Immunology Letters 123 (2009) 1–8

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/

Modulation of TNFα, IL-10 and IL-12p40 levels by a ceramide-1-phosphate analog, PCERA-1, *in vivo* and *ex vivo* in primary macrophages

Dorit Avni[a,1], Meir Goldsmith[a,1], Orna Ernst[a], Roi Mashiach[b], Tove Tuntland[c], Michael M. Meijler[b], Nathanael S. Gray[d,2], Hugh Rosen[e], Tsaffrir Zor[a,*]

[a] *Department of Biochemistry, Life Sciences Institute, Tel-Aviv University, Tel-Aviv 69978, Israel*
[b] *Department of Chemistry, Ben-Gurion University of the Negev, Be'er-Sheva, Israel*
[c] *Department of Pharmacology, Genomics Institute of the Novartis Research Foundation, La Jolla, CA, USA*
[d] *Department of Biological Chemistry, Genomics Institute of the Novartis Research Foundation, La Jolla, CA, USA*
[e] *Department of Immunology, The Scripps Research Institute, La Jolla, CA, USA*

ARTICLE INFO

Article history:
Received 27 August 2008
Received in revised form 10 December 2008
Accepted 23 December 2008
Available online 29 January 2009

Keywords:
TNFα
Peritoneal macrophages
IL-12
IL-10
Anti-inflammatory drugs

ABSTRACT

Phospho-ceramide analog-1 (PCERA-1) has been described as a potent *in vivo* suppressor of the pro-inflammatory cytokine tumor necrosis factor α (TNFα), and thus as a putative drug for the treatment of inflammatory diseases. However, the *in vivo* cell target of PCERA-1 has not been identified, and its *in vivo* effect on secretion of other relevant cytokines has not been reported. We have previously shown that PCERA-1 suppresses lipopolysaccharide (LPS)-induced TNFα production in RAW264.7 macrophages *in vitro*. We therefore hypothesized that PCERA-1 targets TNFα production by primary macrophages. In this study we thus investigated the effect of PCERA-1 on LPS-induced release of TNFα, interleukin (IL)-10 and IL-12p40, *in vivo*, and *ex vivo*. We found that PCERA-1 suppressed production of the pro-inflammatory cytokines, TNFα and IL-12p40, and increased production of the anti-inflammatory cytokine, IL-10, in LPS-challenged mice, and in primary peritoneal macrophages as well as bone marrow-derived macrophages (BMDM) stimulated with LPS and interferon (IFN)-γ. These activities of PCERA-1 were independent of each other. In contrast, PCREA-1 only slightly affected TNFα production in the whole blood assay, where LPS-induced cytokines are mainly produced by monocytes. Moreover, isolated blood monocytes were inert to PCERA-1, but acquired responsiveness to PCERA-1 upon macrophage colony stimulating factor (M-CSF)-induced differentiation into macrophages. Pharmacokinetic analysis in mice showed that while the volume of distribution of PCERA-1 is low, the drug was rapidly exchanged between the peritoneum and the systemic circulation. Together, these results suggest that sensitivity to PCERA-1 increases upon differentiation of blood monocytes into tissue macrophages, and imply a mechanistic role for peritoneal macrophages in the *in vivo* anti-inflammatory activity of PCERA-1. Finally, we show that the mechanism of activity of PCERA-1 and prostaglandin E2 (PGE2) is distinct, and that PCERA-1 signaling is not mediated by EP2, a PGE2 receptor which is also activated by oxidized phospholipids. The independent and reciprocal modulation of production of TNFα and IL-12p40, vs. IL-10, suggests that PCERA-1 may be a candidate drug for the treatment of inflammation-linked diseases.

1. Introduction

Cytokines are regulatory proteins that are produced and released by immune cells in response to tissue injury or infection, sensed by a toll like receptor (TLR) [1]. While cytokines are crucial in orchestrating an effective acute inflammatory response against invading pathogens, their activity may also bear negative consequences, as evident in chronic inflammatory autoimmune diseases [2].

Of particular significance, the pro-inflammatory cytokine tumor necrosis factor α (TNFα), secreted mainly by monocytes and macrophages immediately after pathogen recognition, plays an instrumental role in innate immunity, both directly and indirectly

Abbreviations: PCERA-1, phospho-ceramide analog-1; CERA-1, ceramide analog-1; TLR, toll like receptor; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; IL, interleukin; IFN, interferon; PGE2, prostaglandin E2; GPCR, G-protein coupled receptor; RA, rheumatoid arthritis; IBD, inflammatory bowel disease; IV, intravenous; IP, intra-peritoneal; ED, effective dose; SEM, standard error mean; MAP, mitogen-activated protein; NFκB, nuclear factor κB; BMDM, bone marrow-derived macrophages; M-CSF, macrophage colony stimulating factor.

* Corresponding author. Tel.: +972 3 640 7192; fax: +972 3 640 7192.
E-mail address: tsaffyz@tauex.tau.ac.il (T. Zor).

[1] These authors contributed equally to this work.

[2] Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 250 Longwood Ave., Boston, MA 02115, USA.

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doi:10.1016/j.imlet.2008.12.011

through augmentation of TLR-induced production of additional pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and IL-8 [3]. However, unregulated production of TNFα plays a key pathological role in development and progression of chronic inflammation and autoimmune diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and psoriasis [2]. Accordingly, a successful strategy for improvement of clinical symptoms is based on repeated administration of TNFα blockers, like the monoclonal antibodies Infliximab and Adalimumab and the fusion protein Ethanercept [2]. Therapeutic application of these approved drugs is, however, hampered by the general disadvantages of protein drugs, including, nonexistence of oral application, immunogenic response, restricted distribution and high cost [3]. Considering these limitations, a small molecular weight TNFα suppressor may have the benefits, while lacking the disadvantages, of the protein drugs.

The pro-inflammatory cytokine interleukin-12 (IL-12), composed of p35 and p40 subunits, is produced by macrophages and other immune cells as a late response to TLR activation, and plays an instrumental role in adaptive immunity, mainly by inducing Th1 responses [4]. As such, it has also been implicated in the progression of autoimmune diseases, including RA, IBD and psoriasis [5]. Accordingly, IL-12 blockade is currently being clinically tested in treatments of some of the above inflammatory states, in particular with patients that do not respond to TNFα blockers [6]. While this alternative strategy is potentially promising, it should be kept in mind that IL-12 blockers and TNFα blockers share similar protein drug-associated disadvantages.

The p40 subunit of IL-12 is also a component of another pro-inflammatory cytokine, IL-23 [7]. These two cytokines are essential for the maturation and proliferation of pro-inflammatory T cells subsets, Th1 and the newly discovered Th17, respectively [8]. Those T-cell subsets have distinct roles in the pathogenesis of various auto-immune diseases such as multiple sclerosis [9]. Moreover, the exact roles of IL-12 and Th1 in the progression of several inflammatory diseases including RA [10], IBD [11], and psoriasis [12], are now re-evaluated, in light of the partial overlap in molecular composition and suggested cellular function, with IL-23 and Th17, respectively.

The inflammatory process is followed by an anti-inflammatory response that prevents excessive damage to the host. Impairment of this balance can lead to disproportionate pathology or immunosuppression. TNFα and IL-10 are two key players in these processes, usually acting in opposition. IL-10 suppresses TNFα production by macrophages [13], thereby contributing to resolution of the inflammation. In addition, IL-10 down-regulates Th1 responses by blocking the production of several other pro-inflammatory cytokines, including IL-12 and IFNγ [14]. The necessity of these activities for the inflammatory balance is evident in the IL-10 knockout mouse which spontaneously acquires IBD [15], as well in the development of RA in human due to impaired IL-10 production [16]. In light of these findings, IL-10 administration was considered as an alternative therapy to anti-TNFα for the treatment of RA [17], IBD [18] and psoriasis [19].

