European Journal of Pharmacology 609 (2009) 132–139

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and Inflammation

Sphingosine-1-phosphate increases human alveolar epithelial IL-8 secretion, proliferation and neutrophil chemotaxis

Javier Milara [a,b,*,1], Manuel Mata [a,b,c,1], Maria Dolores Mauricio [b], Eva Donet [c], Esteban J. Morcillo [a,c,d], Julio Cortijo [a,b,c]

[a] Department of Pharmacology, Faculty of Medicine, University of Valencia, Spain
[b] Research Unit, University General Hospital, Valencia, Spain
[c] CIBERES, Health Institute Carlos III, Valencia, Spain
[d] Clinical Pharmacology Unit, University Clinic Hospital, Valencia, Spain

ARTICLE INFO

Article history:
Received 5 November 2008
Received in revised form 27 January 2009
Accepted 3 March 2009
Available online 12 March 2009

Keywords:
Sphingoshine-1-phosphate
Human airway epithelial cell
Epithelium–leukocyte interaction

ABSTRACT

Sphingosine-1-phosphate (S1P) has been presented recently as a pro-inflammatory agent in the airway epithelium since S1P levels are increased in bronchoalveolar lavage fluid of human asthmatics. However, the effects of S1P over the alveolar epithelium and neutrophil interactions are poorly understood. Here, we show that S1P increased interleukin 8 (IL-8) gene expression and protein secretion and proliferation in alveolar epithelial cells A549 at physiological concentrations (1 μM). At the same time, S1P increased intracellular Ca^{2+} concentration (potency 17.91 μM, measured by epifluorescence microscopy), phospholipase D (PLD) activity (measured by chemiluminiscence method) and extracellular matrix-regulated kinase1/2 (ERK1/2) phosphorylation (measured by western blot) via G_i-coupled receptor (inhibited by pertussis toxin 100 ng/ml) in A549 cells. Both, IL-8 secretion and A549 proliferation were dependent of PLD activity (inhibited by 1-butanol 0.5%), intracellular Ca^{2+} (inhibited by acetoxymethyl 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM) 100 μM), ERK1/2 phosphorylation (inhibited by 2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one (PD98059) 10 μM) and G_i-coupled receptors (blocked by pertussis toxin 100 ng/ml). Moreover, S1P increased intercellular adhesion molecule I (ICAM-1) expression and failed in vascular cell adhesion molecule I (VCAM-1) modification (measured by flow cytometer) in A549. Indirectly, A549 supernatant fluids arising from A549-S1P 1 μM stimulation decreased L-selectin expression without CD11b/CD18 integrin modification in human neutrophils. In the same way, A549-S1P supernatant fluids increased neutrophil chemotaxis (Boyden chamber), which was inhibited by antibody against IL-8. This study demonstrates for the first time that S1P participates in the alveolar epithelial interactions *in vitro*.

1. Introduction

The airway epithelium plays a crucial role in initiating and augmenting pulmonary host defense mechanisms by synthesizing and releasing a variety of inflammatory mediators. Interleukin-8 (IL-8), a member of the CXC family of chemokines, is a potent chemoattractant and activator of neutrophils at sites of acute inflammation (Fuke et al., 2004). Studies supporting the involvement of IL-8 in pulmonary inflammatory diseases reported elevated levels of IL-8 in the bronchoalveolar lavage fluid of patients with chronic obstructive pulmonary disease and asthma among other pulmonary disorders (Car et al., 1994). Alveolar epithelium–neutrophil interactions are partly mediated by adhesion molecules such as selectins, and CD11b/CD18 integrin in neutrophils (Burns et al., 2003), but adhesion of neutrophil granulocytes to epithelial intercellular adhesion molecule I (ICAM-1) has also been reported (Colgan et al., 1995). ICAM-1 is expressed on type I and type II pneumocytes (Cunningham et al., 1994) and is up-regulated in the presence of pro-inflammatory cytokines (Barton et al., 1995).

In addition to inflammatory changes, pulmonary disorders present a balance between cellular proliferation and cell death of airway epithelial cells that may explain the structural remodelling changes noted in severe asthma or chronic obstructive pulmonary disease (Cohen et al., 2007; Pilette et al., 2007). The proliferative response in the airway epithelium may be triggered by viral infection or injury in the genetically susceptible individual, but this mechanism remains unknown.

Recently, the bioactive phospholipid Sphingosine-1-phosphate (S1P) has been presented as a pro-inflammatory agent since S1P levels are increased in the bronchoalveolar lavage fluid of asthmatics after antigen challenge (Ammit et al., 2001). S1P is found in

* Corresponding author. Fundacion de Investigacion, Hospital General Universitario de Valencia (HGUV). Avda. tres cruces, s/n.46014 Valencia, Spain. Tel.: +34 9619721146; fax: +34 961972145.
E-mail address: xmilara@hotmail.com (J. Milara).
[1] These authors contributed equally to this work.

0014-2999/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2009.03.012

nanomolar to micromolar concentrations in human plasma and serum (Yatomi et al., 1997), and activates intracellular signalling pathways by ligating 5 members of a G protein-coupled lysophospholipid receptor family (SIP_1, SIP_2, SIP_3, SIP_4, SIP_5), regulating calcium levels and cell growth and survival among other functions. Actually there is growing evidence that S1P is involved in the complex tissue-specific process of inflammation, but the role of S1P on the alveolar epithelium and in the neutrophil–epithelial interaction remains unknown. In the present work we have investigated the effect of physiologic concentrations of S1P over IL-8 secretion and proliferation in human alveolar epithelial cells A549, as well as the role in the alveolar epithelial–neutrophil interactions.

2. Materials and methods

2.1. Materials

Sphingosine-1-phosphate (S1P), 1-butanol, fura 2-Acetoxy-Methyl esther (AM), pertussis toxin, N-formylmethionyl-leucyl-phenylalanine (fMLP), α-actinin and acetoxymethyl 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM) were obtaioned from Sigma Chemical (Madrid, Spain). 2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one (PD 98059) was obtained from Calbiochem Corp. (BioNova, Madrid, Spain). Polyclonal antibodies against phospho-extracellular-signal-regulated kinase 1/2 (ERK 1/ 2) and β-actin were purchased from Cell signaling (Beverly, MA, U.S.A). All other reagents were of analytical grade and obtained commercially. S1P was dissolved in methanol to reach a final concentration of 0.1% v/v. fMLP and BAPTA-AM were dissolved in dimethyl sulfoxide (DMSO) with a final concentration of 0.01% v/v that it didn't affect any o f the experimental results of this work (data not shown). Pertussis toxin and α-actinin were dissolved in phosphate buffered saline.

2.2. Cell culture and cell isolation

A549 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and were cultured in supplemented Roswell Park Memorial Institute (RPMI) 1640 medium at 37 °C in a humidified atmosphere of 5% CO_2 in air as outlined (Mata et al., 2005). Cells at 70–80% confluence were serum deprived by incubation for 12–18 h in RPMI-1640 containing 0.1% (v/v) fetal bovine serum (FBS) prior to stimulation with S1P or other agents.

Samples of peripheral venous blood were obtained in heparin from healthy volunteers as outlined (Dalli et al., 2008). Polymorphonuclear leukocytes were separated by standard laboratory procedures (Boyum, 1968). The purity of neutrophil preparations used was >97%, and viability measured by vital dye trypan blue exclusion was >98%. Cell viability was not affected in the different experimental conditions. The protocol was approved by the local Ethics Committee, and written informed consent was obtained from each donor.

