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Exogenous ceramide-1-phosphate (C1P) and phospho-ceramide analogue-1 (PCERA-1) regulate key macrophage activities via distinct receptors

Sebastián Katz[a,1], Orna Ernst[a,1], Dorit Avni[a], Muhammad Athamna[a], Amir Philosoph[a], Lide Arana[b], Alberto Ouro[b], L. Alexis Hoeferlin[c,d,e], Michael M. Meijler[f], Charles E. Chalfant[c,d,e], Antonio Gómez-Muñoz[b], Tsaffrir Zor[a,*]

[a] Department of Biochemistry & Molecular Biology, Life Sciences Institute, Tel-Aviv University, Tel-Aviv 69978, Israel
[b] Department of Biochemistry & Molecular Biology, University of the Basque Country, P.O. Box 644, Bilbao 48080, Spain
[c] Department of Biochemistry & Molecular Biology, Virginia Commonwealth University, Richmond, VA 23298-0614, United States
[d] Hunter Holmes McGuire Veterans Administration Medical Center, Richmond, VA 23249, United States
[e] The Massey Cancer Center, Richmond, VA 23298, United States
[f] Department of Chemistry, Ben-Gurion University of the Negev, Be'er-Sheva 84105, Israel

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ABSTRACT

Inflammation is an ensemble of tightly regulated steps, in which macrophages play an essential role. Previous reports showed that the natural sphingolipid ceramide 1-phosphate (C1P) stimulates macrophages migration, while the synthetic C1P mimic, phospho-ceramide analogue-1 (PCERA-1), suppresses production of the key pro-inflammatory cytokine TNFα and amplifies production of the key anti-inflammatory cytokine IL-10 in LPS-stimulated macrophages, via one or more unidentified G-protein coupled receptors. We show that C1P stimulated RAW264.7 macrophages migration via the NFκB pathway and MCP-1 induction, while PCERA-1 neither mimicked nor antagonized these activities. Conversely, PCERA-1 synergistically elevated LPS-dependent IL-10 expression in RAW264.7 macrophages via the cAMP-PKA-CREB signaling pathway, while C1P neither mimicked nor antagonized these activities. Interestingly, both compounds have the capacity to additively inhibit TNFα secretion; PCERA-1, but not C1P, suppressed LPS-induced TNFα expression in macrophages in a CREB-dependent manner, while C1P, but not PCERA-1, directly inhibited recombinant TNFα converting enzyme (TACE). Finally, PCERA-1 failed to interfere with binding of C1P to either the cell surface receptor or to TACE. These results thus indicate that the natural sphingolipid C1P and its synthetic analog PCERA-1 bind and activate distinct receptors expressed in RAW264.7 macrophages. Identification of these receptors will be instrumental for elucidation of novel activities of extra-cellular sphingolipids, and may pave the way for the design of new sphingolipid mimics for the treatment of inflammatory diseases, and pathologies which depend on cell migration, as in metastatic tumors.

1. Introduction

Sphingolipids play structural roles as ubiquitous components of membranes in eukaryotes. In addition, it is also well established that sphingolipids regulate diverse cellular events, including inflammatory responses [1]. Ceramide is the precursor of all sphingolipids and can be generated de novo, or alternatively by the action of sphingomyelinases (SMases) [2,3]. The bioactive sphingolipid ceramide 1-phosphate (C1P) is produced by phosphorylation of ceramide, a reaction catalyzed by ceramide kinase (CERK) [4,5]. While the parent molecule ceramide stimulates apoptosis, intracellular C1P stimulates proliferation, and inhibits apoptosis [6–9]. Interestingly, C1P was found in serum, suggesting it may also act

Abbreviations: C1P, ceramide 1-phosphate; CERA-1, ceramide analog-1; CERK, ceramide kinase; CRE, cAMP response element; LPA, lysophosphatidic acid; MCP-1, monocyte chemoattractant protein-1; PCERA-1, phospho-ceramide analog-1; SM, sphingomyelin; SMase, sphingomyelinase; TACE, TNFα converting enzyme.

* Corresponding author. Tel./fax: +972 3 640 7192.
E-mail addresses: seba120777@gmail.com (S. Katz), ornaerns@post.tau.ac.il (O. Ernst), davni@vcu.edu (D. Avni), athamnam@gmail.com (M. Athamna), amir.philo@gmail.com (A. Philosoph), lide.arana@ehu.eus (L. Arana), alber.ouro@gmail.com (A. Ouro), alhoeferlin@vcu.edu (L.A. Hoeferlin), meijler@bgu.ac.il (M.M. Meijler), cechalfant@vcu.edu (C.E. Chalfant), antonio.gomez@ehu.eus (A. Gómez-Muñoz), tsaffyz@tauex.tau.ac.il (T. Zor).
[1] These authors contributed equally to this work.

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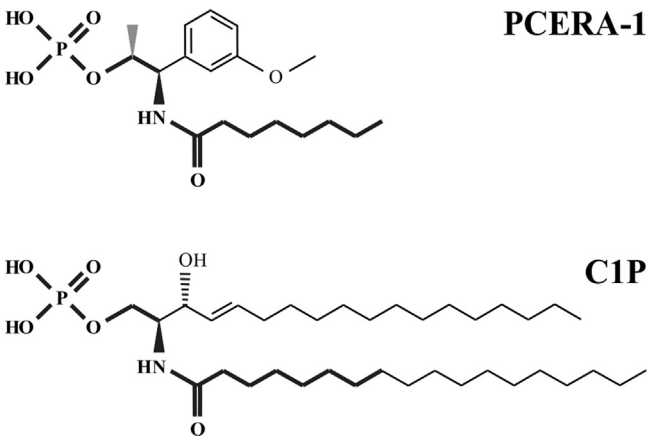

Fig. 1. Structures of C1P and PCERA-1.
The common structure of these molecules is highlighted by bold lines and letters.

as an extra-cellular mediator [10]. Indeed, Gómez-Muñoz and co-workers reported the existence of an as-of-yet unidentified C1P receptor, residing in the plasma membrane. This C1P receptor stimulates macrophages migration via the concerted activation of the ERK1/2, PI3K/Akt and NFκB pathways, and subsequent expression and release of monocyte chemoattractant protein-1 (MCP-1) [11,12]. In addition, extracellular C1P acting via a cell-surface receptor also stimulates glucose uptake and metabolism in macrophages in a PI3K/Akt-dependent manner [13]. Importantly, these cellular effects were blocked by pertussis toxin, suggesting the involvement of a C1P receptor that belongs to the $G\alpha_i$ protein-coupled receptor sub-family [11–13]. In contrast to these receptor-mediated effects of exogenous C1P, Chalfant and co-workers demonstrated that endogenous C1P acts as a second messenger in stimulating the production of inflammatory lipid mediators, by directly binding and activating cytosolic phospholipase A_2 ($cPLA_2$) [14]. More recently, Chalfant and co-workers reported that C1P directly binds TNFα converting enzyme (TACE) and inhibits its in-vitro proteolytic activity [15]. Furthermore, BMDM derived from CERK knockout mice demonstrated reduced intra-cellular C1P levels and a concomitant increase in TNFα secretion [15]. Yet, whether extracellular C1P can also inhibit TNFα secretion was not addressed in that study. In a heterologous system, HEK293T cells that stably express the TLR4/MD-2/CD14 complex, exogenous C1P inhibited activation of the NFκB pathway, upstream of TNFα expression [16]. Yet, this effect of exogenous C1P was observed only when NFκB was stimulated by the TLR4 ligand, and not by a different endogenous receptor, suggesting that in this system C1P specifically interfered with TLR4 signaling and not with TACE [16]. The question of whether exogenous C1P can also inhibit TNFα secretion in macrophages was therefore addressed in the present study.

