\*\*; p< 0.01 vs. sham # p< 0.05 vs. vehicle), while did not modify rolling velocity (panel C; \* p< 0.05 vs. sham). DTD (1mg/kg) did not affect any of the parameters measured. Data have been analyzed with one way ANOVA

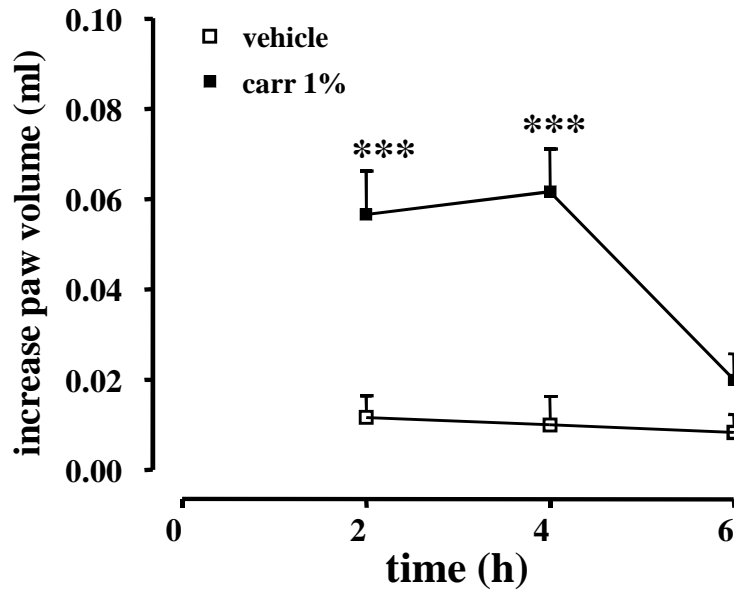
**Figure 6. Effect of L-cycl or DTD on cell infiltration and vascular permeability.** Panel A. L-cycl but not DTD significantly inhibited cell migration into the pouch induced by zymosan (\*\* p<0.01vs vehicle) . Panel B L-cycl but not DTD significantly inhibited MPO levels in skin harvested from mice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

**Figure 7. Effect of antagonists of S1P<sub>2</sub> or S1P<sub>3</sub> on cell infiltration and vascular permeability**

Panel A: JTE-013 (0.3-3mg/Kg) and BML-241(0.3-3mg/Kg) reduced in a dose dependent manner cell accumulation in mouse air pouch (\* p<0.05 \*\* p<0.01; versus vehicle two way ANOVA analysis). Panel B: JTE 013 (1mg/Kg) and BML 241 (1mg/Kg) did not significantly inhibit CM 48/80- induced extravasations as determined by Blue Evans determination (\*\* p<0.01; versus sham; one way ANOVA analysis).