A novel phospholipid-like drug was described by Matsui et al. as a potent *in vivo* suppressor of lipopolysaccharide (LPS)-induced TNFα secretion, while the identity of the cells responding to the drug has remained unknown [20,21]. Further research showed that this putative anti-inflammatory drug, named by us phosphoceramide analog-1 (PCERA-1, Fig. 1), when exogenously added to LPS-stimulated macrophages of the RAW264.7 cell line, inhibits the production of TNFα and increases the production of IL-10, at both the mRNA and protein levels, presumably via the cAMP pathway [22]. The main objectives of the research described here was to determine the effect of PCERA-1 on *in vivo* production of IL-12p40 and IL-10, in addition to TNFα, and to identify primary cells that respond to PCERA-1 *ex vivo*. An additional objective was to compare the anti-inflammatory activity of PCERA-1 and PGE2, in particular with respect to the G_s-coupled receptor, EP2, which has been shown to be activated by either PGE2 or oxidized phospholipids [23]. We show here that PCERA-1 has a robust *in vivo* anti-inflammatory activity, as it inhibits LPS-induced TNFα and IL-12p40 production, while increasing the production of IL-10. We found that PCERA-1 only weakly affected TNFα production by LPS-stimulated monocytes in a whole blood assay, and had no significant effect on isolated monocytes. In contrast, PCERA-1 modulated *ex vivo* production of TNFα, IL-12p40 and IL-10 in stimulated primary macrophages, in an independent manner. Our results further indicate that the PCERA-1 receptor is distinct from EP2 which is common to PGE2 and oxidized phospholipids.

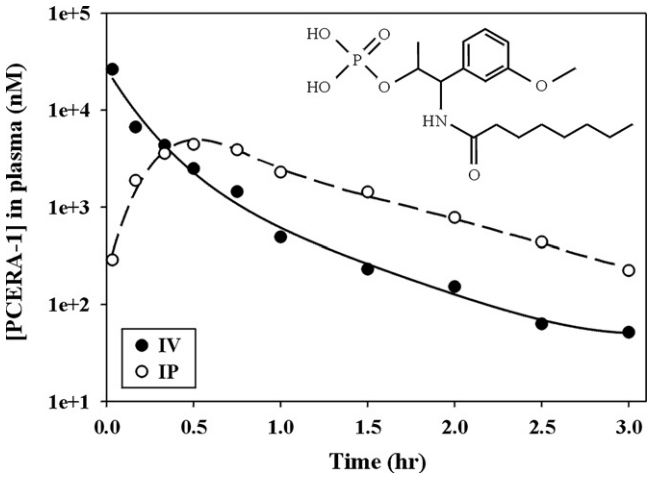

Fig. 1. Pharmacokinetic profile of PCERA-1. BALB/c mice ($n = 8$) were IV-injected (solid circles and line) or IP-injected (open circles and dashed line) with PCERA-1 (1 mg/kg). Blood samples were serially collected into heparin at the indicated times. PCERA-1 concentration in the plasma was measured by LC–MS/MS. The structure of PCERA-1 is depicted.

2. Materials and methods

2.1. Reagents and cell culture

Lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5), AH6809 and thioglycolate were purchased from Sigma (St. Louis, MO). Trypsin, L-glutamine, penicillin and streptomycin were purchased from Biological Industries (Beit Haemek, Israel). DMEM and FBS were purchased from Gibco (Carlsbad, CA). Bovine serum albumin (BSA) was purchased from Amresco (Solon, OH). PGE2 was purchased from Biomol International (Plymouth Meeting, PA). Interferon-γ (IFN-γ) and macrophage colony stimulating factor (M-CSF) were purchased from PeproTech (Rocky Hill, NJ). A neutralizing monoclonal anti-mouse IL-10 antibody, rat IgG isotype control, recombinant mouse TNFα (rTNFα) and ELISA reagents sets for TNFα, IL-10, and IL-12p40 were purchased from R&D Systems (Minneapolis, MN). PCERA-1 and its non-phosphorylated analog, CERA-1, were synthesized according to published procedures [24,25]. PCERA-1 was dissolved in sterile PBS, while CERA-1 was initially dissolved in ethanol and then diluted in sterile PBS containing 4% fatty acid-free BSA. Mouse RAW264.7 macrophage cells, obtained from ATCC (Rockville, MD), were grown to 80–90% confluence in DMEM medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (hereafter culture medium) and 10% FBS, at 37 °C in a humidified incubator with 5% CO_2.

2.2. RAW264.7 macrophages activation assay

RAW264.7 macrophages were maintained for 48 h prior to the experiment in 96-well plates (0.2 ml/well), at 2×10^5 cells/well, in culture medium supplemented with 5% FBS, at 37 °C in a humidified incubator with 5% CO_2. The cells were stimulated with LPS (100 ng/ml) at 37 °C for 2 h in the presence of PCERA-1 (1 μM), PGE2 (0.1 μM) or vehicle. TNFα secretion to the medium was measured by ELISA.

2.3. Animal care

Male BALB/c mice (8–13 weeks, 23 ± 2 g), obtained from the animal breeding centers of Tel-Aviv University (TAU), The Scripps Research Institute (TSRI), or Genomics institute of the Novartis Research Foundation (GNF) were housed in a pathogen-free room under controlled temperature (22–23 °C), humidity, and lighting (12 h light–dark cycles), and were given access to food and water ad libitum. Animal care and experimentation was carried out in accordance with TAU, TSRI and GNF guidelines.

2.4. Pharmacokinetic (PK) studies

PCERA-1, formulated as a 0.5 mg/ml solution in sterile PBS, was administered intravenously (IV) or intraperitoneally (IP) to groups of 8 mice at a dose of 1.0 mg/kg. Blood samples (50 μl) were serially drawn by retro-orbital bleeding at 2, 10, 20, 30 and 45 min after dosing in the first satellite group of 4 mice; and at 1, 1.5, 2, 2.5 and 3 h in the second satellite group of 4 mice. Blood samples were centrifuged to obtain plasma, and total (free and protein-bound) plasma concentrations of PCERA-1 were quantified using a liquid chromatography/mass spectrometry (LC–MS/MS) assay. Pharmacokinetic parameters were calculated by non-compartmental regression analysis of the mean ($n = 4$) plasma concentration data using Winnonlin 4.0 software (Pharsight, Mountain View, CA, USA).

2.5. In vivo cytokine production measurements

The mice received an IP injection (0.1 ml) of either PCERA-1 at doses of up to 8 mg/kg, CERA-1 at 1 mg/kg, or saline as vehicle, followed after 40 min by an IP injection (0.1 ml) of LPS (5 mg/kg). Blood was obtained by cardiac puncture at 1.5 h (for TNFα and IL-10 measurements), or at 4 h (for IL-12p40 measurement), and plasma cytokine levels were determined by ELISA. The data were expressed as the mean ± standard error mean (SEM) of 3–4 animals per group.