The synthetic C1P analog, 1-methyl-2-(3-methoxyphenyl)-2-(octanoylamino) ethyl-disodium-phosphate, suppresses LPS-induced TNFα secretion *in vivo* [17,18]. Consequently, this synthetic C1P analog and closely-related compounds potently and efficiently block mice mortality in a LPS-induced sepsis model and in a galactosamine/LPS-induced hepatitis model [18,19], and prevent lethal liver failure following extensive hepatectomy [20]. The potent anti-inflammatory activity of this molecule, named by Zor and co-workers phospho-ceramide analogue-1 (PCERA-1, Fig. 1), has been demonstrated in vivo as well as in vitro in primary and cultured macrophages [17,21,22]. Extracellular PCERA-1 suppresses secretion of the pro-inflammatory cytokine TNFα and synergistically induces secretion of the anti-inflammatory cytokine interleukin-10 (IL-10) in LPS-stimulated RAW264.7 and primary macrophages [17,21,22]. PCERA-1 elevates cAMP production in a GTP-dependent manner, resulting in PKA-mediated phosphorylation of CREB [23]. These evidences point to the existence of a GPCR responding to PCERA-1, upstream to adenylyl cyclase. Yet, a causative relationship between the cAMP pathway and cytokine modulation by PCERA-1 has so far only been suggested based on the use of a pharmacological PKA inhibitor, H89, which has limited specificity [23–25]. We addressed this open question in the present study, using a molecular biology approach of CREB silencing.

The different receptor-mediated cellular activities for C1P and PCERA-1 in macrophages, suggest the existence of different receptor entities for these two molecules, a G_i-coupled receptor for C1P and a G_s-coupled receptor for PCERA-1 [26]. However, taking into account the high structural similarity between these two compounds (Fig. 1), the possibility of a common receptor should not be excluded. It is possible that PCERA-1 acts as an inverse agonist of the G_i-coupled C1P receptor, thus inhibiting the negative effect of endogenous C1P on basal or constitutive cAMP production. In a manner previously reported for other GPCRs [27–29], the same receptor can also stimulate macrophages migration in response to C1P via $G\beta_\gamma$-mediated ERK1/2 activation.

The objectives of the current study were to determine whether: (i) a single common receptor or distinct receptors mediate the effects of exogenous C1P and PCERA-1 in macrophages; (ii) the cAMP-PKA-CREB pathway mediates PCERA-1 effects; (iii) exogenous C1P can suppress TNFα secretion; (iv) TNFα suppression by C1P and PCERA-1 is mediated by overlapping or alternative mechanisms. We show that the distinct biological responses induced by exogenous C1P and PCERA-1 are mediated by the activation of different plasma membrane receptor entities. We also demonstrate that PCERA-1 up-regulates IL-10 expression and suppresses TNFα expression in LPS-stimulated macrophages via transcriptional activation of CREB, while exogenous C1P additively inhibits TNFα secretion in a CREB-independent manner by direct inhibition of TACE.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5), sphingomyelin (SM), lysophosphatidic acid (LPA), natural bovine brain-derived C1P, phenylmethylsulphonyl fluoride (PMSF), and the antibodies against ERK and phospho-ERK were purchased from Sigma-Aldrich (St Louis, MO). The PCERA-1 was synthesized according to published procedures [22], dissolved in phosphate-buffered saline (PBS) and freshly diluted in culture media. C16:0 C1P was from Matreya (Pleasant Gap, PA) and C8:0 C1P and sphingosine-1-phosphate (S1P) were from Avanti Polar Lipids (Alabasber, AL). C1P vesicles, used in all experiments except for TACE, were prepared as a 2.62 mM stock in ultrapure water by sonication on ice using a probe sonicator until a clear dispersion was observed. Sonicated ultrapure water, served as the appropriate vehicle. [γ-^{32}P]-ATP was purchased from Perkin-Elmer (Waltham, MA) and used for the preparation of [^{32}P]-C1P as previously described [12]. L-Glutamine, penicillin-streptomycin-nystatin was purchased from Biological Industries (Beit Haemek, Israel). Dulbecco's modified Eagle's minimum essential medium (DMEM), Opti-MEM and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA). Bovine serum albumin was purchased from Amresco (Solon, OH). Human recombinant TACE enzyme, Fluorogenic Peptide Substrate III and the enzyme-linked immunosorbent assay (ELISA) reagent sets for IL-10 and TNFα were purchased from R&D Systems (Minneapolis, MN) and that of MCP-1 was from Peprotech (Rocky Hill, NJ). A CRE-containing EVX-1 promoter luciferase reporter gene construct (hereafter CRE reporter) was a kind gift from Dr. Marc

Montminy (Salk Institute, La-Jolla, CA). A plasmid carrying four copies of a consensus NFκB enhancer site upstream to a luciferase reporter gene (hereafter NFκB reporter) was purchased from Clontech (Mountain View, CA). The mouse IL-10 reporter plasmid was generously given by Dr. Smale (UCLA, CA). DH5α bacteria were from Invitrogen (Carlsbad, CA). Endofree Plasmid Maxi Kit was from Qiagen (Hilden, Germany). TransIT2020 transfection reagent was from Mirus Bio LLC (Madison, WI). Dual-luciferase reporter assay kit was from Promega (Madison, WI). PD98059 was from A.G. Scientific (San Diego, CA). Infrared dye-labelled secondary antibodies and blocking buffer were obtained from Li-Cor Biosciences (Lincoln, NE). Immobilon-FL polyvinylidene difluoride (PVDF) membranes were from Millipore (Billerica, MA). COmplete protease inhibitors mixture and PhosSTOP phosphatase inhibitor cocktail were purchased from Roche (Mannheim, Germany).

2.2. Cell culture

Mouse RAW264.7 macrophage cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD), were grown to 80–90% confluence in DMEM supplemented with 8 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 1250 U/ml nystatin (hereafter culture medium) with 10% FBS. The cells were grown and maintained at 37 °C in a humidified incubator with 5% CO_2. A RAW264.7 cell line stably expressing shRNA against CREB1a was a kind gift from Dr. Iain D.C. Fraser (NIH/NIAID, MD) [30].