**Figure 1**

**A**



**B**

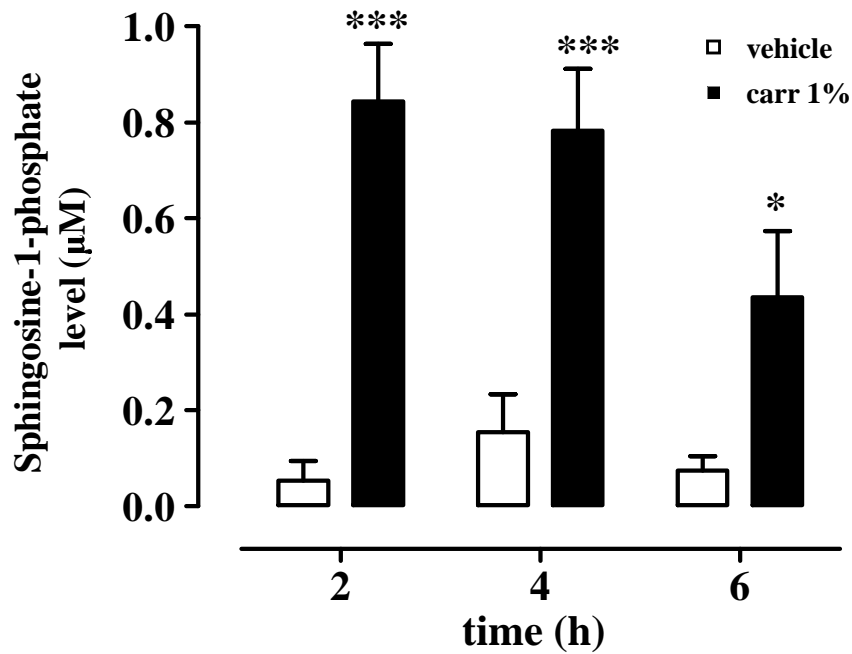
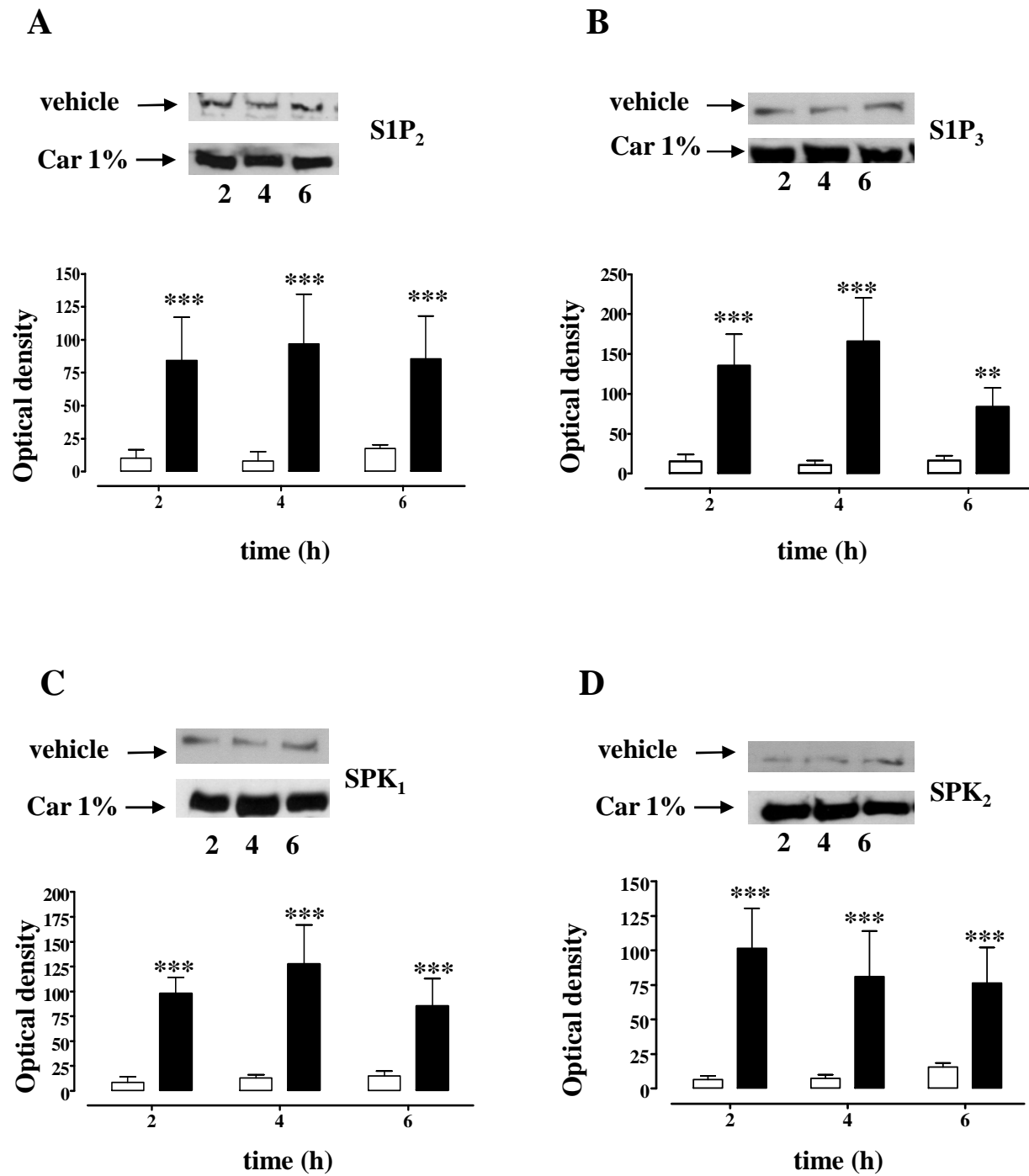