2.6. Ex vivo whole blood assay

Mouse blood was drawn in via the retro-orbital plexus under anesthesia. Whole blood was pooled together, mixed with RPMI (20% volume) containing 10 units/ml of heparin, and aliquoted 300 μl per assay. LPS (1 μg/ml), and PCERA-1 (10 μM) or PGE2 (1 μM) were added for 5 h at 37 °C. The plasma was then collected by centrifugation (1500 × *g* for 3 min), and secreted TNFα was measured by ELISA.

2.7. Isolation, culture, and ex vivo activation of primary mouse monocytes

Mouse blood was drawn in via the retro-orbital plexus under anesthesia into heparin (15 units/ml). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, using Histopaque 10831 (Sigma) according to the manufacturer's instructions, and washed twice with PBS. Monocytes were purified by positive selection using mouse CD11b microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purity of monocytes (>98%) was verified by cell morphology. The monocytes were washed, re-suspended in culture medium supplemented with 10% FBS, seeded in a 96-well culture plate (0.1 ml/well) at 5×10^4 cells/well, and stimulated with LPS (1 μg/ml) in the presence of either PCERA-1 (10 μM), PGE2 (0.1 μM), or vehicle at 37 °C for 12 h. Cytokine levels in the culture medium were determined by ELISA.

2.8. Ex vivo differentiation of monocytes into macrophages, and activation

Blood monocytes were isolated and purified as described above, re-suspended in culture medium supplemented with 10% FBS, and seeded in a 12-well plate at 0.3×10^6 cells/well in a humidified incubator (37 °C, 5% CO_2). After 24 h the medium was gently removed and replaced with fresh culture medium supplemented with 10% FBS and 50 ng/ml of macrophage colony stimulating factor (M-CSF). The cells were allowed to grow and differentiate for 11 days under these conditions while the medium was replaced every 2 days. At day 12, when >95% of the cells acquired macrophage morphology, the cells were removed by scraping and seeded in a 96-well culture plate at 1×10^5 cells/well under the same conditions. At day 14 the cells were treated with LPS (1 μg/ml) in the presence or absence of PCERA-1 (1 μM) for 16 h. Cytokine levels in the culture medium were then determined by ELISA.

2.9. Isolation, culture, and ex vivo activation of mouse peritoneal macrophages

Mouse peritoneal macrophages were elicited by injection of 2 ml 4% Brewer's thioglycollate into the peritoneal cavities of the BALB/c mice, 5 days prior to harvest of peritoneal exudate cells. The cells were washed, re-suspended in culture medium supplemented with 10% FBS, and seeded in a 96-well culture plate (0.2 ml/well) at 5×10^5 cells/well. Following incubation of 4 h at 37 °C, non-adherent cells were removed by repeated washing with warm culture medium and the adherent cells (~98% homogenous by appearance) were further cultured overnight at 37 °C. Stimulation was then performed with IFN-γ (10 ng/ml) and LPS (1 μg/ml) in the presence or absence of PCERA-1 (1 μM) at 37 °C for 12 h. Cytokine levels in the culture medium were determined by ELISA. All incubations were carried out in a humidified incubator with 5% CO_2.

2.10. Isolation and ex vivo activation of mouse bone marrow-derived macrophages (BMDM)

BALB/c mice were sacrificed and the femoral and tibial marrow was flushed with sterile PBS using a 27-gage needle. Red blood cells were removed by osmotic shock. The cells were re-suspended in culture medium supplemented with 15% FBS and 10 ng/ml M-CSF. The cells were seeded in Petri dishes at a density of 2.9×10^5 cells/cm^2 and were incubated at 37 °C in a humidified incubator with 5% CO_2. After 1 day, adherent cells were discarded, while non-adherent cells were re-suspended in fresh medium and then allowed to further differentiate. On day 3, half of the supernatant was collected, centrifuged (1000 × *g* for 5 min), and cells pellet was re-suspended in fresh medium and returned to the culture dish. On day 6, the culture medium was replaced and non-adherent cells were discarded. On day 7, the adherent cells (differentiated BMDM, ~98% homogenous by appearance) were transferred to 96-well culture plates (0.2 ml/well), at 2×10^5 cells/well, for 1 day. Stimulation was then performed with LPS (1 μg/ml) and IFN-γ (10 ng/ml) in the presence or absence of PCERA-1 (1 μM) at 37 °C for 4 h (for TNFα and IL-10

determinations) or for 12 h (for IL-12p40 determination). Cytokine levels in the medium were determined by ELISA.

2.11. Enzyme-linked immunosorbent assay (ELISA)

Measurements of TNFα, IL-10 and IL-12p40 levels in mice plasma and in culture medium of peritoneal macrophages were performed with commercially available ELISA reagents sets, according to the manufacturer's instructions, using a microplate reader (Bio-Tek, Winooski, VT). The samples were stored at −80 °C until used.

2.12. Statistical analysis

All the data were analyzed using Student's *t*-test wherever applicable. In all cases, differences of $p < 0.05$ were considered to be significant. All experiments were repeated at least three times.

3. Results

3.1. Pharmacokinetic analysis of PCERA-1

To evaluate the pharmacokinetic (PK) properties of PCERA-1, the compound was injected, IV or IP, to BALB/c mice, and the blood plasma concentration of PCERA-1 was determined at various time points by LC–MS/MS (Fig. 1). The PK parameters were estimated by fitting the mean plasma concentration ($n = 4$) against time. PCREA-1 demonstrated low clearance (6.6 ml/(min*kg), 7% of hepatic blood flow) and a very small volume of distribution (0.12 l/kg, 50% higher than the blood volume), which resulted in a short half-life of elimination of 0.6 h. The initial plasma concentration after administration of the IV dose was 22 μM, while at 3 h the concentration had decreased to 50 nM. PCERA-1 was readily absorbed from the peritoneum to the systemic circulation, as nearly 90% of the IP-administered PCERA-1 shortly appeared in the plasma (AUC = 5847 and 5228 h*nM, for IV and IP administration, respectively), with maximal plasma concentration of 4.6 μM observed within 0.5 h of the IP injection. These results indicate that PCERA-1 was rapidly exchanged between the peritoneum and the systemic circulation; however, its distribution was largely limited to the blood compartment.

3.2. In vivo activity of PCERA-1

Administration of 1.0 mg/kg PCERA-1 to LPS-challenged BALB/c mice reduced the level of TNFα released to the serum, by up to 75%, with an effective dose $(ED)_{50}$ of approximately 0.1 mg/kg (Fig. 2A), in accordance with Matsui et al. [24]. Similar results were obtained with C57BL/6J and ICR mice strains (data not shown). The effect of PCERA-1 on *in vivo* LPS-induced production of IL-10 and IL-12p40 was also studied. Administration of PCERA-1 enhanced production of the anti-inflammatory cytokine IL-10, by up to 5-fold (Fig. 2B), while it inhibited production of the p40 subunit of the pro-inflammatory cytokine IL-12, by up to 80% (Fig. 2C). Dose-dependency was similar for modulation of the three cytokines (Fig. 2A–C), and a higher dose had no significant additional effect (data not shown). We have verified that CERA-1, a non-phosphorylated derivative of PCERA-1 is inactive *in vivo* (Fig. 2D). These results imply that PCERA-1 displays a robust *in vivo* anti-inflammatory activity.