2.3. Intracellular free Ca^{2+} measurements

Intracellular free calcium concentration ($[Ca^{2+}]_i$) was measured by epifluorescence microscopy (Spectramaster System, Perkin Elmer, Life Sciences, Cambridge, UK) in cultured A549 cells using the Ca^{2+} indicator dye fura-2-AM as outlined (Dalli et al., 2008). Briefly, cell fluorescence of fura-2-AM (5 μM)-loaded cells was measured by using continuous rapid alternating excitation from monochromators (340 and 380 nm) and emission at 510 nm in a fluorescence spectrophotometer equipped with a xenon lamp (Nikon XB0 100, Tokyo, Japan) and a CCD camera CoolSNAPfx photometrics 193 (20 MHz, 1300×1030 pixels). The fluorescence ratio was recorded every 0.1 s using Lambda 10-2 Sutter Instrument (Nikon CO. Tokyo, Japan) and Fluorescence Analysis was performed with the Software Metafluor® 5.0.

2.4. PCR and real time RT-PCR

Total RNA was isolated from cultured A549 cells by using TriPure® Isolation Reagent (Roche, Indianapolis, USA). The reverse transcription was performed in 1 μg of total RNA with TaqMan reverse transcription reagents kit (Applied Biosystems, Perkin-Elmer Corporation, CA, USA). 1.5 μl of the cDNA was employed for the different PCR reactions. Primers used for PCR amplification were as follows: 5′to 3′: human $S1P_1$ (GenBank accession no. NM_001400) forward CTGGATCACTCATCGAACCACC, reverse CCGTGTAGTTGTAATGCCGGAC (210 bp), human $S1P_2$ (GenBank accession no. NM_005226) forward TTCATCGTGTTGGCTGTGCTC, reverse CCTTCGGAGAGTGGCTGCTATT (205 bp), human $S1P_3$ (GenBank accession no. NM_004230) forward CCAAGGTCAAGCTGTATGGCA, reverse GCCAACAGGATGATGGAGAAGA (208 bp), human $S1P_4$ (GenBank accession no NM_003775) forward CCAAGCGCTACATCCTCTTC, reverse CAGAGGTTGGAGCCAAAGAC (200 bp), human $S1P_5$ (GenBank accession no. NM_0030760) forward AAGGAGTAGTTCCCGAAGGACC, reverse CATCATTGTTCCTGTGGCTC (202 bp), and glyceraldehyde-3-phosphate dehydrogenase (GenBank accession no. NM_002046) forward GTATTGGGCGCCTGGTCACC, reverse CGCTCCTGGAAGATGGTGATGG (202 bp).

PCR reactions were performed for 35 cycles, with denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s, and elongation at 72 °C for 30 s, with a final extension at 72 °C for 10 min. PCR products were separated by 2% agarose gel electrophoresis and photographed under UV illumination. Band intensities were quantified by laser densitometry scanning. The results were expressed as a ratio of the band intensity relative to the corresponding GAPDH band. For real time PCR, 300 ng of total RNA was used for reverse transcription (RT) and amplification of target cDNA in the 7900HT Fast Real-Time PCR System (Applied Biosystem). The primers used for amplification of cDNA were obtained from TaqMan Gene Expression Assays (Applied Biosystem, Assay-On-Demand) for IL-8 (Hs00174103_m1, amplicon length 101 bp) and GAPDH (endogenous control; 4352339E, amplicon length 107 bp). The basal IL-8 mRNA expression was measured at 24 h of cell culture, and compared with the IL-8 mRNA expression of each time of S1P stimulation. Relative quantification of IL-8 transcripts was determined with the $2^{-\Delta\Delta Ct}$ method as outlined (Sellitti et al., 2007).

2.5. Measurement of IL-8 secretion

A549 cells (~80% confluence) in 35 mm dishes were pre-treated with specified agents for the indicated time periods prior to stimulation. After S1P stimulation, cell supernatants were removed, centrifuged at 5000 g for 5 min at 4 °C and stored at −80 °C. The basal IL-8 determination was measured at 48 h of cell culture. The IL-8 concentration in the medium was measured by ELISA, which was performed according to the manufacturer's instructions (R&D Systems).

2.6. Cell proliferation

Cell proliferation was measured by luminometric immunoassay based on BrdU incorporation during DNA synthesis using a cell proliferation enzyme-linked immunosorbent assay BrdU kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, A549 cells were treated with different proliferation modulators. Cells were then exposed to S1P (1 μM) for 12 h. For each condition as well as the controls, five wells were used. Subsequently, 20 μl of BrdU-labeling solution were added to each well, and the cells were incubated again for 12 h. BrdU labelling was measured at 370 and 490 nm and quantified using a microplate spectrophotometer (Victor 1420 Multilabel Counter, Perkin Elmer). Proliferation data refer to the absorbance values of BrdU-labeled cellular DNA content per well. Stimulation is expressed as *x*-fold proliferation over basal growth of the untreated control set as unity.

2.7. Phospholipase D activity

Phospholipase D (PLD) activity was performed as outlined (Pedruzzi et al., 1998). This method is based in the determination of amount of choline (CHO) mediated by the conversion of phosphatidylcholine in phosphatidic acid (PA) and CHO, that is catalysed by PLD. CHO was measured by using a chemiluminiscence assay. Aliquots of 10^7 A549 cells were incubated in Eppendorf tubes at 37 °C in the absence or presence of stimuli or different modulators of PLD activity as described in Results. To stop reactions, tubes were incubated in ice-cold methanol for 5–10 s, to allow a decrease in temperature to 2–4 °C. Cells were centrifuged (30 s, 10,000 g), and the pellets were resuspended in 100 mM glycine buffer (pH 10.2) containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$ and sonicated at 4 °C (three strokes of 10 s each with sonicator Sonorex® (Bandelin Electronic S.A)). CHO was oxidized in 200 µl of 200 mM phosphate buffer (pH 8.5) containing 0.02 mM luminol, 5 U peroxidase, 1 U CHO oxidase. The reaction was initiated in the dark by injecting 10 µl of different A549 supernatants and light emission was monitored every 3 s in a Wallac 1420 Victor® Multilabel Counter (EG&G, Turku, Finland). Moreover, exogenous CHO-chloride was used alone from 5 pmol to 75 pmol as standard curve. The PLD activity was quantified by calculation of produced choline according to a standard curve, and was expressed as ΔPLD *vs.* control (*x*-fold of control) in CHO pmol/10^7cells.

2.8. Preparation of cell lysates and western blot analysis for ERK 1/2

A549 cells grown in 100 mm dishes (~80% confluence) were stimulated with S1P with or without previous treatment with different extracellular matrix-regulated kinase1/2 (ERK1/2) modulators, then medium was removed and cells were lysed by the addition of 0.5 ml of buffer containing 10 mM Tris/HCl pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol (DTT), 1 mM orthovanadate, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin. The samples (20 µg of protein) were subjected to SDS/PAGE, and proteins from the gel were transferred to a PVDF membrane, blocked with 5% (w/v) non-fat dry milk in TBST (25 mM Tris base, pH 7.4, 137 mM NaCl and 0.1% Tween 20) for 1 h and incubated with primary antibodies against phospho-ERK 1/2 (1:1000 dilution) in TBST overnight at 4 °C. The membranes were washed four times with TBST at 20 min intervals and then incubated with mouse horseradish peroxidase-conjugated secondary antibody (1:3000 dilution) for 2 h at room temperature. The membranes were developed with an enhanced chemiluminiscence with ECL according to the manufacturer's recommendation.