Figure 2



A

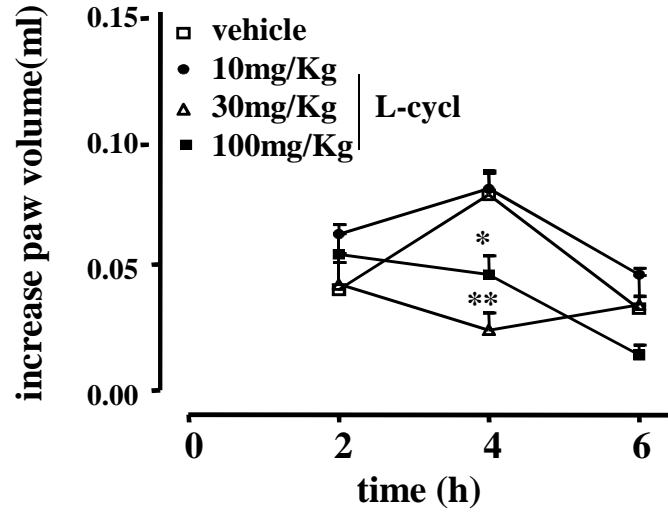
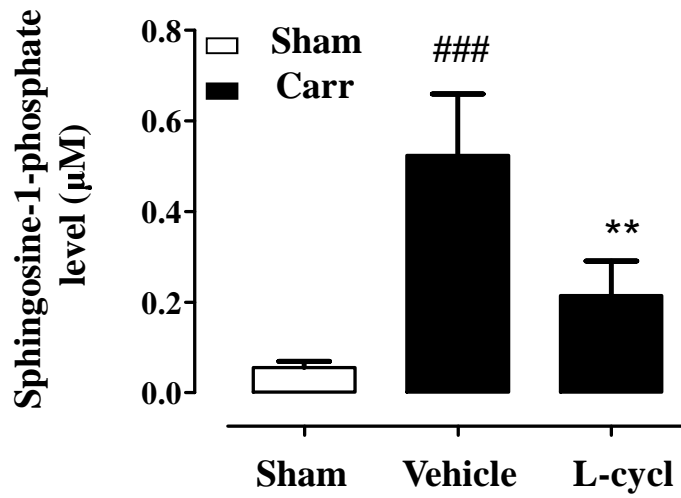