3.3. Differential activity of PCERA-1 in primary monocytes, compared to macrophages

The effect of PCERA-1 on LPS-stimulated TNFα production was also measured in an *ex vivo* whole blood assay. Fig. 3A shows that

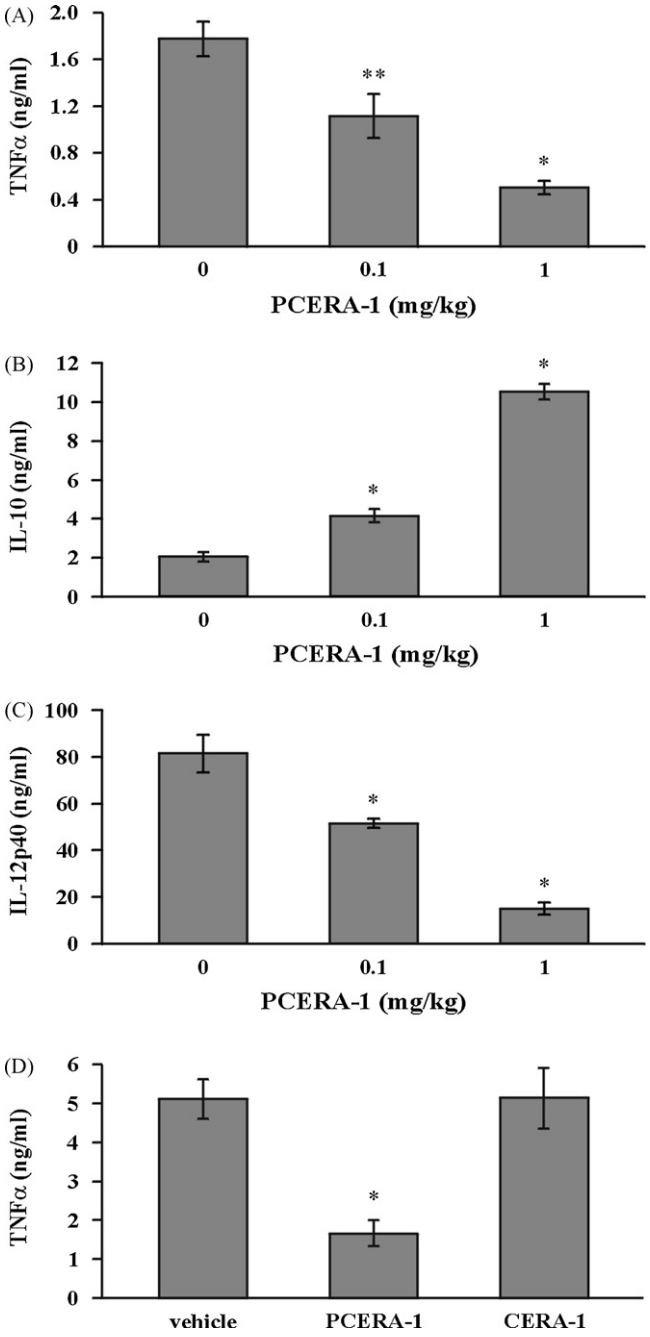

Fig. 2. Dose response for the *in vivo* effect of PCERA-1 on cytokine production. BALB/c mice were IP-injected with PCERA-1 at the indicated dose (A–C) or at 1 mg/kg (D), with CERA-1—a non-phosphorylated derivative (D, 1 mg/kg), or with vehicle, 40 min prior to IP administration of LPS (5 mg/kg). Blood was collected after 1.5 h (A, B and D), or after 4 h (C). TNFα (A and D), IL-10 (B), and IL-12p40 (C) serum levels were measured by ELISA. The data are expressed as mean ± standard error mean (SEM) ($n = 4$). *$p < 0.001$ and **$p < 0.03$.

LPS-induced TNFα production in whole blood was only slightly inhibited by PCERA-1. In contrast, the endogenous TNFα suppressor PGE2, serving as a positive control, greatly suppressed TNFα production in the whole blood assay. This experiment was repeated several times with blood withdrawn from BALB/c, C57BL/6J and ICR mice strains, and yielded similar results (data not shown). As monocytes represent the major LPS-sensitive TNFα producing cells in the blood [26], these results suggest that monocytes are largely insensitive to PCERA-1. In order to prove this suggestion, we measured the effect of PCERA-1 on LPS-stimulated TNFα production

by blood monocytes that were isolated and purified from all other blood cells via positive selection using mouse CD11b microbeads. Fig. 3B clearly shows that PCERA-1 had no significant effect on TNFα production by the purified blood monocytes, whereas PGE2 reduced LPS-stimulated TNFα production down to background levels, as it did also in the whole blood assay. In contrast, PCERA-1 and PGE2, suppressed TNFα production by LPS-stimulated RAW264.7 macrophages to a comparable level (Fig. 3C). Therefore, these results indicate that monocytes are only slightly, if at all, sensitive to PCERA-1.

Considering the contrast between the sensitivity of RAW264.7 macrophages to PCERA-1, and the insensitivity of monocytes to PCERA-1, we decided to determine whether isolated blood monocytes could become sensitive to PCERA-1 following M-CSF-induced differentiation into macrophages. Fig. 3D clearly shows that the differentiated cells, when stimulated with LPS in the presence of PCERA-1, produced a diminished level of the pro-inflammatory TNFα and an elevated level of the anti-inflammatory IL-10. These results thus indicate that macrophages acquired responsiveness to PCERA-1 during their differentiation from the inert monocytes.

3.4. Evaluation of the putative role of the PGE2 receptor, EP2, in PCERA-1 signaling

Suppression of LPS-induced TNFα production by LDL-derived oxidized phospholipids [27] is mediated via the PGE2 receptor, EP2 [23], and subsequently the second messenger cAMP [28]. As PCERA-1, a phospholipid-like molecule (Fig. 1), is also suggested to inhibit LPS-induced TNFα production via cAMP [22], we decided to examine whether EP2 mediates PCERA-1 signaling. To this end, we measured IL-10 induction by PCERA-1 in LPS-stimulated RAW264.7 macrophages, in the presence and absence of the specific EP2 antagonist AH6809. IL-10 induction, rather than TNFα suppression, was chosen as a probe for PCERA-1 activity, due to its higher signal to noise ratio. PGE2 served as a positive control. We found that while the EP2 antagonist blocked 40% of the PGE2-induced IL-10 production, it had no measurable effect on IL-10 induction by PCERA-1 (Fig. 4). These results thus specify that, in contrast to PGE2 and to oxidized phospholipids, PCERA-1 does not signal via EP2.

3.5. Modulation of TNFα, IL-10 and IL-12p40 production in primary macrophages by PCERA-1

In vitro activity of PCERA-1 was demonstrated in the RAW264.7 macrophages cell line (Fig. 3C). To better correlate between the *in vitro* and *in vivo* effects of PCERA-1, murine peritoneal macrophages were elicited, harvested, and incubated *ex vivo* with LPS and IFN-γ at 37 °C for 12 h in the presence or absence of PCERA-1. The levels of TNFα, IL-10 and IL-12p40, secreted to the culture medium, were measured by ELISA. This experiment was repeated in parallel, once with the addition of a neutralizing anti-IL-10 antibody to eliminate suppression of TNFα and IL-12p40 production, potentially mediated by PCERA-1-induced IL-10, and again with the addition of rTNFα to eliminate the potential effect of PCERA-1-suppressed TNFα level on IL-10 and IL-12p40 production. Fig. 5 shows that PCERA-1 directly modulated the production of all three studied cytokines in the primary culture of peritoneal macrophages. Our data also show that in the presence or absence of PCERA-1, an αIL-10 antibody did not affect production of IL-12p40 (Fig. 5A) or TNFα (Fig. 5B), although it fully neutralized IL-10 (data not shown). An IgG isotype-matched control antibody also did not change the release of these cytokines (data not shown). Exogenous rTNFα, added to peritoneal macrophages in large excess of the LPS-induced level, did not significantly alter the accumulation of IL-12p40 (Fig. 5A) or IL-10 (Fig. 5C). These results indicate that the effect of PCERA-1 on IL-12p40 production in peritoneal macrophages does not depend