2.3. Cytokine expression measurement

RAW264.7 macrophages were seeded in 96-well plates at 1.5×10^5 cells per well and maintained for 48 h in DMEM medium supplemented with 5% FBS. The culture medium was replaced 2 h before treatment to avoid the artifact of medium replacement on signaling [31]. The cells were stimulated with LPS, PCERA-1 or C1P, separately or in combination, as indicated, at 37 °C for 2 h (for IL-10 analysis), 5 h (for TNFα analysis) or 24 h (for MCP-1 analysis). Cytokine secretion into the medium was measured with commercially available ELISA reagents sets, according to the manufacturer's instructions, using a microplate reader (Bio-Tek, Winooski, Vermont).

2.4. ERK1/2 phosphorylation assay

RAW264.7 macrophages seeded at 4×10^5 cells per well in 12-well plates were starved in culture medium supplemented with 0.1% FBS for 24 h before the experiment. The cells were stimulated with PCERA-1, C1P or LPS, separately or in combination, as indicated, at 37 °C for 20 min. The cells were then washed twice with cold PBS and lysed for 1 h at 4 °C with buffer containing Triton X-100 (1%), Tris–HCl buffer pH 8.0 (50 mm), NaCl (100 mm), β-glycerophosphate (50 mm), sodium orthovanadate (1 mm), EDTA (1 mm), EGTA (1 mm), glycerol (30%), PMSF (1 mm), PhosSTOP and a cOmplete protease inhibitor mixture. Cell extracts were centrifuged (14,000 × *g*, 15 min at 4 °C), the supernatants (30 μg protein) were subjected to 10% SDS–PAGE, and proteins were transferred to Immobilon-FL PVDF membrane, that was then incubated with antibodies raised against doubly phosphorylated ERK1/2 and general ERK. Two-color imaging and quantitative analysis of western blots were performed using the Odyssey infrared imaging system (Li-Cor Biosciences), according to the manufacturer's instructions.

2.5. Transfection and reporter gene assay

The CRE, NFκB and IL-10 luciferase reporter plasmids were amplified using DH5α bacteria, and purified using an Endofree Plasmid Maxi Kit. The resulting plasmid preparations showed no ability to induce TNFα production in RAW264.7 macrophages, indicating that it was indeed free of LPS contamination (<0.1 ng/ml). RAW264.7 macrophages were grown for 24 h in 12-well plates, at 3×10^5 cells per well, in culture medium supplemented with 10% FBS. The cells were then co-transfected with 0.4 μg reporter plasmid and 0.2 μg pRL-TK vector which contains the herpes simplex virus thymidine kinase (HSV-TK) promoter to provide low to moderate levels of *renilla* luciferase expression (for normalization). The two plasmids were pre-incubated with the transfection reagent in Opti-MEM medium for 15 min at room temperature, before addition to the cells. Following a 24 h transfection, the cells were stimulated with C1P and/or PCERA-1 at 37 °C for 3 h. Luciferase activity in cell extracts was determined following the manufacturer's (Promega) instructions. Data were expressed as a ratio of firefly luciferase activity divided by the *renilla* luciferase activity, relative to unstimulated control cells. Transfection with the empty reporter vector, performed as a control, yielded no detectable activity.

2.6. Migration assay

Migration of RAW264.7 macrophages was measured by a transwell assay, using 24-well chemotaxis chambers (Transwell, Corning Costar). A volume of 100 μl serum-free culture medium containing 1×10^5 cells was placed in the upper well. C1P and/or PCERA-1 were placed in the lower compartment in 300 μl of culture medium supplemented with 0.1% fatty acid-free BSA and 0.2% FBS pre-treated with activated carbon. After 4 h at 37 °C, non-migrated cells were removed with a cotton swab and the filters were fixed with 5% formaldehyde and stained with hematoxylin–eosin. Migrating cells were counted under the microscope at 40x magnification in 6 randomly selected fields per well.

2.7. C1P binding assay

Fresh RAW264.7 macrophages membranes were prepared and the assay was performed as previously described [12]. In brief, C1P and other sphingolipids were sonicated in a binding buffer containing Tris–HCl buffer pH 7.5 (50 mm), NaCl (150 mm), leupeptin (10 μg/ml), aprotinin (10 μg/ml), PMSF (0.2 mm), and fatty acid-free BSA (0.8%). The membranes were gently mixed for 30 min with 8 μM of [^{32}P]-C1P and with the competing sphingolipids at a concentration that was 50-fold higher than the radioactive ligand, in a 150 μl volume in borosilicate tubes at 37 °C. The membranes were then poured on GF/C filters in a 1225 sampling manifold (Millipore). The filters were quickly washed 3 times with 350 μl of a washing buffer containing Tris–HCl buffer pH 7.5 (10 mm) and NaCl (15 mm) at 4 °C. The radioactivity was measured by liquid scintillation counting.

2.8. In vitro TACE activity measurement

The assay was performed as previously described [15]. In brief, Triton X-100 at 20X CMC in TACE assay buffer was added to the phospholipid (C1P and/or PCERA-1, dried under nitrogen) to produce mixed micelles where the phospholipid is at 0.35 mol% (compared to Triton X-100). The phospholipid-containing micelles (or control Triton X-100 micelles) were pre-mixed for 5 min at 37 °C with an equal volume of recombinant human TACE (0.01 μg). The assay was initiated by the addition of 10 μM fluorogenic substrate III (peptide sequence derived from pro-TNFα), and the enzymatic

activity was measured for 60 min by the fluorescence emitted from the cleavage product (excitation and emission wavelength of 320 and 405 nm, respectively), according to the manufacturer's instructions (R&D systems).

2.9. Protein determination

Protein was determined by a modification of the Bradford procedure, which yields linear results, increased sensitivity and reduced detergent interference, as Zor and co-workers previously described [32,33]. Bovine serum albumin served as standard.

2.10. Statistical analysis

Error bars were calculated for at least three replicates within a single experiment. All experiments were repeated at least twice. Data were analyzed using Student's *t*-test wherever applicable. In all cases, differences of $p < 0.05$ were considered to be significant. At Fig. 7B, the reported p value compares shCREB and control cells for the suppressive effect of PCERA-1 on LPS-stimulated TNFα expression (LPS + PCERA-1 value divided by LPS alone value). This p value represents the sum of two *p* values calculated for: (1) individual values of the numerator (i.e., LPS + PCERA-1 values) divided by average value of denominator (i.e., LPS alone values). (2) average value of the numerator divided by individual values of the denominator. This p value calculation therefore takes into account all data upon which the effect of PCERA-1 on LPS activity was calculated.

3. Results

The possibility of antagonism between C1P and its synthetic analog, PCERA-1, was investigated in order to evaluate whether these two molecules exert their cellular effects through a common receptor. In each of the assays described below, we enabled efficient competition by incubating the RAW264.7 macrophages with the known agonist at a sub-optimal concentration and with the putative antagonist at a saturating concentration, based on our previous determinations of $EC_{50} = 0.1$ μM for cytokine modulation by PCERA-1 [22], and $K_d = 8$ μM for binding of C1P delivered in a sonicated vesicular form to achieve maximal receptor-mediated activities [12].