Figure 3

B



C

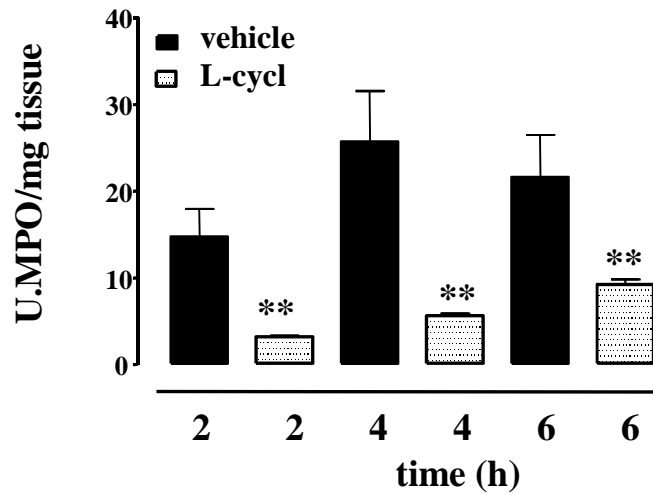


Figure 4

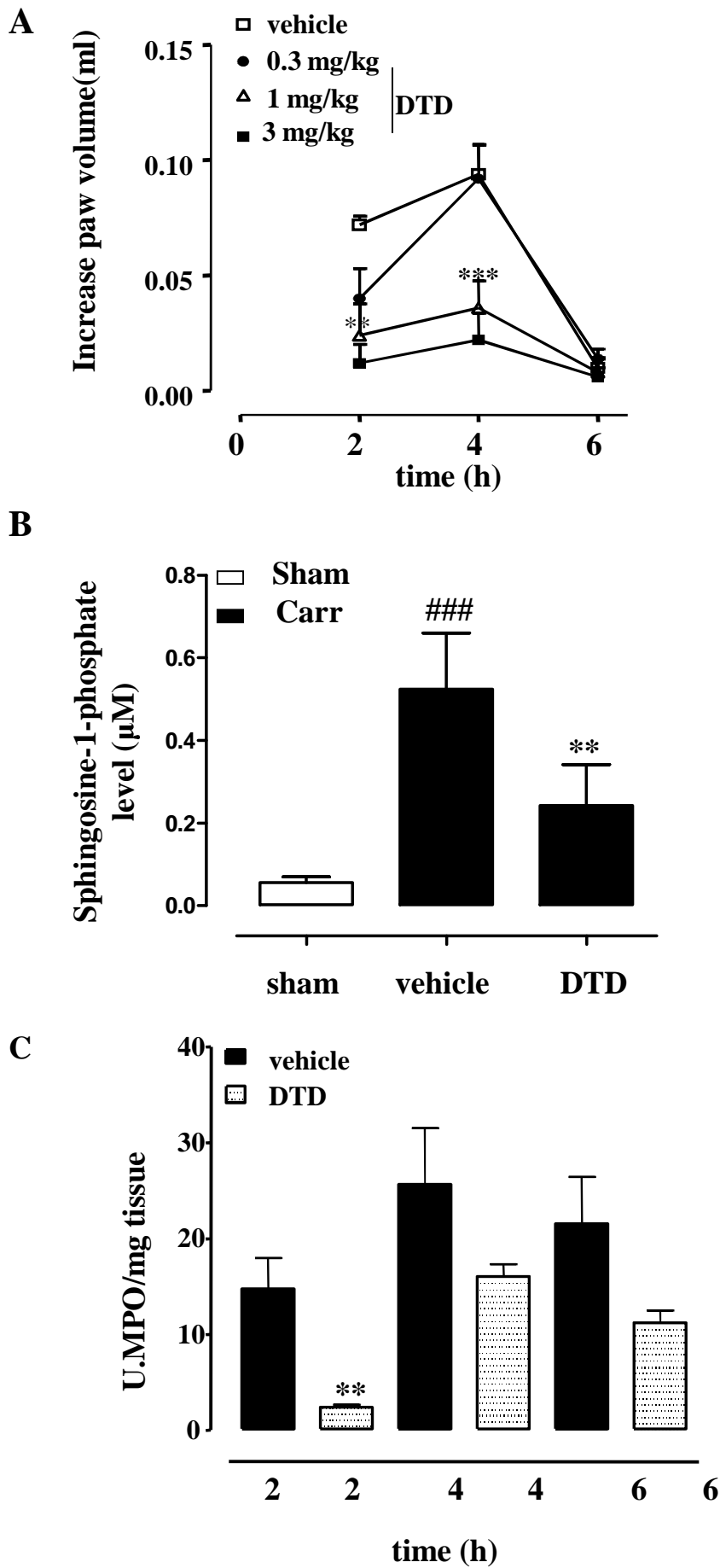


Figure 5

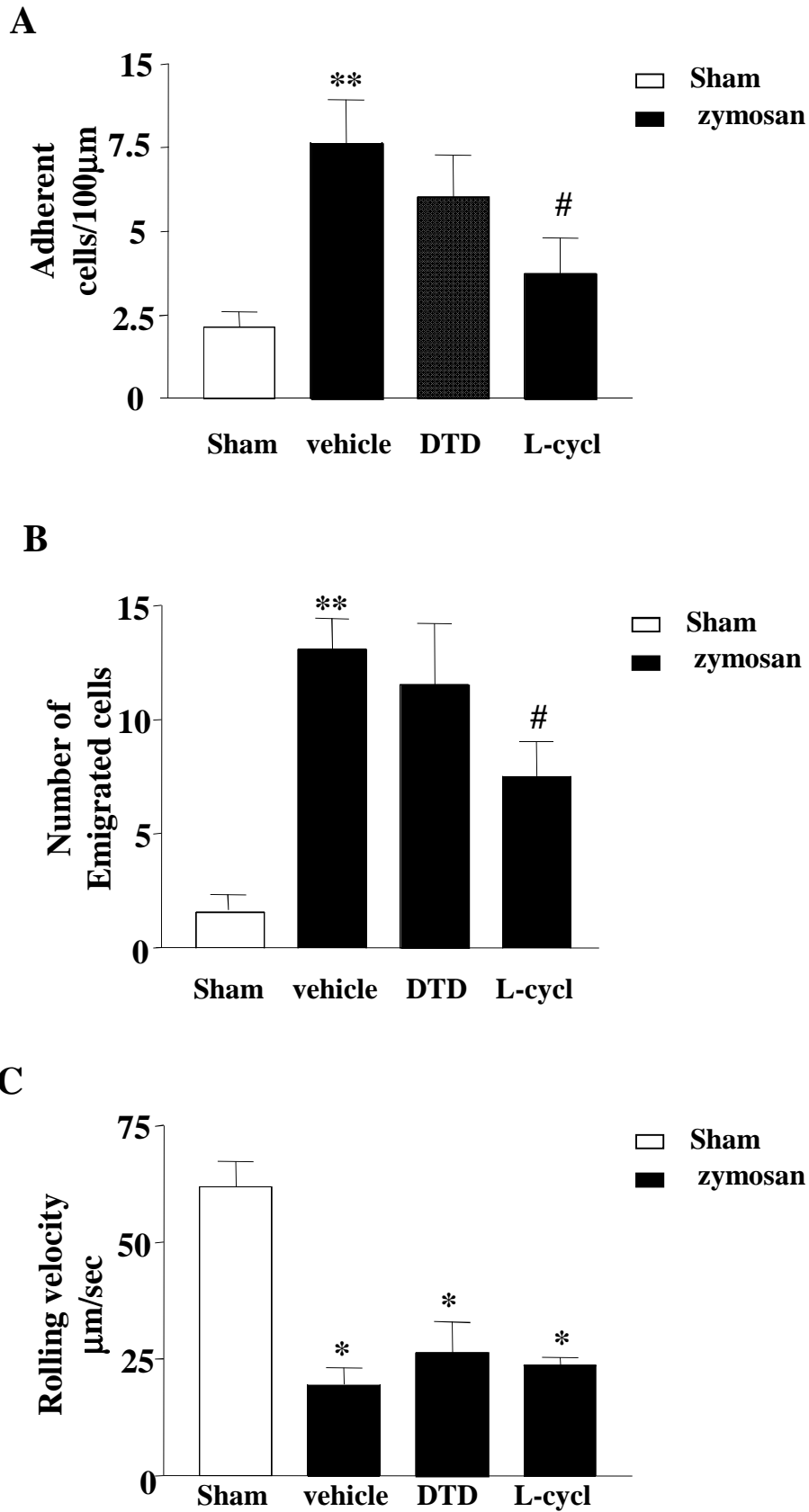