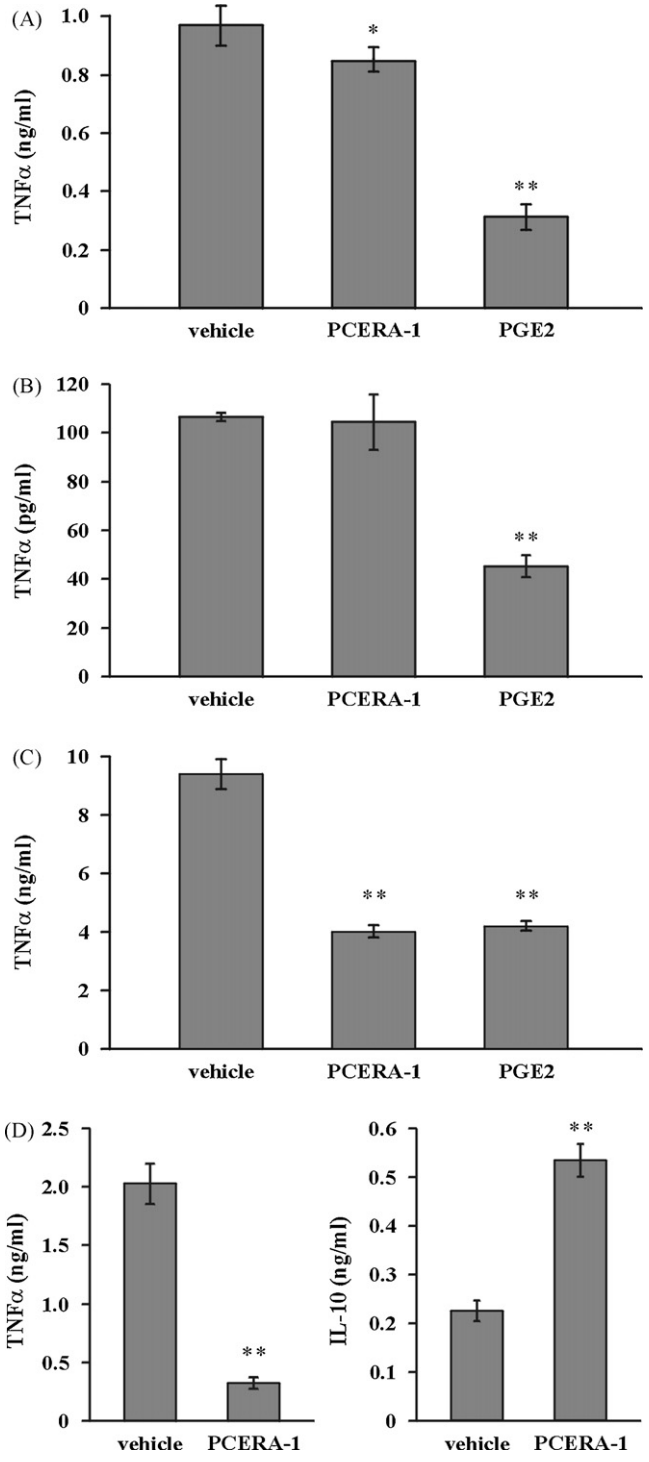

Fig. 3. Differential activity of PCERA-1 in monocytes and macrophages. (A and B) Heparinized whole blood (A) or isolated blood monocytes (B) from BALB/c mice were incubated at 37 °C for 5 h (A) or 12 h (B) with LPS (1 μg/ml) in the presence of either PCERA-1 (10 μM), PGE2 (0.1 μM) or vehicle. TNFα release was measured by ELISA. Each data point represents the mean ± standard deviation (S.D.) of 5 (A) or 6 (B) replicates. TNFα level in the absence of LPS was 0.4 ng/ml (A) or 50 pg/ml (B). (C) Mouse macrophage RAW264.7 cells were incubated at 37 °C for 2 h with LPS (100 ng/ml) in the presence of either PCERA-1 (1 μM), PGE2 (0.1 μM) or vehicle. TNFα release to the medium was measured by ELISA. Each data point represents the mean ± S.D. of 6 replicates. (D) Isolated blood monocytes were differentiated into macrophages as described in Section 2. The macrophages were then incubated at 37 °C for 16 hr with LPS (1 μg/ml) in the presence or absence of PCERA-1 (1 μM). TNFα and IL-10 release was measured by ELISA. Each data point represents the mean ± S.D. of 6 replicates. The cytokines were undetectable (lower than 20 pg/ml) in the absence of LPS. $^{*}p < 0.05$ and $^{**}p < 0.001$.

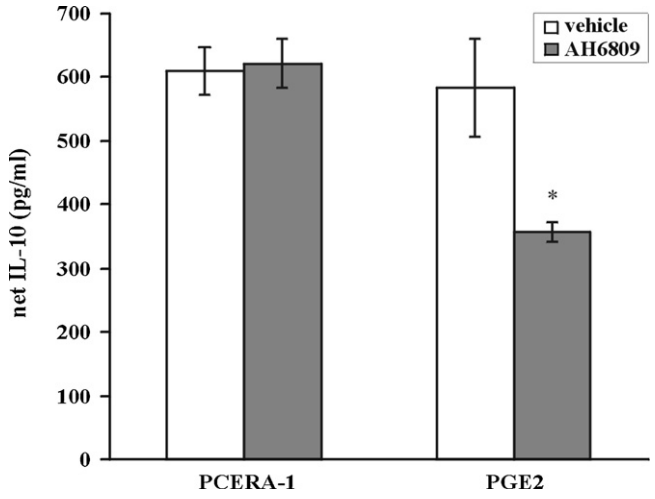

Fig. 4. PCERA-1 and PGE2 have distinct mechanisms of activity in RAW264.7 macrophages. Mouse macrophage RAW264.7 cells were pre-incubated with the EP2 antagonist AH6809 (30 μM, solid bars), or with vehicle (0.2% DMSO, open bars) for 15 min, prior to incubation at 37 °C for 2 h with LPS (100 ng/ml) in the presence of either PCERA-1 (1 μM), PGE2 (0.1 μM) or vehicle. IL-10 release to the medium was measured by ELISA. Each data point represents the mean ± S.D. ($n = 6$) of net PCERA-1-induced or PGE2-induced IL-10 production, following reduction of IL-10 level in the presence of LPS only (183 ± 19 pg/ml and 143 ± 22 pg/ml, in the absence and presence of AH6809, respectively). *$p < 0.002$.