3.1. PCERA-1, but not C1P, induces IL-10 expression in LPS-stimulated macrophages via the cAMP-PKA-CREB pathway

3.1.1. IL-10 induction by PCERA-1

Fig. 2A shows that C1P alone did not significantly affect IL-10 release, and also could not antagonize the positive effect of PCERA-1 on LPS-stimulated IL-10 release, consistent with a previous study performed with the less efficient formulation of fatty acid-free BSA-bound C1P [26]. Similarly, C1P slightly elevated LPS-stimulated IL-10 promoter reporter activity but was unable to antagonize the synergistic IL-10 transcription activation induced by PCERA-1 and LPS (Fig. 2B). These results suggest that PCERA-1-stimulated IL-10 expression is mediated by a different receptor from that of C1P.

3.1.2. CREB-mediated transcription activation

Next, RAW264.7 macrophages were transfected with a consensus CRE reporter, to measure stimulation of the cAMP pathway [23,24]. Fig. 3A shows that C1P neither stimulated the CRE reporter, nor antagonized the transcriptional activation by PCERA-1, further implying the existence of distinct C1P and PCERA-1 receptors.

Stimulation of LPS-induced IL-10 expression by PCERA-1 was shown to be blocked by a pharmacological PKA inhibitor [23]. Due to the limited selectivity of pharmacological inhibitors in general, and of this inhibitor in particular [24,34], we set a goal to affirm the involvement of the cAMP-PKA-CREB pathway in IL-10 expression stimulation by PCERA-1, using a RAW264.7 macrophage cell line that stably expresses shRNA against CREB1a (hereafter shCREB) resulting in 80% silencing efficiency [30]. Fig. 3B shows that CREB silencing indeed inhibited 75% of IL-10 induction by PCERA-1 in LPS-stimulated cells. As above, C1P had only a minor effect on LPS-stimulated IL-10 expression. These results thus confirm that PCERA-1 up-regulates LPS-stimulated IL-10 expression in macrophages via the cAMP-PKA-CREB pathway.

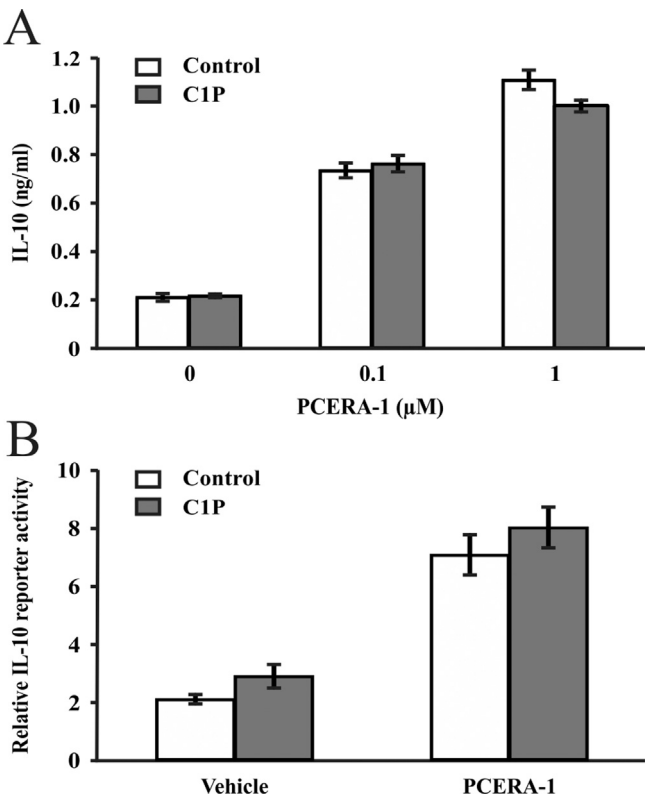

Fig. 2. PCERA-1-induced IL-10 transcription and release are unaffected by C1P. Mouse macrophage RAW264.7 cells, either naive (A) or pre-transfected with a mouse IL-10 promoter reporter (B), were treated for 2 h (A) or 3 h (B) at 37 °C with LPS (100 ng/ml), alone or in combination with PCERA-1 at the indicated concentration (A) or at 1 μM (B), in the presence or absence of C16:0 C1P (50 μM). (A) IL-10 release into the medium was measured by ELISA. Data expressed as mean ± SD ($n = 6$). (B) Luciferase reporter data expressed as mean ± SD ($n = 3$) of values normalized against renilla luciferase activity, relative to unstimulated cells. $p < 0.0001$ (A) or $p < 0.002$ (B) for cells co-stimulated with LPS + PCERA-1 compared to cells stimulated with LPS alone. The results are representative of 3 independent experiments.

3.2. C1P, but not PCERA-1, stimulates macrophages migration via ERK1/2, NFκB and MCP-1

3.2.1. Stimulation of macrophages migration by C1P

Fig. 4A shows that C1P stimulated macrophages migration over 4-fold, whereas PCERA-1 had an insignificant effect by itself and was unable to affect C1P-stimulated macrophages migration in a statistically-significant manner. Furthermore, C1P induced MCP-1 release in a dose-dependent manner while PCERA-1 neither induced, nor was able to significantly antagonize C1P-stimulated MCP-1 release (Fig. 4B). The very low and statistically marginal inhibition of MCP-1 release by PCERA-1 was unspecific, as it was observed also for basal as well as LPS-stimulated MCP-1 release (Fig. 4B). These results support the exclusive function of the C1P receptor in stimulating expression of MCP-1, which then mediates macrophages migration in an autocrine/paracrine manner [11].