2.9. Expression of adhesion molecules by A549 and human neutrophils

A total of 5×10^5 A549 cells were cultured in six-well tissue culture plates for 7 days, and cells were washed twice and incubated in fresh medium or were stimulated by the addition of S1P (1 µM) or TNF-α (10 ng/ml). The medium was removed and cells were detached by a short incubation in trypsin solution (Life Technologies). Immunofluorescence labelling of cultured A549 was performed by incubation with human mAbs VCAM-1 and ICAM-1-Alexa fluor 488 directed against VCAM-1 and ICAM-1 (Dako cytomation, Heidelberg, Germany) or isotype control antimouse IgG-Alexa fluor 488 (Dako cytomation, Heidelberg, Germany) (data not shown).

A total of 2.5×10^6 human neutrophils were stimulated with S1P, IL-8, or A549 supernatant cell culture (treated with or without 1 µM S1P for 24 h) at different times. Then, Immunofluorescence labelling of human neutrophils was performed by incubation with human mAbs L-selectin-FITC and CD11b/CD18-PECy5 (Dako cytomation, Heidelberg,

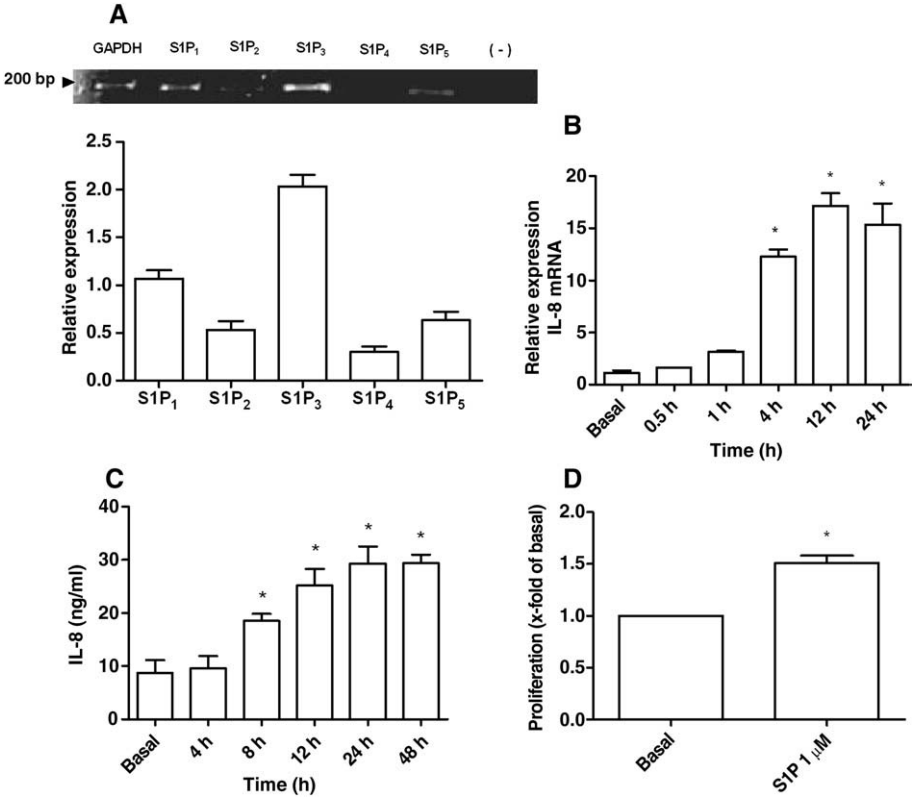

Fig. 1. Distribution of S1P receptors and effect of S1P on A549 cell proliferation and IL-8 secretion. A) Relative Sphingosine-1-phosphate receptor 1, 2, 3, 4 and 5 mRNA expression levels were determined by PCR in human A549 cells as described in Materials and methods. B) RT-PCR kinetic expression of IL-8 gene was made for $n = 4$ different extractions and expressed as $2^{-\Delta\Delta Ct}$. C) IL-8 secreted into the medium was quantified by ELISA in $n = 4$ per condition. D) A549 cells were stimulated with 1 µM S1P, after 12 h, 10 µM BrdU-labeled was added, and DNA synthesis was monitored for 12 h. Values (means ± S.E.M. of 4 independent experiments run five times) are expressed as *x*-fold of BrdU incorporation over basal ($x = 1$). Error bars represent mean ± S.E.M. (*$P < 0.05$ from basal).

Germany) or the respective isotype controls antimouse IgG-FITC (Dako cytomation, Heidelberg, Germany) (data not shown). The different fluorescence measures were performed in EPICS XL cytometer (Beckman-Coulter, Hialea, USA). Results were expressed like representative histograms and mean fluorescence intensity (arbitrary units).

2.10. Chemotaxis

Cell migration was measured with the Boyden chamber technique as previously described (Wilkinson, 1998) with modifications (Buenestado et al., 2006). The two compartments of the chamber were separated by a cellulose nitrate Millipore filter with a pore size of 3 μm (Sartorius, Goettingen, Germany). Neutrophils (2.5×10^6 cells ml^{-1} in RPMI 1640 containing 20 mmol l^{-1} HEPES and supplemented with 10 mg m^{-1} bovine serum albumin (BSA); pH 7.4) were placed in a volume of 200 μl in the upper wells.

The lower wells were filled with 200 μl of RPMI 1640 supplemented as indicated above (spontaneous movements) or with S1P (1 μM), IL-8 (20 ng/ml), fMLP (100 nM), antibody to IL-8 or A549 supernatant cell culture (treated with or without 1 μM S1P for 24 h), supernatant plus antibody to IL-8 or supernatant plus antibody to IL-8 and fMLP (100 nM). Then, chambers were incubated for 30 min at 37 °C and 5% CO_2. After migration, the filters were fixed and stained with Diff Quick (Baxter Diagnostics AG) and the distance (μm) travelled into the filter was determined according to the leading front technique. Chemotactic assays were carried out in triplicate and the migration distance of the neutrophils was determined at five different filter sites. The results are expressed as mean ± S.E.M. of the chemotaxis index, which is the distance of stimulated migration divided by that random migration (spontaneous movement).

2.11. Statistics

Data are presented as mean ± S.E.M. of n experiments. Statistical analysis of data was carried out by analysis of variance (ANOVA) followed by Bonferroni test (GraphPad Software Inc, San Diego, CA, U. S.A.). Significance was accepted when $^*P<0.05$.

3. Results

3.1. Expression of S1P receptors in alveolar epithelial cells A549

We examined the mRNA expression of different S1P receptors in A549. The semiquantitative amount of each one referred to housekeeping, GAPDH, was quantified as different band intensities as shown Fig. 1A. S1P receptor SIP_3 was the most highly expressed with lesser amount of SIP_1, SIP_2, SIP_4 and SIP_5.

3.2. S1P induces IL-8 expression and alveolar epithelial cells A549 proliferation

A549 treatment with S1P at physiologic concentrations (1 μM) resulted in a time-dependent IL-8 mRNA increase (Fig. 1B, $n=4$), with meaningful expression at 4 h, maximal expression at 12 h and plateaued for 24 h. The release of IL-8 into the medium by A549 cells upon stimulation with S1P significantly increased after 8 h and plateaued for 24 h (Fig. 1C, $n=3$). On the other hand, when A549 cells were stimulated with S1P (1 μM) for 24 h, cells showed an increase in 1.5 times respect the basal growth (Fig. 1D, $n=3$, $P<0.05$). Methanol 0.1% v/v was added as vehicle of S1P without effects in IL-8 secretion and proliferation (data not shown).