Figure 6

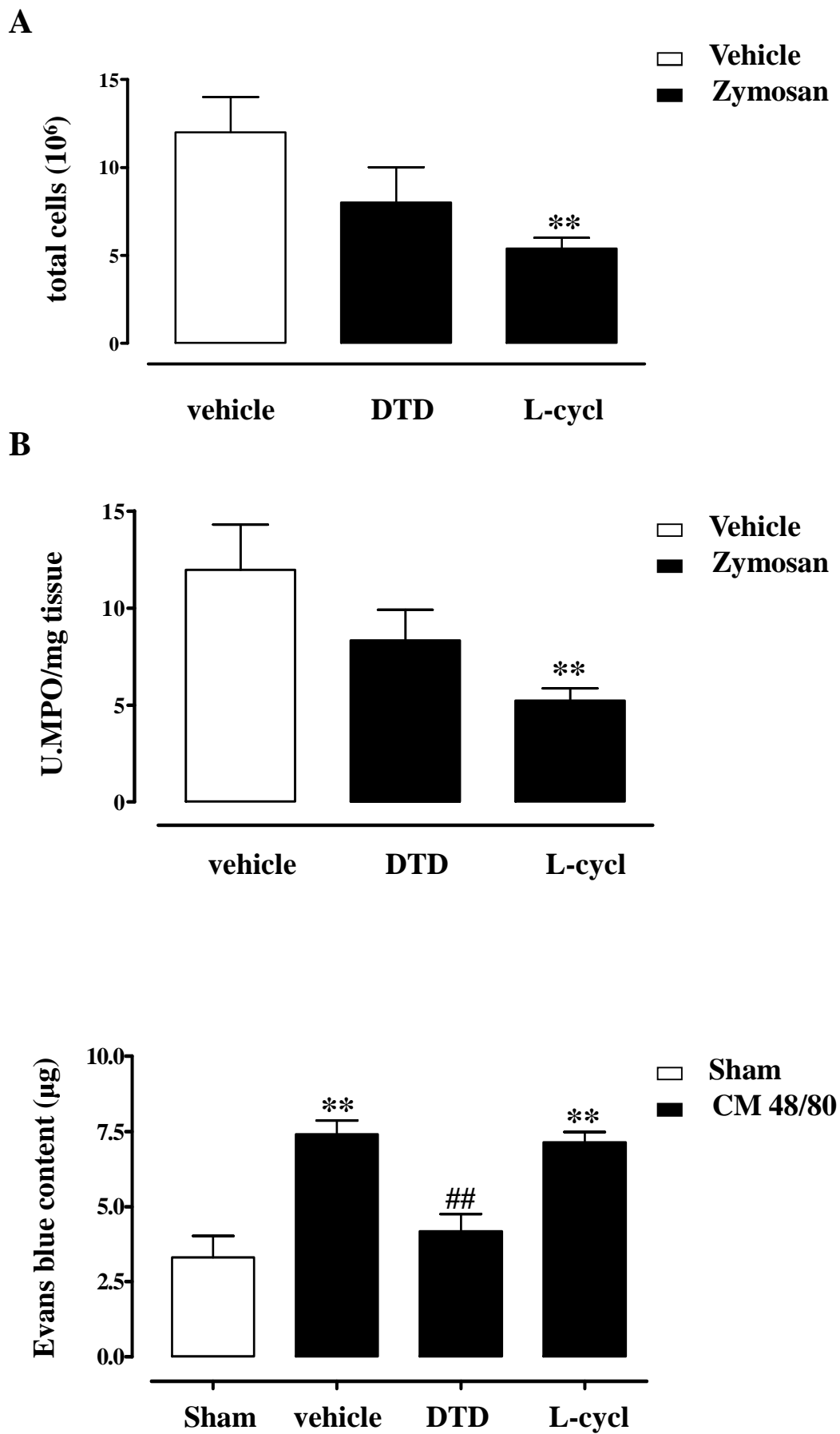


Figure 7