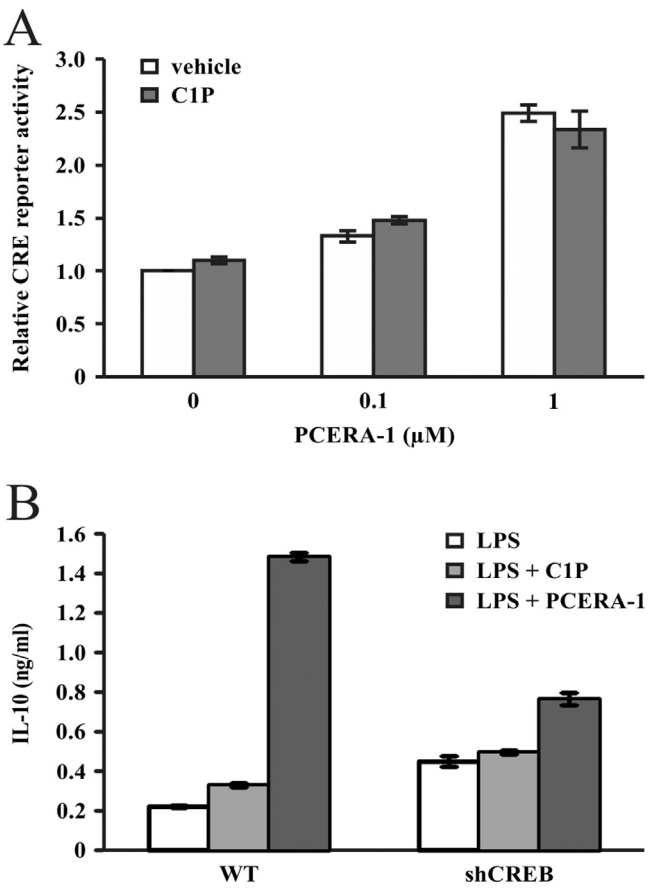

Fig. 3. PCERA-1 stimulates LPS-induced IL-10 expression via the PKA-CREB pathway, which is unaffected by C1P.
(A) RAW264.7 macrophages, pre-transfected with a CRE reporter construct and with renilla luciferase for normalization, were incubated for 3 h at 37 °C with increasing concentrations of PCERA-1, in the presence or absence of natural bovine brain-derived C1P (50 μM). Luciferase reporter data expressed as mean ± SD (n = 3) of values normalized against renilla luciferase activity, relative to unstimulated cells. $p < 0.05$ for treatment of PCERA-1 relative to resting cells. The results are representative of 3 independent experiments. (B) Stably CREB-silenced (shCREB) and control RAW264.7 macrophages were incubated for 5 h at 37 °C with LPS (100 ng/ml) in the presence or absence of PCERA-1 (1 μM) or C16:0 C1P (50 μM). IL-10 secretion to the medium was measured by ELISA. Data expressed as mean ± SD ($n = 6$). $p < 0.00001$ for shCREB cells compared to control cells regarding PCERA-1. The results are representative of 2 independent experiments.

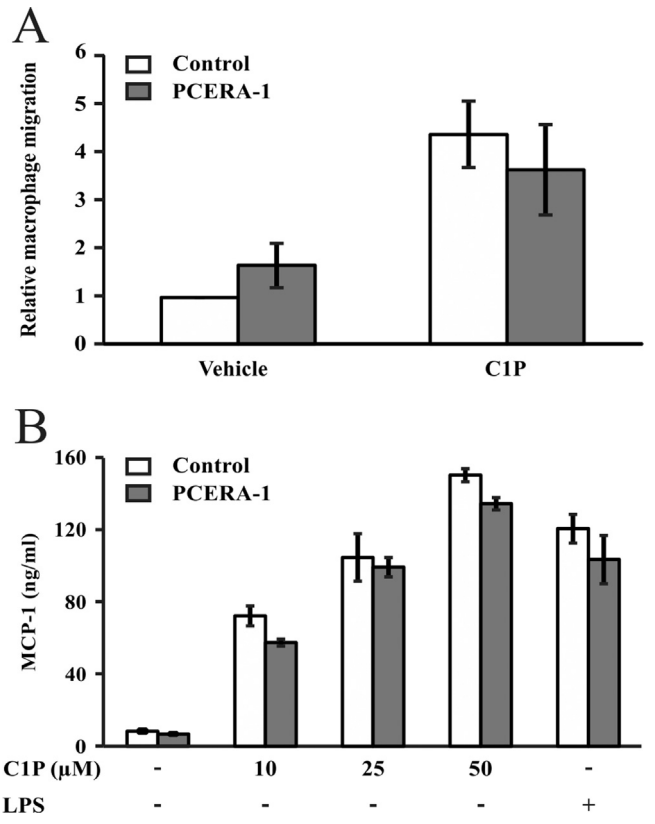

Fig. 4. C1P-stimulated MCP-1 release and macrophages migration are unaffected by PCERA-1.
(A) RAW264.7 cells were placed at the upper compartment of chemotaxis chambers, while C16:0 C1P (30 μM) and/or PCERA-1 (2.5 μM) were added to the lower compartment. After 4 h at 37 °C, cell migration was determined by counting the number of cells present at 6 randomly selected microscopy fields on the filter. Data expressed as mean ± SEM of independent measurements at different wells (n = 5), relative to control resting cells. $p < 0.05$ for treatment of C1P relative to resting cells. The results are representative of 3 independent experiments. (B) RAW264.7 macrophages were treated for 24 h at 37 °C with increasing concentrations of C16:0 C1P at the indicated concentration, or with LPS (100 ng/ml), in the presence or absence of PCERA-1 (1 μM). MCP-1 release into the medium was measured by ELISA. Data expressed as mean ± SD ($n = 6$). $p < 0.006$ for cells stimulated with C1P or LPS, relative to resting cells. The results are representative of 3 independent experiments.

3.2.2. *ERK1/2 phosphorylation and NFκB transcriptional activation by C1P*

Fig. 5A shows that C1P stimulated ERK1/2 phosphorylation, which was as expected blocked by PD98059, an inhibitor of the ERK1/2 kinase, MEK. PCERA-1 alone was unable to significantly activate ERK1/2, as previously reported [22]. Interestingly, C1P-induced ERK1/2 phosphorylation was inhibited by PCERA-1; however, ERK1/2 inhibition by PCERA-1 was also observed when the cells were stimulated with lysophosphatidic acid (LPA), as a control. These results suggest that PCERA-1 does not specifically antagonize ERK1/2 activation by C1P, and are therefore consistent with the hypothesis of separate receptors for these molecules. Furthermore, PCERA-1 neither stimulated NFκB reporter activity, nor affected the transcriptional activation of the reporter by C1P (Fig. 5B), providing further evidence for the existence of a distinct C1P receptor.

3.3. *PCERA-1 does not bind the C1P receptor*

A binding assay using radiolabeled C1P was performed with RAW264.7 macrophage membranes in order to directly examine whether C1P and PCERA-1 can bind a joint receptor. Fig. 6 shows that only unlabeled C1P, but not PCERA-1, sphingosine-1-phosphate (S1P) or sphingomyelin (SM), was able to displace [^{32}P]-C1P-specific binding. This result reinforced the data shown above, and confirmed that the C1P receptor does not bind PCERA-1, and thus establish that distinct GPCRs expressed in RAW264.7 macrophages recognize these similar molecules.