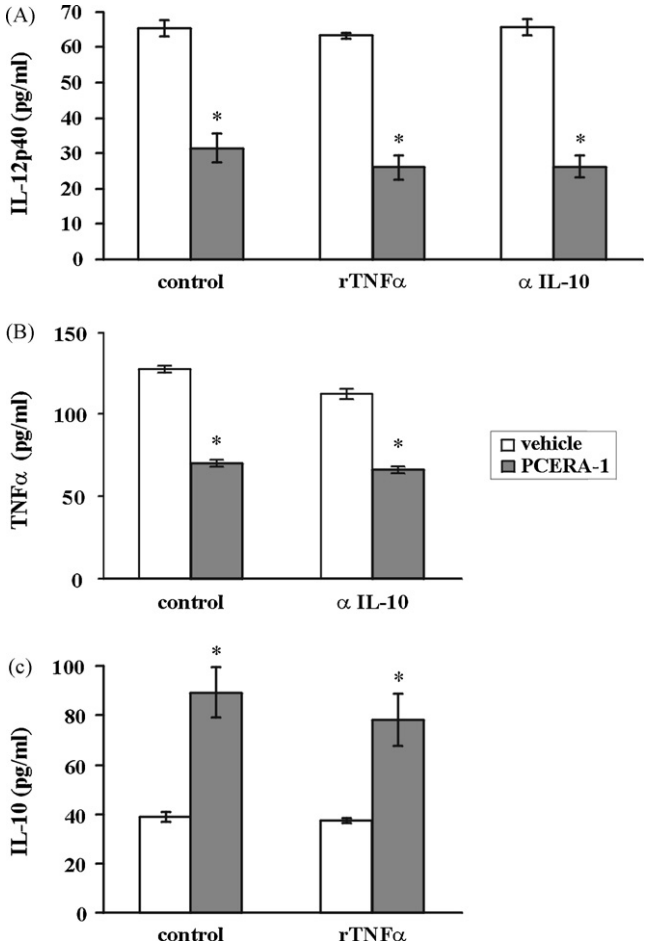

Fig. 5. PCERA-1 independently modulates the production of IL-12p40, TNFα and IL-10 in peritoneal macrophages. Peritoneal macrophages were incubated at 37 °C for 12 h with LPS (1 μg/ml) and IFN-γ (10 ng/ml), in the presence (solid bars) or absence (open bars) of PCERA-1 (1 μM). A neutralizing monoclonal anti-IL-10 antibody (3 μg/ml) or recombinant TNFα (1.4 ng/ml), was added as indicated. The protein levels of IL-12p40 (A), TNFα (B), and IL-10 (C) secreted to the culture medium were measured by ELISA. Each data point represents the mean ± S.D. of 6 replicates. *$p < 0.01$.

on its prior modulation of TNFα or IL-10 production, and that these early effects are also independent of each other.

To further establish the discovery of macrophages as a target cell for PCERA-1, murine bone marrow cells were harvested, differentiated *ex vivo* into BMDM using M-CSF for 8 days, and then stimulated with LPS and IFN-γ for up to 12 h in the presence or absence of PCERA-1. The levels of TNFα, IL-10 and IL-12p40, secreted to the culture medium, were measured by ELISA. As in peritoneal macrophages, PCERA-1 suppressed production of the pro-inflammatory cytokines and elevated production of the anti-inflammatory cytokine also in the primary culture of BMDM (Fig. 6).

4. Discussion

The successful application of anti-cytokine biological agents for the treatment of various chronic inflammatory diseases, along with the known shortcomings of protein-drugs, has stimulated the search for novel oral anti-cytokine small-molecules. Such putative drugs include for example, inhibitors of TNFα converting enzyme (TACE) [29], p38 mitogen-activated protein (MAP) kinase [29], nuclear factor κB (NFκB) [30], and cAMP phosphodiesterase 4 (PDE4) [31]. PCERA-1 was described by Matsui et al. [20,21] as a potent *in vivo* TNFα suppressor, but its effect on production of cytokines other than TNFα, has not been reported. In light of the key opposing roles of IL-10 and IL-12 in linking innate and adaptive immunity, as well as in the progression of inflammatory autoimmune diseases, we decided to study whether PCERA-1 regulates *in vivo* and *ex vivo* production of these cytokines. We show here that PCERA-1 was able to down-regulate the release of both pro-inflammatory cytokines, TNFα and IL-12p40, and to up-regulate the release of the anti-inflammatory IL-10, in LPS-challenged mice, as well as in stimulated primary macrophages. These findings are important for elucidation of the mechanism of action of PCERA-1.

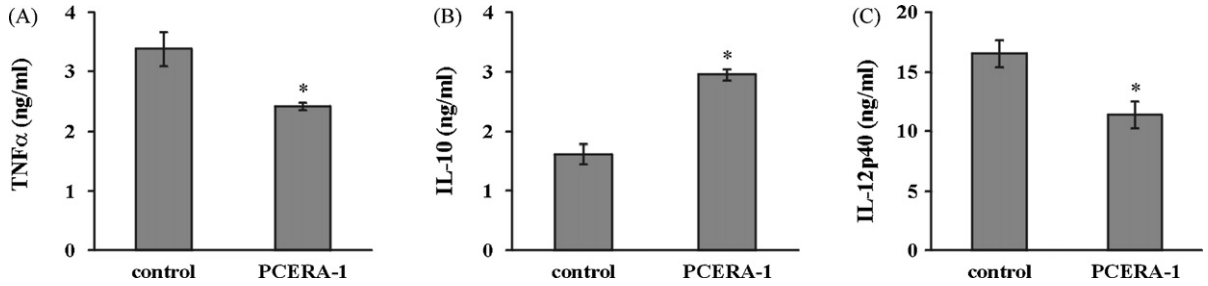

Fig. 6. PCERA-1 modulates the production of IL-12p40, TNFα and IL-10 in bone marrow-derived macrophages (BMDM). BMDM were incubated at 37 °C for 4 h (A and B) or for 12 h (C) with LPS (1 μg/ml) and IFN-γ (10 ng/ml), in the presence or absence of PCERA-1 (1 μM). The protein levels of TNFα (A), IL-10 (B) and IL-12p40 (C) secreted to the culture medium were measured by ELISA. Each data point represents the mean ± S.D. of 6 replicates. *$p < 0.01$.

First, the up-regulation of IL-10 release by PCERA-1 is inconsistent with either a general toxic effect or a simplified mechanism of LPS-TLR4 signaling blockade. Additionally, time course analysis indicated that PCERA-1 truly modulated the release of TNFα and IL-10, rather than increased or decreased, respectively, the lag time until their release (data not shown). We have previously reported that PCERA-1 elevates cAMP level in RAW264.7 macrophages, and that PKA inhibition reverses the effect of PCERA-1 on TNFα and IL-10 production in these cells [22]. Observations in peritoneal macrophages have indicated that cAMP suppresses production of LPS-induced TNFα and IL-12p40 [32], and enhances production of LPS-induced IL-10 [33]. Thus, taken together, our findings strongly suggest that cAMP elevation by PCERA-1 accounts, at least partially, for the observed regulation of cytokine production in peritoneal macrophages.

IL-10 and TNFα have a reciprocal relationship. While TNFα promotes the production of IL-10 [34], IL-10 inhibits the expression of TNFα [13]. In addition, IL-10 inhibits the expression of IL-12p40 as well [14]. We have therefore decided to determine whether TNFα and IL-12p40 suppression by PCERA-1 is dependent on the prior elevation of IL-10 in the mouse peritoneal macrophages. Using a neutralizing anti-IL-10 antibody we show that the effect of PCERA-1 on TNFα and IL-12p40 release is independent of IL-10 activity. The lack of effect of IL-10 neutralization on LPS-induced IL-12p40 production may be explained by the low magnitude and/or kinetics of IL-10 release. Similarly, addition of exogenous TNFα to the PCERA-1-treated peritoneal macrophages, demonstrated that IL-10 and IL-12p40 levels were modulated by PCERA-1 regardless of the TNFα level. These results indicate that reduced TNFα and IL-12p40 secretion and increased IL-10 production are independent consequences of PCERA-1 treatment.