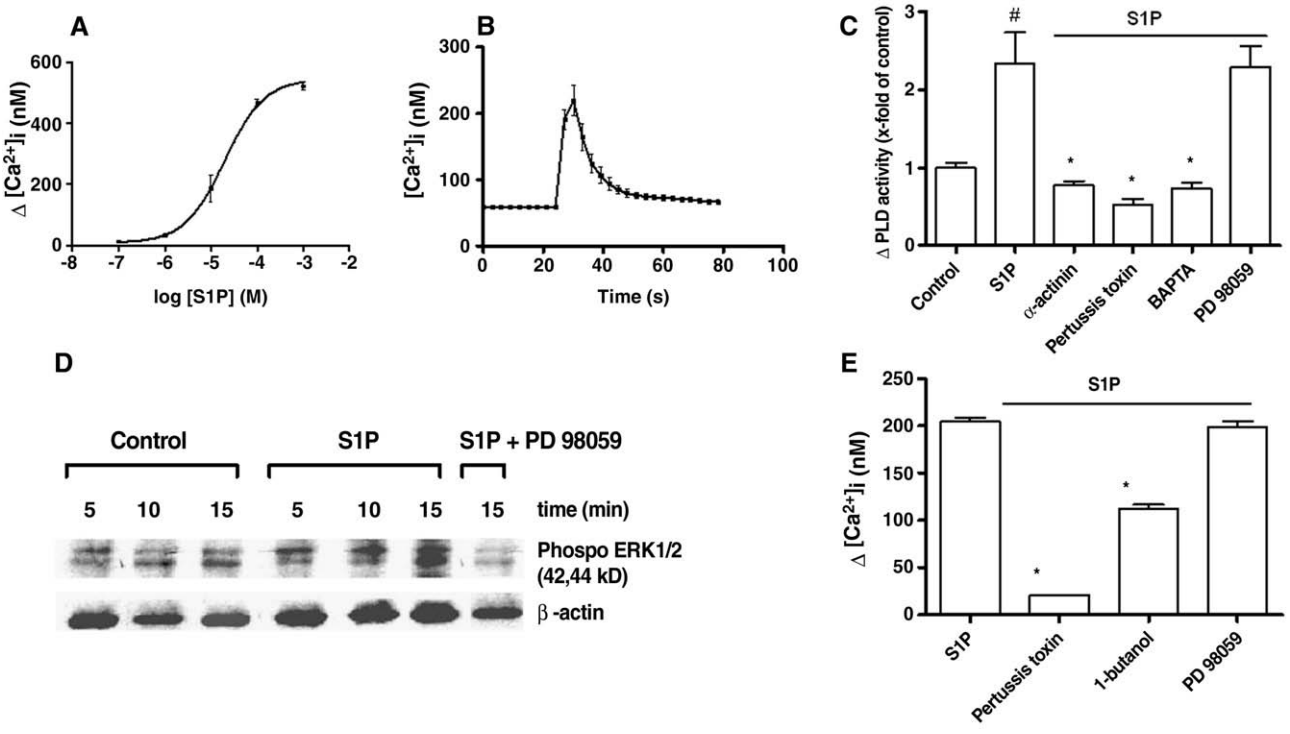

Fig. 2. S1P increase $[Ca^{2+}]_i$, PLD activity and ERK1/2 phosphorylation. A) The increase in $[Ca^{2+}]_i$ evoked by the addition of the indicated total [S1P] gave rise to a EC_{50} of 17.91 μM in A549 cells. B) A549 treatment with S1P 10 μM resulted in a rapid increase of $[Ca^{2+}]_i$ followed by a prolonged plateau over the basal $[Ca^{2+}]_i$. C) A549 were incubated in the absence (basal PLD activity) or presence of stimuli (S1P 1 μM for 20 min) with the previous treatment of α-actinin 300 nM for 20 min, pertussis toxin 100 ng/ml for 24 h, BAPTA/AM 100 μM for 30 min or PD 98059 10 μM for 15 min. D) A549 cells were challenged with S1P 1 μM for various time periods as indicated, and cell lysates were analysed (20 μg of protein per line) by western blot for phosphorylation of ERK1/2 and β-actin protein. Images are representative of three independent experiments. E) The increase of $[Ca^{2+}]_i$ induced by S1P 10 μM was significantly reduced in the presence of pertussis toxin 100 ng/ml for 24 h or 1-butanol 0.5% for 15 min, and unaffected in the presence of PD98059 10 μM for 15 min. The bars give the mean ± S.E.M. of $\Delta[Ca^{2+}]_i$ for 3 independent experiments of measurements made in 5–7 cells by experiment ($^*P<0.05$ from S1P treatment, $\#P<0.05$ from control).

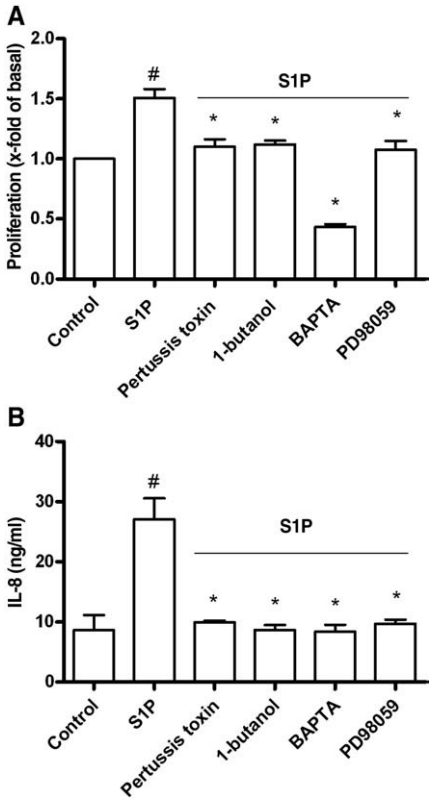

Fig. 3. Effect of intracellular Ca^{2+}, PLD, protein G_i, and ERK1/2 on S1P-induced IL-8 secretion and proliferation in A549 cells. A549 cells were pretreated in presence or absence of pertussis toxin 100 ng/ml for 24 h, 1-butanol 0.5% for 15 min, PD 98059 10 μM for 15 min or BAPTA-AM 100 μM for 30 min, and then, cells were stimulated with S1P 1 μM for 24 h. A) A549 proliferation was determined with BrdU assay at 24 h. Value are expressed as *x*-fold stimulation of BrdU incorporation over basal ($x=1$). B) IL-8 secreted into the medium was quantified by ELISA. Data are mean ± S.E.M. of triplicate determinations and are representative of at least three independent experiments (*$P<0.05$ from S1P treatment, #$P<0.05$ from control).

3.3. S1P activates intracellular Ca^{2+}, PLD activity and ERK1/2 phosphorylation in alveolar epithelial cells A549

S1P (100 nM to 1 mM) produced a concentration-dependent increase of $[Ca^{2+}]_i$ in A549 cells, with a potency of 17.91 μM ($-\log EC_{50}=4.743\pm0.06$; $n=5$, Fig. 2A). The S1P-induced increase of $[Ca^{2+}]_i$ consisted of an initially rapid increase of $[Ca^{2+}]_i$ to reach a peak in ~5 s followed by a subsequent decline that reached in ~25 s a plateau slightly above baseline (Fig. 2B, $n=5$).

On the other hand, the addition of S1P (1 μM) to A549 cells for 2 min at 37 °C increased the product of PLD activity, CHO, in 2.32 ± 0.39-fold *vs.* control, and it was inhibited by α-actinin, a natural inhibitor of PLD activity (Lee et al., 2001) to basal levels (Fig. 2C, $n=5$, $P<0.05$) without affecting PLD basal activity (data not show).