3.4. *C1P and PCERA-1 inhibit TNFα secretion by distinct mechanisms*

3.4.1. *TNFα secretion inhibition by C1P and PCERA-1*

While exogenous PCERA-1 down-regulates TNFα expression in LPS-stimulated macrophages [22], TNFα secretion (but not expression) is elevated in macrophages from CERK knockout mice, suggesting that endogenous C1P inhibits TACE activity [15]. In the present study we asked whether exogenous C1P can also inhibit TNFα secretion in LPS-stimulated macrophages or whether it can antagonize PCERA-1. Fig. 7A shows that treatment of LPS-stimulated RAW264.7 macrophages with exogenous C1P inhibited TNFα secretion by 22%; PCERA-1 treatment inhibited 52% of TNFα secretion, while the co-treatment with C1P and PCERA-1 resulted in an additive effect—67% inhibition. These results suggest that C1P

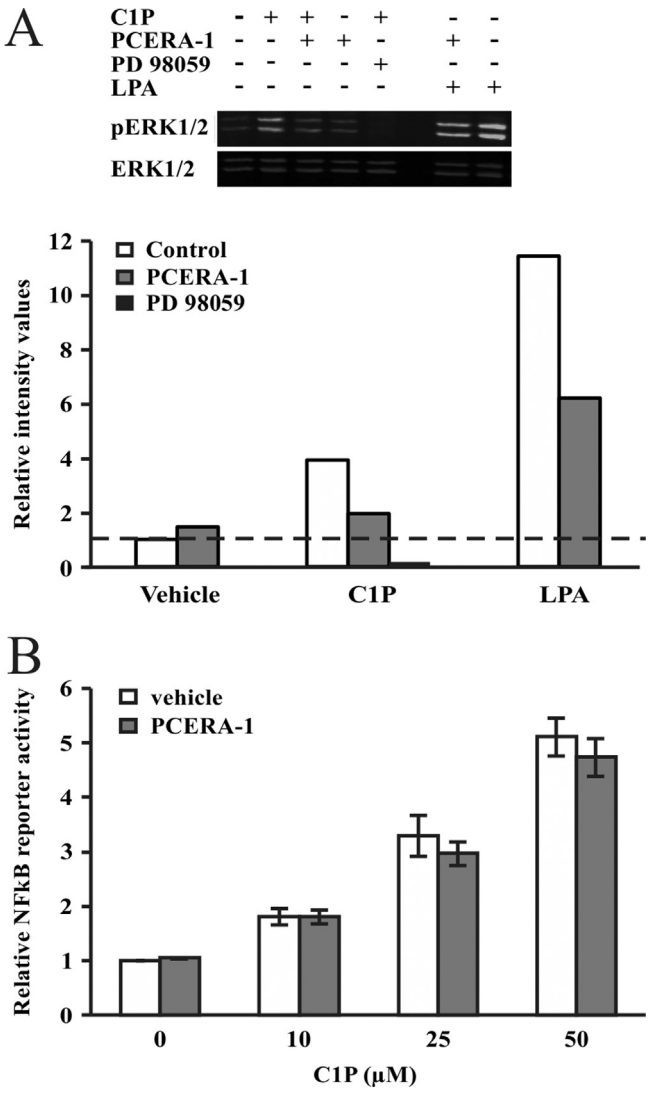

Fig. 5. C1P, but not PCERA-1, stimulates the ERK1/2 and NF κB pathways.
(A) RAW264.7 macrophages were treated for 20 min at 37 °C with natural bovine brain-derived C1P (50 μM), PCERA-1 (10 μM) and LPA (10 μM), separately, or in combinations. Where indicated, the cells were pre-incubated with the MEK inhibitor PD98059 (10 μM) for 30 min. Cell lysates were subjected to SDS-PAGE and transferred to a PDVF membrane, which was simultaneously probed with antibodies against the doubly phosphorylated form of ERK1/2 and general ERK1/2. Quantitative western blot analysis is shown as the ratio of intensities of pERK and total ERK, relative to unstimulated control cells. The results are representative of 4 independent experiments. (B) RAW264.7 cells were transiently transfected with a NFκB luciferase reporter construct and with renilla luciferase for normalization. The cells were incubated for 3 h at 37 °C with increasing concentrations of natural bovine brain-derived C1P at the indicated concentration, in the presence or absence of PCERA-1 (10 μM). Luciferase reporter data expressed as mean ± SD ($n = 3$) of values normalized against renilla luciferase activity, relative to unstimulated cells. $p < 0.01$ for cells stimulated with C1P relative to resting cells. The results are representative of 3 independent experiments.

and PCERA-1 suppress TNFα secretion via distinct mechanisms, and further indicate that C1P does not bind the PCERA-1 receptor.

3.4.2. Mechanism of TNFα expression inhibition by PCERA-1

The shCREB stable cell line was used again to determine whether the cAMP-PKA-CREB pathway mediates TNFα suppression by PCERA-1. Fig. 7B shows that CREB silencing indeed partially blocked the suppressive effect of PCERA-1 on LPS-stimulated TNFα expression. Importantly, exogenous C1P modestly suppressed TNFα expression in a CREB-independent manner. These results thus confirm that PCERA-1 inhibits LPS-stimulated TNFα expression in macrophages via the cAMP-PKA-CREB pathway and that C1P inhibits TNFα secretion via a different mechanism.

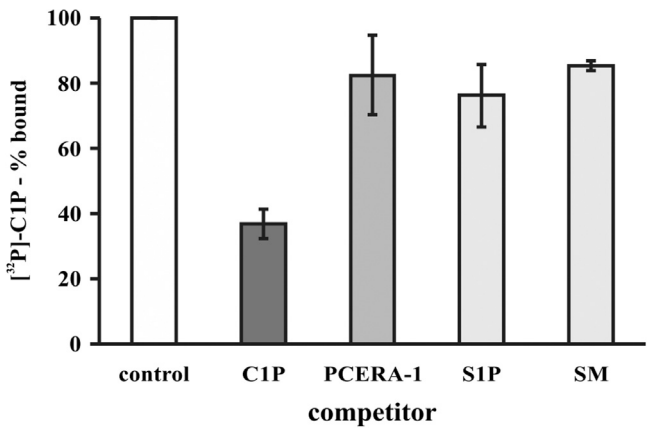

Fig. 6. PCERA-1 does not bind the C1P receptor.
RAW264.7 macrophage membranes were incubated for 30 min with radiolabeled [32P]-C16:0 C1P (8 μM) in the presence or absence of either unlabeled C1P, PCERA-1, sphingosine-1-phosphate (S1P) or Sphingomyelin (SM), at 400 μM. Specific binding was determined by filtration and liquid scintillation counting. Data expressed as mean ± SD ($n = 3$), relative to binding in the absence of a competitor. $p < 0.05$ for competition with C1P relative to control. The results are representative of 4 independent experiments.

3.4.3. TACE inhibition by C1P

As C1P directly interacts with TACE and inhibits its enzymatic activity in a receptor-independent manner [15], we examined in the present study whether PCERA-1 can also directly interact with TACE in an in-vitro assay. To that end, the enzymatic activity of recombinant TACE was determined in the presence of C1P and/or PCERA-1 in Triton X-100 micelles. Fig. 7C shows that C1P inhibited TACE activity by 60%, as expected, while PCERA-1 was unable to affect TACE activity on its own or to antagonize C1P. Thus, we conclude that vesicular C1P modestly inhibits TNFα secretion from macrophages in a CREB-independent manner, while C1P dispersed in mixed Triton X-100 micelles severely inhibits TACE in an in-vitro assay. In contrast, PCERA-1 suppresses TNFα secretion at the transcription level, but does not appreciably inhibit TACE in vitro.