Matsui et al. have reported that while PCERA-1 potently suppressed *in vivo* TNFα release, it failed to do so in *ex vivo* systems which enclose LPS-sensitive TNFα-producing cells: whole blood assay, PBMCs and spleen cells [35]. Thus, the identity of the PCERA-1-sensitive cells remained unknown. Monocytes/macrophages are considered as the major source of TNFα during LPS challenge [36]. We discovered that PCERA-1 suppressed *in vitro* TNFα production in the RAW264.7 macrophage cell line. We thus hypothesized that the *in vivo* activity of PCERA-1 is attributed to tissue macrophages, rather than to monocytes present in blood. To test this hypothesis, we extended our *in vitro* studies to *ex vivo* primary cultures of mouse peritoneal and bone marrow-derived macrophages on one hand, and on the other hand to mouse *ex vivo* whole blood assay. We found that PCERA-1 considerably modulated cytokine production in the peritoneal and bone marrow-derived macrophages, whereas it had a fairly small effect on LPS-induced TNFα production in blood cells. Since monocytes are the major LPS-sensitive TNFα producing cells in the blood [26], and since differentiation to macrophages occurs in the tissues [37], our findings suggested that tissue macrophages, rather than monocytes, are accountable for the *in vivo* effects of PCERA-1. To confirm this conclusion we examined the effect of PCERA-1 on a primary culture of blood monocytes, before and after their differentiation into macrophages by M-CSF. We found that PCERA-1 was unable to affect cytokine production by the isolated monocytes, whereas it was fully active on the differentiated macrophages. These findings suggest that expression of the PCERA-1 receptor is up-regulated as monocytes differentiate into macrophages.

Similar TNFα suppression by PCERA-1 was observed following its IP administration (Fig. 2) and IV administration (data not shown), in accordance with the pharmacokinetic analysis which demonstrated rapid transfer of PCERA-1 between the peritoneum and the blood (Fig. 1), and very low volume of distribution (Fig. 1 and [25]). Taken together, these findings point out at peritoneal macrophages as an *in vivo* active site for PCERA-1. The insensitivity of spleen macrophage cells to PCERA-1 [35] may be explained by their different differentiation state. Macrophages display extreme functional and phenotypic heterogeneity, resulting from adaptation to different tissue microenvironment [38,39]. Moreover, peritoneal and spleen macrophages might originate from different precursor cells [37]. The heterogeneity between these two macrophage subtypes was exemplified by considerable phenotypic differences in expression of multiple macrophage cell-surface markers [38]. We have previously found that PCERA-1 acts in an extra-cellular manner, presumably via a cell-surface receptor [22]. It is thus suggested that an expression of a putative PCERA-1 receptor is up-regulated as monocytes differentiate into some, but not necessarily all, macrophage subtypes.

LDL-derived oxidized phospholipids can also inhibit LPS-induced TNFα production [27]. This activity was found to be mediated by cAMP [28], partially via the PGE2 receptor, EP2 [23]. As PCERA-1 is a phospholipid-like molecule (Fig. 1), that is suggested to inhibit LPS-induced TNFα production via cAMP [22], the existence of a common receptor could not be excluded. However, we showed here that PGE2 strongly suppressed LPS-induced TNFα production in whole blood and in isolated monocytes, whereas PCERA-1 was almost completely inactive in those systems. This divergence, specifically observed in monocytes, and not in RAW264.7 macrophages, could be indicative of distinct receptors for PGE2 and PCERA-1, or could alternatively be explained by low expression of EP2, relative to other PGE2 receptor subtypes, in blood monocytes. To distinguish between these two possibilities, we evaluated the sensitivity of PCERA-1 and PGE2 signaling in RAW264.7 macrophages, to the specific EP2 antagonist AH6809. The contrast between the effect of AH6809 on PGE2 signaling, and its lack of effect on PCERA-1 signaling, thus indicates that the PCERA-1 receptor is distinct, rather than shared with PGE2, and other EP2 agonists.

An appropriate inflammatory response to infection depends on a delicate balance between pro- and anti-inflammatory cytokines secreted by immune cells such as macrophages, whereas an imbalance plays an important role in the initiation and perpetuation of autoimmune diseases [40]. Substantial progress is being made in the medical field of cytokine-based immuno-intervention. Essentially, this is implemented by neutralization of pro-inflammatory cytokines such as TNFα, IL-12, IFN-γ, IL-2, IL-6 and IL-1, or by administration of anti-inflammatory cytokines such as IL-10, IFN-β, transforming growth factor (TGF)-β, and IL-4 [41]. Among these strategies, TNFα neutralization was the first therapy to be approved, and is considered as the most effective treatment currently available for its indications [41]. However, therapeutic application of these, as well as of the other clinically trialed cytokine-based immuno-interventions, is hampered by the general disadvantages of protein drugs [3], and by the inherent limitation of being specifically directed against a single target, which is in conflict with the complex nature of the inflammatory process. We show here that PCERA-1 independently suppresses production of the pro-inflammatory cytokines TNFα, and IL-12p40, and induces the anti-inflammatory cytokine IL-10. This multi-cytokine modulating feature is expected to be advantageous over the conventional clinical approach represented by the existing anti-TNFα therapy. Suppression of p40 by PCERA-1 is expected to down-regulate the activity of both IL-12 and IL-23, and in particular the maturation and proliferation of pro-inflammatory T cells subsets, Th1 and Th17, respectively. Thus, in addition to the suppression of innate immune responses, carried out by macrophages, PCERA-1 is anticipated to block also adaptive immune responses, carried out by these specific T cells. Further study of PCERA-1 mechanism of action may lead to development of novel anti-inflammatory drugs with exceptional potency resulting from a long-term synergistic effect on both sides of the inflammatory balance.

Note added in proof

The IV pharmacokinetic data closely match those obtained also by Matsui et al. [25].

Acknowledgments

This work by supported by grants from the European Commission (IRG #021862), from Teva Pharmaceutical Industries Ltd., from the public committee for allocation of Estate funds at Israel's ministry of justice (#3223), and from the Israel Science Foundation (#907/07). T.Z. was financially supported by Israel's Ministry of Absorption. We are grateful to Mrs. Nava Silberstein for superb technical assistance, to Dr. Bernhard Geierstanger for a multi-cytokine analysis that oriented us to the relevant cytokines, and to Dr. Noam Kariv for expert technical advices regarding *in vivo* experiments. Ms. Angelina Luzader, Mr. John Nikpur, and Mr. Jonathan Chang are acknowledged for technical assistance with the PK study. We thank Mr. Peter Ding and Dr. Mark Parnell for chemical synthesis of PCERA-1 and Dr. Germana Sanna, Dr. Samuel Goldsmith, and Dr. Galit Levy-Rimler for helpful discussions. We are grateful to Dr. Uriel Zor, Dr. Meir Shinitzky, and Dr. Dan Frenkel, for critical reading of the manuscript.