Conversely, S1P stimulation resulted in a rapid ERK1/2 phosphorylation in A549 cells (peak at 15 min), which was markedly inhibited by PD 98059 (10 μM) (Fig. 2D, $n=3$). On the other hand, pertussis toxin pre-treatment (24 h, 100 ng/ml) nearly abolished the S1P-induced $[Ca^{2+}]_i$ mobilization and PLD activation (Fig. 2E and C, $n=5$, $P<0,05$) without affecting the basal levels of both (data not show). Moreover, pre-treatment with 1-butanol (15 min, 0.5%), an inhibitor of PLD (Cummings et al., 2002b), decreased by ~50% the increase of $[Ca^{2+}]_i$ induced by S1P (Fig. 2E, $n=5$, $P<0.05$). At the same time, the intracellular Ca^{2+} chelator, BAPTA-AM (100 μM, 15 min before S1P addition), repressed the PLD activity induced by S1P (1 μM) (Fig. 2C, $n=5$, $P<0.05$). However, the inhibition of ERK1/2 phosphorylation with PD 98059 (10 μM) failed to modify the $[Ca^{2+}]_i$ and PLD activity. Neither 1-butanol nor BAPTA-AM nor PD 98059 affected the basal levels of the $[Ca^{2+}]_i$ and PLD activity (data not shown).

3.4. Effect of intracellular Ca^{2+}, PLD, G_i protein, and ERK1/2 on S1P-induced IL-8 secretion and proliferation in alveolar epithelial cells A549

As shown in Fig. 3A and B, pertussis toxin pretreatment effectively blocked IL-8 secretion and proliferation almost to the

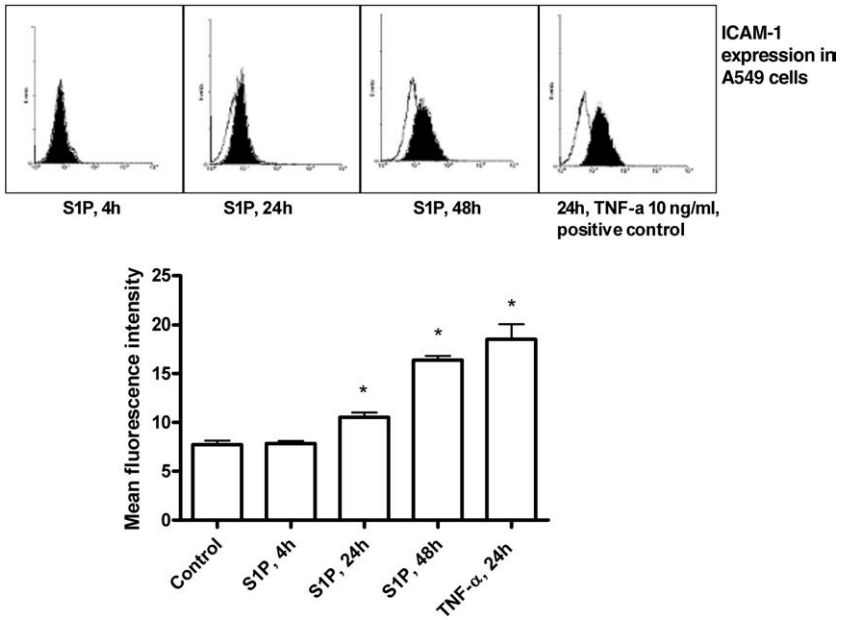

Fig. 4. S1P induces ICAM-1 expression in A549. A) Expression of ICAM-1 by A549 cells after 4 h, 24 h, and 48 h of S1P 1 μM and TNF-α 10 ng/ml for 24 h (black histograms) respect control measured at 48 h (white histograms). Results are presented as histogram (10,000 events = ordinate; abscissa = fluorescence intensity) and graphic bars of mean fluorescence intensity ± S.E.M. Immunofluorescence was performed by the use of mouse anti-human ICAM-1 (Alexa fluor 488-labelled). Cells were analyzed on EPICS XL cytometer (Beckman-Coulter, Hialea, U.S.A) ($n=4$ each; *$P<0.05$).

basal levels ($n=4$, $P<0.05$). The inhibition of PLD activity by 1-butanol (15 min, 0.5%), the intracellular Ca^{2+} chelation (BAPTA-AM, 100 μM, 15 min before SIP addition) and the inhibition of the ERK1/2 phosphorylation by PD 98059 (10 μM) resulted in a significant reduction of the IL-8 secretion and proliferation (Fig. 3A and B, $P<0.05$) induced by S1P. The treatment of A549 cells with pertussis toxin or PD 98059 didn't affect to the basal IL-8 secretion or A549 proliferation, while BAPTA-AM treatment reduced proliferation in 0.5 ± 0.04 fold respect to the basal proliferation (data not show).

3.5. Effect of S1P over expression of adhesion molecules by alveolar epithelial cells A549

A549 treatment with TNF-α (24 h, 10 ng/ml) increased the expression of ICAM-1 as positive control, and to a minor extent, S1P (1 μM for 4 h, 24 h, and 48 h) showed a time dependent increase of ICAM-1 protein expression (Fig. 4). The expression of VCAM-1 by epithelial cells has been reported in bronchial and renal epithelium (Nakajima et al., 1994) whereas contradictory results have been reported for alveolar epithelial cells (Cunningham et al., 1994). Our results indicate very low levels of VCAM-1 expression on A549 (data not shown). Stimulation of A549 with TNF-α (24 h, 10 ng/ml) or S1P (1 μM, 4 h, 24 h, and 48 h) did not modify the VCAM-1 expression (data not shown).

3.6. Effect of S1P and the A549 supernatant in adhesion molecules of human neutrophils

Human neutrophils incubation with S1P (1 μM) for 15 min and 30 min did not show any change in the L-selectin and CD11b/CD18 surface expression (Fig. 5, $n=4$). In contrast, human neutrophils treated with A549 supernatant (from A549 treated with S1P (1 μM for 24 h) for a period of 15 min and 30 min (black histograms, Fig. 5), resulted in a decrease of the L-selectin surface expression compared with human neutrophils exposed to A549 supernatant without S1P stimulation (white histograms, Fig. 5). Moreover, the expression of human neutrophil CD11b/CD18 was unaffected by the A549 supernatant treated with S1P (Fig. 5, $n=4$).