4. Discussion

In this report we present evidence that C1P and its synthetic analog PCERA-1 display distinct cellular effects, mediated through the activation of different receptors expressed in macrophages. The structural similitude between these molecules (Fig. 1) motivated us to test the hypothesis that a single receptor binds the two molecules, and that the distinct cellular effects observed are mediated by different downstream signaling cascades. This hypothetical signaling split could have emerged for example from the simultaneous activity of Gα versus Gβγ. In this scenario, the two molecules should exert similar cellular effects which were apparently over-looked. However, our data clearly show that C1P and PCERA-1 do not share any agonist GPCR-mediated effects. Alternatively, we hypothesized that a signaling split from a common receptor may have emerged from inverse agonism or from distinct receptor conformations acquired upon binding of the different molecules, a phenomenon named “biased agonism” or “agonist-directed trafficking of receptor signaling” [35–37]. However, the observed lack of antagonism in the various assays described here argues against that common receptor hypothesis and in favor of distinct receptors for the endogenous sphingolipid, C1P, and for its synthetic analog, PCERA-1. Furthermore, PCERA-1 failed to compete with radiolabeled C1P on binding to macrophages membranes,

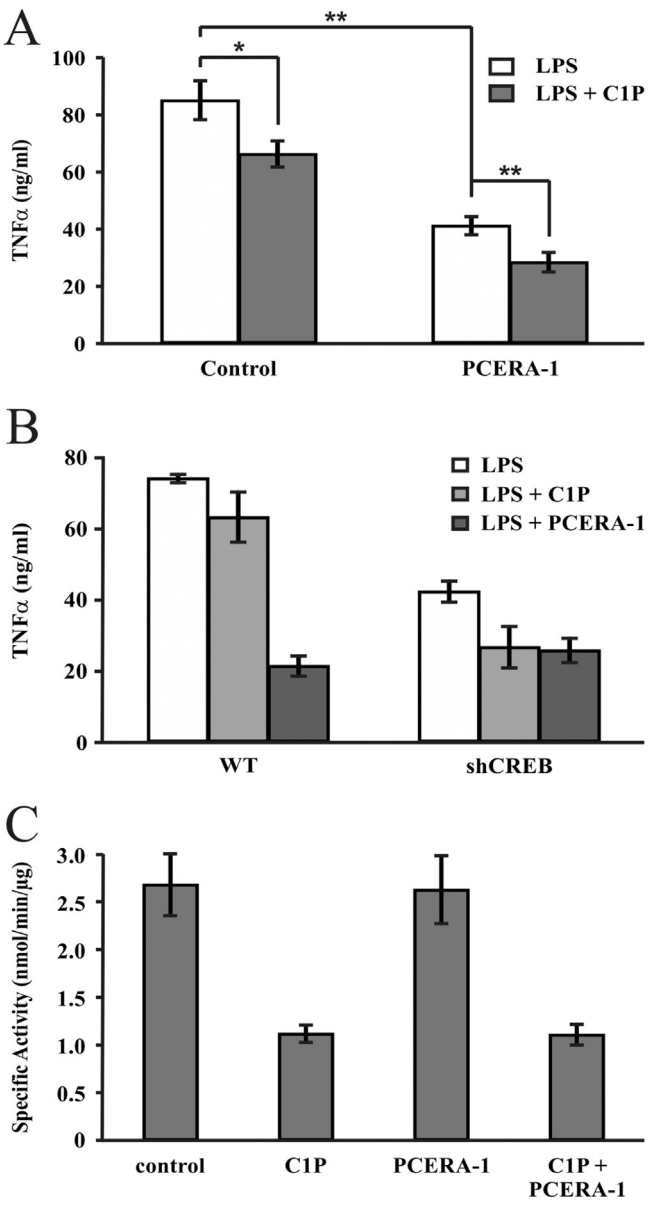

Fig. 7. PCERA-1 suppresses TNF α expression via the PKA-CREB pathway, while C1P directly inhibits TACE.
(A and B) Control RAW264.7 macrophages (A and B) and a stably CREB-silenced (shCREB) derivative cell line (B) were incubated for 5 h at 37 °C with LPS (100 ng/ml) and PCERA-1 at 0.1 μM (A) or 1 μM (B), C16:0 C1P (50 μM) or vehicle, as indicated. TNFα secretion to the medium was measured by ELISA. Data expressed as mean ± SD ($n = 6$). (A) $^{*}p < 0.003$, $^{**}P < 0.0002$. (B) $p < 0.008$ for the suppressive effect of PCERA-1 in shCREB cells compared to control cells (see materials and methods for calculation method). The results are representative of 3 independent experiments. (C) In vitro TACE activity was measured as described [11] using recombinant human TACE (0.01 μg/well), fluorogenic peptide substrate III (10 μM) and Triton X-100 mixed micelles containing C8:0 C1P and/or PCERA-1 at 0.35 mol% in regard to the Triton X-100. Data expressed as mean of TACE specific activity values (nmoles of cleaved fluoregenic product/min/μg of recombinant TACE) ± SD ($n = 3$).

directly indicating that the C1P receptor does not interact with PCERA-1, and thus also concluding that a distinct PCERA-1 receptor is also expressed in macrophages.

At present, the identity of the endogenous ligand for the PCERA-1 receptor is unclear. Theoretically, there are over 50 different species of C1P, differing in chain length, oxidation state and additional chemical modifications. In our studies we used a specific C1P sub-species, i.e., C16:0, as well as natural bovine brain-derived C1P, which is a mixture of endogenous C1P molecules. The finding that this mixture did not antagonize CRE-dependent transcriptional activation by PCERA-1 suggests that the endogenous activator of the PCERA-1 receptor is either a sphingolipid that is similar, yet distinct from C1P, or a structurally unrelated molecule. Yet, we can't exclude the possibility that the PCERA-1 receptor is activated by a particular C1P sub-species which is endogenously produced in a spatio-temporal specific manner, and is not present at high proportion in the natural bovine brain-derived C1P mixture used here.

The phospholipid like-molecule, PCERA-1, has potent anti-inflammatory actions both in vivo and in vitro [17,21–23,26]. Zor and co-workers have previously reported that PCERA-1 down-regulates the pro-inflammatory cytokine TNFα and up-regulates the anti-inflammatory cytokine IL-10 in LPS-stimulated macrophages [22,23]. These previous reports suggested that the cAMP pathway may mediate cytokine modulation by PCERA-1, based on circumstantial evidence [22], and on the use of a PKA inhibitor with a rather poor selectivity [23,24,34]. That suggestion was also based on the notion that activation of the cAMP pathway by extra-cellular mediators binding to G_s-coupled receptors is regarded as a central common anti-inflammatory mechanism [38,39]. In the present report we used a macrophage cell line with stably-silenced CREB, to show that indeed the cAMP-PKA-CREB pathway is essential for regulation of expression of both cytokines by PCERA-1, confirming causative relationship.