References

[1] Chen K, Huang J, Gong W, Iribarren P, Dunlop NM, Wang JM. Toll-like receptors in inflammation, infection and cancer. Int Immunopharmacol 2007;7:1271–85.
[2] Williams RO, Paleolog E, Feldmann M. Cytokine inhibitors in rheumatoid arthritis and other autoimmune diseases. Curr Opin Pharmacol 2007;7:412–7.
[3] Beutler BA. The role of tumor necrosis factor in health and disease. J Rheumatol Suppl 1999;57:16–21.
[4] Del Vecchio M, Bajetta E, Canova S, Lotze MT, Wesa A, Parmiani G, et al. Interleukin-12: biological properties and clinical application. Clin Cancer Res 2007;13:4677–85.
[5] Adorini L. Interleukin-12, a key cytokine in Th1-mediated autoimmune diseases. Cell Mol Life Sci 1999;55:1610–25.
[6] Wittig BM. Drug evaluation: CNTO-1275, a mAb against IL-12/IL-23p40 for the potential treatment of inflammatory diseases. Curr Opin Investig Drugs 2007;8:947–54.
[7] Reiner SL. Development in motion: helper T cells at work. Cell 2007;129:33–6.
[8] Bettelli E, Korn T, Kuchroo VK. Th17: the third member of the effector T cell trilogy. Curr Opin Immunol 2007;19:652–7.
[9] Weiner HL. A shift from adaptive to innate immunity: a potential mechanism of disease progression in multiple sclerosis. J Neurol 2008;255(Suppl. 1):3–11.
[10] Lubberts E. IL-17/Th17 targeting: on the road to prevent chronic destructive arthritis? Cytokine 2008;41:84–91.
[11] Maloy KJ. The Interleukin-23/Interleukin-17 axis in intestinal inflammation. J Intern Med 2008;263:584–90.
[12] Ghoreschi K, Weigert C, Rocken M. Immunopathogenesis and role of T cells in psoriasis. Clin Dermatol 2007;25:574–80.
[13] Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol 1991;147:3815–22.
[14] Cao S, Liu J, Chesi M, Bergsagel PL, Ho IC, Donnelly RP, et al. Differential regulation of IL-12 and IL-10 gene expression in macrophages by the basic leucine zipper transcription factor c-Maf fibrosarcoma. J Immunol 2002;169:5715–25.
[15] Rennick DM, Fort MM, Davidson NJ. Studies with IL-10 −/− mice: an overview. J Leukoc Biol 1997;61:389–96.
[16] Hajeer AH, Lazarus M, Turner D, Mageed RA, Vencovsky J, Sinnott P, et al. IL-10 gene promoter polymorphisms in rheumatoid arthritis. Scand J Rheumatol 1998;27:142–5.
[17] van Roon J, Wijngaarden S, Lafeber FP, Damen C, van de Winkel J, Bijlsma JW. Interleukin 10 treatment of patients with rheumatoid arthritis enhances Fc gamma receptor expression on monocytes and responsiveness to immune complex stimulation. J Rheumatol 2003;30:648–51.
[18] Schreiber S, Fedorak RN, Nielsen OH, Wild G, Williams CN, Nikolaus S, et al. Safety and efficacy of recombinant human interleukin 10 in chronic active Crohn's disease. Crohn's Disease IL-10 Cooperative Study Group. Gastroenterology 2000;119:1461–72.
[19] Asadullah K, Docke WD, Sabat RV, Volk HD, Sterry W. The treatment of psoriasis with IL-10: rationale and review of the first clinical trials. Expert Opin Investig Drugs 2000;9:95–102.
[20] Matsui T, Kondo T, Nishita Y, Itadani S, Nakatani S, Omawari N, et al. Highly potent inhibitors of TNF-α production. Part 1. Discovery of chemical leads. Bioorg Med Chem Lett 2002;12:903–5.
[21] Matsui T, Kondo T, Nishita Y, Itadani S, Tsuruta H, Fujita S, et al. Highly potent inhibitors of TNF-α production. Part 2. Identification of drug candidates. Bioorg Med Chem Lett 2002;12:907–10.
[22] Goldsmith M, Avni D, Levy-Rimler G, Mashiach R, Ernst O, Levi M, et al. A ceramide-1-phosphate analogue, PCERA-1, simultaneously suppresses tumour necrosis factor (TNF)-α and induces interleukin (IL)-10 production in activated macrophages. Immunology 2008;127:103–15.
[23] Li R, Mouillesseaux KP, Montoya D, Cruz D, Gharavi N, Dun M, et al. Identification of prostaglandin E2 receptor subtype 2 as a receptor activated by OxPAPC. Circ Res 2006;98:642–50.
[24] Matsui T, Kondo T, Nishita Y, Itadani S, Tsuruta H, Fujita S, et al. Highly potent inhibitors of TNF-α production. Part 2. Metabolic stabilization of a newly found chemical lead and conformational analysis of an active diastereoisomer. Bioorg Med Chem 2002;10:3787–805.
[25] Matsui T, Kondo T, Nakatani S, Omawari N, Sakai M, Mori H, et al. Synthesis, further biological evaluation and pharmacodynamics of newly discovered inhibitors of TNF-α production. Bioorg Med Chem 2003;11:3937–43.
[26] Cuturi MC, Murphy M, Costa-Giomi MP, Weinmann R, Perussia B, Trinchieri G. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. J Exp Med 1987;165:1581–94.
[27] Mackman N. How do oxidized phospholipids inhibit LPS signaling? Arterioscler Thromb Vasc Biol 2003;23:1133–6.
[28] Berliner JA, Subbanagounder G, Leitinger N, Watson AD, Vora D. Evidence for a role of phospholipid oxidation products in atherogenesis. Trends Cardiovasc Med 2001;11:142–7.
[29] Wagner G, Laufer S. Small molecular anti-cytokine agents. Med Res Rev 2006;26:1–62.
[30] Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-κB and IκB proteins: implications in cancer and inflammation. Trends Biochem Sci 2005;30:43–52.
[31] Dastidar SG, Rajagopal D, Ray A. Therapeutic benefit of PDE4 inhibitors in inflammatory diseases. Curr Opin Investig Drugs 2007;8:364–72.
[32] Liu J, Chen M, Wang X. Calcitonin gene-related peptide inhibits lipopolysaccharide-induced interleukin-12 release from mouse peritoneal macrophages, mediated by the cAMP pathway. Immunology 2000;101:61–7.
[33] Feng WG, Wang YB, Zhang JS, Wang XY, Li CL. Chang ZL. cAMP elevators inhibit LPS-induced IL-12 p40 expression by interfering with phosphorylation of p38 MAPK in murine peritoneal macrophages. Cell Res 2002;12:331–7.
[34] Katsikis PD, Chu CQ, Brennan FM, Maini RN, Feldmann M. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J Exp Med 1994;179:1517–27.
[35] Matsui T, Takahashi S, Matsunaga N, Nakamura K, Omawari N, Sakai M, et al. Discovery of novel phosphonic acid derivatives as new chemical leads for inhibitors of TNF-α production. Bioorg Med Chem 2002;10:3807–15.
[36] Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA 1975;72:3666–70.
[37] Gordon S. Pathogen recognition or homeostasis? APC receptor functions in innate immunity. C R Biol 2004;327:603–7.
[38] Liu G, Xia XP, Gong SL, Zhao Y. The macrophage heterogeneity: difference between mouse peritoneal exudate and splenic F4/80+ macrophages. J Cell Physiol 2006;209:341–52.
[39] Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. J Leukoc Biol 2004;76:509–13.
[40] Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. Cytokine Growth Factor Rev 2002;13:413–21.
[41] Adorini L. Cytokine-based immunointervention in the treatment of autoimmune diseases. Clin Exp Immunol 2003;132:185–92.