3.7. Effect of S1P and A549 supernatant in human neutrophil chemotaxis

Direct action of S1P (1 μM over human neutrophil chemotaxis resulted without effect. In contrast, IL-8 (20 ng/ml) and fMLP (100 nM) positive controls, resulted in a significant increase of chemotaxis (white bars Fig. 6, $n=4$, $P<0.05$). However, the addition of the A549 supernatant stimulated with S1P increased neutrophil chemotaxis compared with the control untreated with S1P (lined bars Fig. 6, $n=4$, $P<0.05$). The A549 supernatant treated with S1P and antibody to IL-8 caused a decrease in chemotaxis, less than the chemotaxis control (Fig. 6, $n=4$, $P<0.05$). In contrast, the addition of the antibody to IL-8 was without effect when we stimulated human

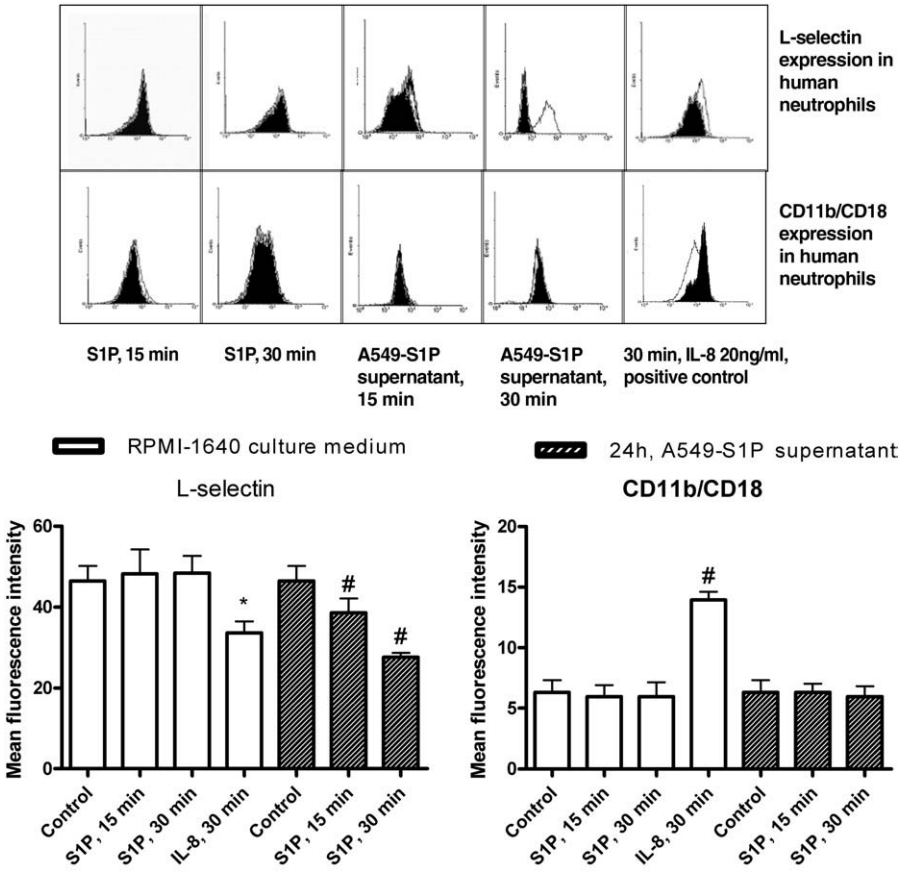

Fig. 5. Effect of S1P on L-selectin and CD11b/CD18 in human neutrophils. Expression of L-selectin and CD11b/CD18 in human neutrophils after 15 min and 30 min of S1P 1 μM addition, and 30 min of 20 ng/ml IL-8 (black histograms, white bars) addition respect to control (white histograms, white bars). Moreover, human neutrophils were treated for 15 min and 30 min with A549 supernatant previously treated with (black histograms, lined bars) and without (white histograms, lined bars) S1P 1 μM for 24 h. Immunofluorescence was performed by the use of L-selectin (FITC) and CD11b/CD18 (PECy5) mAbs. Cells were analyzed on EPICS XL cytometer (Beckman-Coulter, Hialea, USA). Each histogram of this representative experiment represents 10,000 events in the ordinate and fluorescence intensity in the abscissa. Graphics represent the mean fluorescence intensity ± S.E.M. ($n=4$, $^*P<0.05$ respect control white bar, $\#P<0.05$ respect control lined bar).

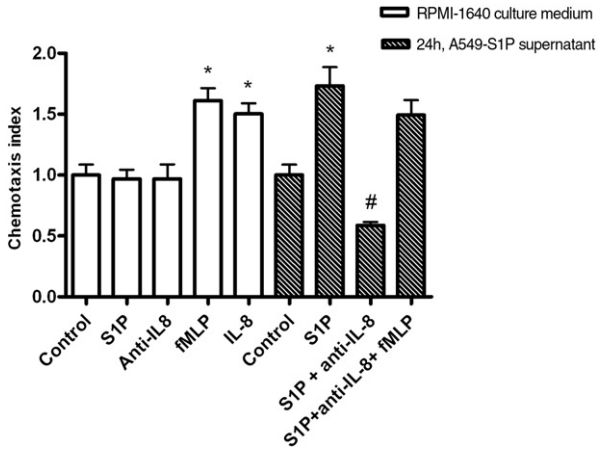

Fig. 6. S1P indirectly induces human neutrophil chemotaxis. Human neutrophil chemotaxis was performed in a Boyden chamber. As chemoattractants, S1P (1 μM), IL-8 (20 ng/ml), fMLP (100 nM) or A549 supernatant cell culture (treated with or without S1P1 μM for 24 h), supernatant plus antibody to IL-8 or supernatant plus antibody to IL-8 and fMLP (100 nM) were added, and then, chambers were incubated for 30 min at 37 °C and 5% CO_2. Data are presented as mean ± S.E.M. of the chemotaxis index ($n = 4$ per condition, $^*P < 0.05$ from control, $\#P < 0.05$ from S1P).

neutrophils with A549-S1P supernatant plus antibody to IL-8 and fMLP (100 nM). On the other hand, we observed that the addition of the antibody to IL-8 to neutrophils in absence of A549 supernatant didn't change the basal chemotaxis, linking the effects of A549-S1P supernatant with the secretion of IL-8 induced by S1P in A549.

4. Discussion

Our findings demonstrate that in human alveolar type II A549 cells, extracellular S1P stimulates proliferation, IL-8 production and adhesion molecule modification in A549, and indirectly, modulates chemotaxis and adhesion molecule expression in human neutrophils. Although human A549 cells have been widely used in studies of the interaction leukocyte–epithelium (Rosseau et al., 2000) these tumour epithelial cells are not phenotypically normal which represents a limitation of this study.

$S1P_1$, $S1P_3$, $S1P_4$, $S1P_5$, and weak $S1P_2$ have been detected in human bronchial epithelial cells, Beas-2B and A549 cells (Chen et al., 2008; Cummings et al., 2002b) similar to our detected pattern of expression in A549 (Fig. 1A). The predominant expression of $S1P_3$ in bronchial and alveolar epithelial cells explains the main effects attributed to S1P in these cells, like the phospholipase A2α activation and the increases in lung permeability via tight junction opening (Chen et al., 2008; Gon et al., 2005). These observations, perhaps, could help to explain the S1P functional effects observed in this work. Moreover, S1P receptors exhibit differential coupling to heterotrimeric G proteins making functional analysis complex. For instance, $S1P_1$ and $S1P_4$ couple directly to the G_i pathway, whereas $S1P_2$ and $S1P_3$ stimulate the G_i, G_q, and $G_{12/13}$ pathways, and $S1P_5$ activates both G_i and $G_{12/13}$ proteins (Spiegel and Milstien, 2000). In this study, pertussis toxin pretreatment nearly abolished IL-8 production and A549 cell proliferation as well as PLD activation and intracellular Ca^{2+} mobilization, so we can affirm that extracellular S1P activates this pathway through G_i-protein activation and maybe by the interaction with the majority $S1P_3$.