Our previous reports also demonstrated that TNFα and IL-10 modulation by PCERA-1 does not involve modulation of ERK1/2 or NFκB activity [22,23]. Importantly, ERK1/2 and NFκB activities are essential for C1P-stimulated cell migration [12]. As demonstrated above, PCERA-1 failed to antagonize C1P in NFκB activation and inhibited ERK1/2 activation in an unspecific manner. These C1P receptor-dependent pathways are regarded as pro-inflammatory as NFκB regulates expression of multiple pro-inflammatory cytokines (i.e., TNFα) and chemokines, such as MCP-1 which is essential for migration of macrophages to the site of inflammation [11]. Thus, the known receptor-mediated activities of C1P and PCERA-1 may be regarded as pro- and anti-inflammatory, respectively.

As opposed to the distinct cell surface receptors revealed here, a common intra-cellular target for C1P and its synthetic analog PCERA-1 is the group IVA calcium-dependent cytosolic phospholipase A2α (cPLA2α) [40]. Intra-cellular C1P has a well-established role as a second messenger directly binding and activating $cPLA_{2\alpha}$, leading to the production of inflammatory lipid mediators, such as PGE_2 [14,41]. C1P interacts with the C2/CaLB domain of cPLA2α and activates the enzyme by increasing its affinity to calcium and promoting translocation from the cytosol to the membranes, where the substrate resides [14,42–45]. The Zor and Chalfant labs have previously shown that both C1P and PCERA-1 stimulate in vitro cPLA2α activity, whereas inhibition was demonstrated for the dephosphorylated derivatives, ceramide and ceramide analog-1 (CERA-1), respectively. Moreover, CERA-1 blocked cPLA2α activity and PGE_2 production in macrophages [40]. While the stimulation of PGE_2 production is generally (but not exclusively) considered as pro-inflammatory, it should be noted that the C1P-stimulated $cPLA_2$ product, arachidonic acid, is a precursor for a variety of lipid mediators, some of them exhibit anti-inflammatory roles [46].

In addition to cPLA2α, C1P can also directly bind several other enzymes and regulate their activity. Chalfant and co-workers have reported that endogenous C1P is a negative regulator of LPS-induced TNFα secretion, via direct binding and inhibition of TACE, an enzyme that processes precursor membrane-bound TNFα into the bioactive pro-inflammatory cytokine [15]. Although similar in structure, PCERA-1 failed to affect TACE proteolytic activity or its inhibition by C1P, indicating that TACE exclusively binds C1P. While mixed micelles containing dispersed C1P severely inhibited recom-

binant TACE in the in-vitro assay, vesicular C1P only modestly inhibited TNFα secretion in LPS-stimulated macrophages, unlike PCERA-1 (Fig. 7). This quantitative difference may be settled by considering the source or formulation of C1P in each assay. TACE inhibition in macrophages was associated with endogenous C1P produced by CERK [15] and in-vitro TACE inhibition was achieved by C1P dispersed in mixed micelles at 0.35 mol% relative to the major constituent, Triton X-100 (Fig. 7C). As the micelles are composed of ~120 molecules of Triton X-100 [15], it is calculated that there is on average a single C1P molecule per 2 micelles, yielding 60% TACE inhibition (Fig. 7C). This is consistent with the earlier report demonstrating over 90% TACE inhibition by C1P present at 0.85 mol% in Triton X-100 micelles, equating to ~1C1P molecule per micelle [15]. In contrast, TNFα secretion was modestly inhibited by exogenous C1P that was brought into solution by sonication producing vesicles made entirely of C1P (Fig. 7A and B). While this formulation is optimal for stimulating the G_i-coupled receptor regulating migration [12], it is apparently not optimal for binding and inhibition of TACE acting on pro-TNFα in macrophages membranes.

Other anti-inflammatory targets of C1P are the sphingomyelinases (SMases), enzymes that convert sphingomyelin into ceramide. The lab of Gómez-Muñoz has previously demonstrated that C1P prevents apoptosis in macrophages by inhibition of acid SMase activity, resulting in reduced levels of the pro-apoptotic ceramide [9]. A recent report showed that C1P can reduce cigarette smoke-induced lung inflammation, apparently by inhibiting cigarette smoke-induced activation of neutral SMase and NFκB and subsequent pro-inflammatory cytokine release in neutrophils [47]. In relation to these findings, a synthetic C1P analog, named SMA-7, suppressed LPS-stimulated NFκB activation and pro-inflammatory cytokines release from intestinal epithelial cells and from human THP-1 macrophages, by inhibiting LPS-stimulated ceramide production through neutral and acid SMase, respectively [48,49]. Taken together, C1P and synthetic C1P analogs regulate inflammation in a complex manner, via cell-surface GPCRs initiating intra-cellular signaling cascades, as well as by directly binding specific enzymes that hold important roles in inflammation.

The results shown here demonstrate that C1P, but not PCERA-1, stimulates MCP-1 release from RAW264.7 macrophages. The Gómez-Muñoz lab has previously reported that C1P promotes MCP-1 release in additional cell types and that this chemokine is a major mediator of C1P-stimulated cell migration [11]. Therefore, regulation of MCP-1 secretion by C1P could have implications for treatment of pathologic inflammatory processes as well as various types of cancer at the stage of metastasis [50]. As an example of the role of C1P-induced MCP-1 in inflammatory auto-immune diseases, it was found that CERK deficiency in mice fed with a high fat diet attenuates MCP-1 signaling in macrophages infiltrating adipose tissue, resulting in the suppression of local inflammation and improvement in the related glucose intolerance and obesity-associated diabetes [51]. With regards to MCP-1 role in tumor metastasis, MCP-1 blockade slowed down prostatic cancer growth in bone by inhibiting infiltration of macrophages and osteoclasts in a tumor dissemination model [52–54].

The evidence presented here that C1P and PCERA-1 affect key macrophages functions through activation of distinct plasma membrane receptors, adds to previous knowledge regarding direct targets of C1P in macrophages, whose location is either intracellular (cPLA2α), in membranes (SMases), or extra-cellular (TACE). While TACE was demonstrated here to exclusively bind C1P, it has been previously shown by us that cPLA2α has a binding site that can accommodate also PCERA-1, and can be inhibited by the respective ceramide analog [40]. Identification of the C1P receptor, and analysis of its tissue-specific expression is expected to reveal additional biological roles for this sphingolipid, that until recently has only been considered to be an intra-cellular second messenger. From the translational aspect, identification of the C1P receptor could be an important strategy in drug development for treatment of inflammatory and cell migration-associated pathologies as in the case of metastatic tumors, while the PCERA-1 receptor could serve as a drug target for the treatment of chronic or acute inflammatory diseases. A structural insight into binding sites of these receptors is required in order to design novel molecules that would selectively target one receptor over the other and also distinguish between the receptor and C1P-binding enzymes.

Conflicts of interest

The authors declare no conflicts of interest.

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