Intracellular Ca^{2+} and PLD are involved in different cellular processes like, exocytosis, secretion, cellular proliferation and mitogenesis among others (Berridge et al., 2000; Cummings et al., 2002a). Previously, it has been shown that PLD activation increases IL-8 secretion through RhoA and protein kinase C (PKC) pathway in bronchial epithelial cells (Cummings et al., 2002b; Meacci et al., 1999). In this study, S1P induced IL-8 secretion and A549 proliferation through a common pathway that implicated a rapid PLD activation and intracellular Ca^{2+} mobilization. Moreover, both PLD and intracellular Ca^{2+} activation were interdependent, since intracellular Ca^{2+} suppression inhibits PLD activation and PLD inhibition reduces intracellular Ca^{2+} increment, maybe due to decrease in diacylglycerol levels generated by the PLD activity. Furthermore, it has been shown that PLD induce ERK1/2 phosphorylation, but PD 98059 does not attenuate S1P-induced PLD activation, suggesting ERK stimulation is downstream of PLD in Beas-2B cells (Wang et al., 2002). In this work, we have observed that S1P-induced IL-8 secretion and A549 proliferation is dependent on the ERK1/2 phosphorylation since PD 98059 inhibits, almost to basal levels, these two processes. At the same time, PD 98059 failed in PLD and intracellular Ca^{2+} inhibition. This fact, suggests that ERK1/2 phosphorylation could be subsequent to PLD and intracellular Ca^{2+} activation.

Since IL-8 plays a key role like a potent neutrophil chemoattractant into the alveolar space, we have studied the implication of S1P in this process. In the lung, a principal site of leukocyte emigration in response to inflammation is the alveolar capillary bed. In this context, different inflammatory mediators like IL-1β and TNF-α increased in the alveolar space during airway inflammation, stimulate the alveolar macrophage S1P releases (Jolly et al., 2002), and at the same time, stimulate the release of chemoattractant factors like IL-8. These sets of signals induce the activation of the systemic neutrophils and the sequestration into the alveolar capillaries (Burns et al., 2003). In this process, neutrophil migration can be divided into four steps: rolling, adhesion, extravasation, and migration. L-selectin constitutively expressed on mature neutrophils plays a key role in establishing the weak adhesive interaction associated with leukocyte rolling (Ley et al., 1991) through the shedding of L-selectin. In contrast to L-selectin, CD11b expression is increased on neutrophil surface under inflammatory conditions, and mediates the adhesion to the alveolar epithelial cells through the ICAM-1 interaction (Burns et al., 2003). Although sequestration of neutrophils within alveolar capillaries is not dependent on L-selectin or β2-integrins, the retention times within this capillary bed are influenced by these adhesion molecules (Kubo et al., 1999). All these sequential processes finish in a further concentration of neutrophils at the alveolar walls with a corresponding increase in airway inflammation.

At the present, no evidence has been shown about the direct effect of S1P on the adhesion molecules in human neutrophils and airway epithelial cells, but the fact that S1P levels are dramatically enhanced in the airways of asthmatics suggests that S1P may play a role in the modulation of these adhesion molecules.

In this study, we observed that human neutrophil stimulation with the A549-S1P supernatant decreased L-selectin surface expression and did not modify in a significant manner CD11b/CD18. We noted that the content of supernatant is not known with the exception of the IL-8 concentration detected by ELISA. In fact, L-selectin decreases in response to IL-8 (20 ng/ml) was lower than L-selectin decreases in response to A549-S1P supernatant. The content of IL-8 in the A549-S1P supernatant after 24 h of S1P stimulation was 29.27 ± 3.2 ng/ml, slightly higher to the IL-8 concentration we used like a positive control. By this reason we can affirm that IL-8 is implicated in L-selectin decreases, but perhaps there are more substances in the supernatant related with this process, although at the present, there is no evidence that S1P can release some factor from the airway epithelial cells related with the L-selectin shedding.

Likewise, S1P-A549 supernatant addition increased neutrophil chemotaxis in contrast to the basal A549 supernatant. Previous reports have shown contradictory effects of S1P over neutrophil chemotaxis. Whereas Kawa et al. (1997) showed an inhibitory chemotactic effect, Rahaman et al. (2006) showed an increase of neutrophil chemotaxis. In this study, we noted an absence of direct effect of S1P. In addition the indirect increase of neutrophil chemotaxis caused by the S1P-A549 supernatant was completely

abolished when IL-8 was removed from supernatant. These results are in agreement with the main function of IL-8 on neutrophil chemotaxis.

Finally, we studied the effect of S1P over ICAM-1 and VCAM-1 expression in alveolar type II epithelial cells A549. ICAM-1, a major ligand of CD11b/CD18, is expressed on type I and type II pneumocytes (Cunningham and Kirby, 1995; Nakajima et al., 1994) and is up-regulated in the presence of proinflammatory cytokines (Cunningham et al., 1994; Rosseau et al., 2000), and VCAM-1 has been detected on bronchial and renal epithelium (Cunningham and Kirby, 1995; Nakajima et al., 1994). Shimamura et al. (2004) (Shimamura et al., 2004) were the first to observe an increment in ICAM-1, VCAM-1 and E-selectin in endothelial cells treated with S1P. However, this effect over the alveolar epithelium is unknown. We show here, that S1P, at physiologic concentrations, enhanced ICAM-1 surface expression in a time dependent fashion, but failed in VCAM-1 modification. The increase in IL-8 secretion and ICAM-1 are in agreement with the increase in neutrophil chemotaxis.

In summary, our findings demonstrate that S1P increases IL-8 secretion and proliferation in alveolar epithelial cells A549 in the same fashion, through G_i protein activation and intracellular Ca^{2+}-PLD-ERK1/2 pathway. In addition, S1P increases ICAM-1 expression and, indirectly, neutrophil chemotaxis by the increases of IL-8 concentration in A549 supernatant. Our data indicate that S1P could augment local inflammatory reactions in alveolar epithelium.

Acknowledgements

This work was supported by grants SAF2006-01002 (EJM), SAF2005-00669 (JC) and SAF20083113 (JC) from CICYT (Ministry of Education and Science, Spanish Government), and GV2007-287 from Regional Government ('Generalitat Valenciana'). JM has a research contract from FIS of Health Institute 'Carlos III' of Ministry of Health (Spain). This work has not received financial support from drug companies.

References

Ammit, A.J., Hastie, A.T., Edsall, L.C., Hoffman, R.K., Amrani, Y., Krymskaya, V.P., Kane, S.A., Peters, S.P., Penn, R.B., Spiegel, S., Panettieri Jr., R.A., 2001. Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. FASEB J. 15, 1212–1214.

Barton, W.W., Wilcoxen, S., Christensen, P.J., Paine, R., 1995. Disparate cytokine regulation of ICAM-1 in rat alveolar epithelial cells and pulmonary endothelial cells in vitro. Am. J. Physiol. 269, L127–135.

Berridge, M.J., Lipp, P., Bootman, M.D., 2000. The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell Biol. 1, 11–21.

Boyum, A., 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand. J. Clin. Lab. Invest. Suppl. 97, 77–89.

Buenestado, A., Cortijo, J., Sanz, M.J., Naim-Abu-Nabah, Y., Martinez-Losa, M., Mata, M., Issekutz, A.C., Marti-Bonmati, E., Morcillo, E.J., 2006. Olive oil-based lipid emulsion's neutral effects on neutrophil functions and leukocyte–endothelial cell interactions. JPEN J. Parenter Enteral Nutr. 30, 286–296.

Burns, A.R., Smith, C.W., Walker, D.C., 2003. Unique structural features that influence neutrophil emigration into the lung. Physiol. Rev. 83, 309–336.

Car, B.D., Meloni, F., Luisetti, M., Semenzato, G., Gialdroni-Grassi, G., Walz, A., 1994. Elevated IL-8 and MCP-1 in the bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. Am. J. Respir. Crit. Care Med. 149, 655–659.

Chen, L.Y., Woszczek, G., Nagineni, S., Logun, C., Shelhamer, J.H., 2008. Cytosolic phospholipase A2{alpha} activation induced by S1P is mediated by the S1P3 receptor in lung epithelial cells. Am. J. Physiol. Lung. Cell. Mol. Physiol.

Cohen, L.E.X., Tarsi, J., Ramkumar, T., Horiuchi, T.K., Cochran, R., DeMartino, S., Schechtman, K.B., Hussain, I., Holtzman, M.J., Castro, M., 2007. Epithelial cell proliferation contributes to airway remodeling in severe asthma. Am. J. Respir. Crit. Care Med. 176, 138–145.

Colgan, S.P., Parkos, C.A., McGuirk, D., Brady, H.R., Papayianni, A.A., Frendl, G., Madara, J.L., 1995. Receptors involved in carbohydrate binding modulate intestinal epithelial–neutrophil interactions. J. Biol. Chem. 270, 10531–10539.

Cunningham, A.C., Kirby, J.A., 1995. Regulation and function of adhesion molecule expression by human alveolar epithelial cells. Immunology 86, 279–286.

Cunningham, A.C., Milne, D.S., Wilkes, J., Dark, J.H., Tetley, T.D., Kirby, J.A., 1994. Constitutive expression of MHC and adhesion molecules by alveolar epithelial cells (type II pneumocytes) isolated from human lung and comparison with immunocytochemical findings. J. Cell. Sci. 107 (Pt 2), 443–449.

Cummings, R., Parinandi, N., Wang, L., Usatyuk, P., Natarajan, V., 2002a. Phospholipase D/phosphatidic acid signal transduction: role and physiological significance in lung. Mol. Cell Biochem. 234–235, 99–109.

Cummings, R.J., Parinandi, N.L., Zaiman, A., Wang, L., Usatyuk, P.V., Garcia, J.G., Natarajan, V., 2002b. Phospholipase D activation by sphingosine 1-phosphate regulates interleukin-8 secretion in human bronchial epithelial cells. J. Biol. Chem. 277, 30227–30235.

Dalli, E., Milara, J., Cortijo, J., Morcillo, E.J., Cosin-Sales, J., Sotillo, J.F., 2008. Hawthorn extract inhibits human isolated neutrophil functions. Pharmacol. Res.

Fuke, S., Betsuyaku, T., Nasuhara, Y., Morikawa, T., Katoh, H., Nishimura, M., 2004. Chemokines in bronchiolar epithelium in the development of chronic obstructive pulmonary disease. Am. J. Respir Cell. Mol. Biol. 31, 405–412.

Gon, Y., Wood, M.R., Kiosses, W.B., Jo, E., Sanna, M.G., Chun, J., Rosen, H., 2005. S1P3 receptor-induced reorganization of epithelial tight junctions compromises lung barrier integrity and is potentiated by TNF. Proc. Natl. Acad. Sci. U. S. A. 102, 9270–9275.

Jolly, P.S., Rosenfeldt, H.M., Milstien, S., Spiegel, S., 2002. The roles of sphingosine-1-phosphate in asthma. Mol. Immunol. 38, 1239–1245.

Kawa, S., Kimura, S., Hakomori, S., Igarashi, Y., 1997. Inhibition of chemotactic motility and trans-endothelial migration of human neutrophils by sphingosine 1-phosphate. FEBS Lett. 420, 196–200.

Kubo, H., Doyle, N.A., Graham, L., Bhagwan, S.D., Quinlan, W.M., Doerschuk, C.M., 1999. L- and P-selectin and CD11/CD18 in intracapillary neutrophil sequestration in rabbit lungs. Am. J. Respir. Crit. Care Med. 159, 267–274.

Lee, S., Park, J.B., Kim, J.H., Kim, Y., Shin, K.J., Lee, J.S., Ha, S.H., Suh, P.G., Ryu, S.H., 2001. Actin directly interacts with phospholipase D, inhibiting its activity. J. Biol. Chem. 276, 28252–28260.

Ley, K., Gaehtgens, P., Fennie, C., Singer, M.S., Lasky, L.A., Rosen, S.D., 1991. Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules in vivo. Blood 77, 2553–2555.

Mata, M., Sarria, B., Buenestado, A., Cortijo, J., Cerda, M., Morcillo, E.J., 2005. Phosphodiesterase 4 inhibition decreases MUC5AC expression induced by epidermal growth factor in human airway epithelial cells. Thorax 60, 144–152.

Meacci, E., Vasta, V., Moorman, J.P., Bobak, D.A., Bruni, P., Moss, J., Vaughan, M., 1999. Effect of Rho and ADP-ribosylation factor GTPases on phospholipase D activity in intact human adenocarcinoma A549 cells. J. Biol. Chem. 274, 18605–18612.

Nakajima, S., Look, D.C., Roswit, W.T., Bragdon, M.J., Holtzman, M.J., 1994. Selective differences in vascular endothelial- vs. airway epithelial-T cell adhesion mechanisms. Am. J. Physiol. 267, L422–432.

Pedruzzi, E., Hakim, J., Giroud, J.P., Perianin, A., 1998. Analysis of choline and phosphorylcholine content in human neutrophils stimulated by f-Met-Leu-Phe and phorbol myristate acetate: contribution of phospholipase D and C. Cell. Signal. 10, 481–489.

Pilette, C., Colinet, B., Kiss, R., Andre, S., Kaltner, H., Gabius, H.J., Delos, M., Vaerman, J.P., Decramer, M., Sibille, Y., 2007. Increased galectin-3 expression and intra-epithelial neutrophils in small airways in severe COPD. Eur. Respir. J. 29, 914–922.

Rahaman, M., Costello, R.W., Belmonte, K.E., Gendy, S.S., Walsh, M.T., 2006. Neutrophil sphingosine 1-phosphate and lysophosphatidic acid receptors in pneumonia. Am. J. Respir. Cell Mol. Biol. 34, 233–241.

Rosseau, S., Selhorst, J., Wiechmann, K., Leissner, K., Maus, U., Mayer, K., Grimminger, F., Seeger, W., Lohmeyer, J., 2000. Monocyte migration through the alveolar epithelial barrier: adhesion molecule mechanisms and impact of chemokines. J. Immunol. 164, 427–435.

Sellitti, D.F., Puggina, E., Lagranha, C., Doi, S.Q., Pithon-Curi, T., Kohn, L.D., Suzuki, K., 2007. TGF-beta-like transcriptional effects of thyroglobulin (Tg) in mouse mesangial cells. Endocr. J. 54, 449–458.

Shimamura, K., Takashiro, Y., Akiyama, N., Hirabayashi, T., Murayama, T., 2004. Expression of adhesion molecules by sphingosine 1-phosphate and histamine in endothelial cells. Eur. J. Pharmacol. 486, 141–150.

Spiegel, S., Milstien, S., 2000. Functions of a new family of sphingosine-1-phosphate receptors. Biochim. Biophys. Acta 1484, 107–116.

Wang, L., Cummings, R., Usatyuk, P., Morris, A., Irani, K., Natarajan, V., 2002. Involvement of phospholipases D1 and D2 in sphingosine 1-phosphate-induced ERK (extracellular-signal-regulated kinase) activation and interleukin-8 secretion in human bronchial epithelial cells. Biochem. J. 367, 751–760.

Wilkinson, P.C., 1998. Assays of leukocyte locomotion and chemotaxis. J. Immunol. Methods 216, 139–153.

Yatomi, Y., Igarashi, Y., Yang, L., Hisano, N., Qi, R., Asazuma, N., Satoh, K., Ozaki, Y., Kume, S., 1997. Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. J. Biochem. 121, 969–973.