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Synergistic IL-10 induction by LPS and the ceramide-1-phosphate analog PCERA-1 is mediated by the cAMP and p38 MAP kinase pathways

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ABSTRACT

Expression of the anti-inflammatory cytokine IL-10 can be induced either by TLR agonists such as lipopolysaccharide (LPS), or by various endogenous stimuli, in particular those acting via a cAMP-dependent signaling pathway. We have previously reported that the synthetic phospho-ceramide analogue-1 (PCERA-1) increases cAMP level and subsequently down-regulates production of TNFα and up-regulates production of IL-10 in LPS-stimulated macrophages. The objective of this study was to determine the mechanism of activity of PCERA-1 and the role of cAMP in LPS-induced IL-10 production. We show here that PCERA-1 induces IL-10 production in synergism with various TLR agonists in mouse RAW264.7 macrophages. Cooperativity is evident both at the mRNA and protein levels. IL-10 production by LPS and PCERA-1 is mediated by the cAMP pathway and by the p38 MAP kinase. Phosphorylation of p38 is cooperatively accomplished by LPS and PCERA-1 or other cAMP inducers. Furthermore, the activity of PCERA-1 can be partially mimicked by a cell-permeable analog of cAMP, and blocked by the protein kinase A (PKA) inhibitor H89. Finally, in the absence of PCERA-1, the residual IL-10 induction by LPS depends on the basal cAMP level as it can be largely elevated by the phosphodiesterase (PDE)-4 inhibitor rolipram. Our results thus indicate that IL-10 induction by LPS critically depends on basal cAMP level, and that a co-stimulus by a TLR agonist and a cAMP-elevating agent results in synergistic PKA-dependent and p38-dependent IL-10 production.

1. Introduction

Inflammation is mediated by cytokines released from immune cells in response to molecular components of pathogens, such as LPS of gram-negative bacteria, which are sensed by a TLR (Chen et al., 2007). Among the key pro-inflammatory cytokines are TNFα, IL-12 and IFN-γ. Their activity is counter-balanced by an anti-inflammatory response that prevents excessive damage to the host. Excessive or deficient production of some cytokines can lead to disproportionate pathology or immuno-suppression. IL-10 is a key anti-inflammatory cytokine which suppresses production of TNFα by macrophages (Fiorentino et al., 1991) and production of IL-12 and IFN-γ by activated Th1 cells (Cao et al., 2002), thereby limiting inflammation and contributing to its resolution. The crucial regulatory role of IL-10 is evident for example in the IL-10 knock-out mouse which spontaneously acquires inflammatory bowel disease (Rennick et al., 1997), in the development of rheumatoid arthritis in human due to impaired IL-10 production (Hajeer et al., 1998), and in the increased susceptibility to LPS-induced sepsis in mice following blockade of IL-10 by neutralizing antibodies (Howard et al., 1993).

A variety of extra-cellular mediators which activate the cAMP pathway contribute to the resolution of inflammation, by down-regulation of TNFα (Kast, 2000). In addition, cAMP also up-regulates IL-10 expression via protein kinase A (PKA)-mediated phosphorylation of CREB (Gonzalez and Montminy, 1989), and subsequent formation of the transcription machinery complex at the IL-10 promoter cAMP responsive element (CRE) site (Brenner et al., 2003). LPS has also been shown to induce IL-10 expression, but in this case regulation of transcription is thought to be mediated mainly by the p38 pathway via the transcription factor Sp1 binding at a more proximal site on the IL-10 promoter (Brightbill et al., 2000; Ma et al., 2001). Enhanced LPS-induced IL-10 production has been observed for several cAMP inducers, including β-adrenergic receptor (β-AR) agonists (Szelenyi et al., 2000), and PGE2 (Takano et al., 1998). However, the interplay between the cAMP and TLR path-

Abbreviations: PCERA-1, phospho-ceramide analogue-1; PKA, protein kinase A; β-AR, β-adrenergic receptor; PDE, phosphodiesterase; HPRT1, hypoxanthin ribosyltransferase 1; BMDM, bone marrow-derived macrophages.

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ways is not well understood. In particular, the extent to which LPS can activate IL-10 expression in the absence of a cAMP inducer has not been well defined.

A novel anti-inflammatory phospholipid-like drug was originally described as a potent *in vivo* suppressor of LPS-induced TNFα secretion (Matsui et al., 2002a,c). The drug, named by us phospho-ceramide analogue-1 (PCERA-1), inhibits the expression of TNFα and increases that of IL-10 when exogenously added to LPS-stimulated RAW264.7 macrophages (Goldsmith et al., 2008) or to LPS-stimulated primary macrophages (Avni et al., 2009). PCERA-1 elevates intracellular cAMP level and was suggested to act via a cell-surface receptor (Goldsmith et al., 2008). The main objective of the research described here was to determine the mechanism of IL-10 production by PCERA-1, and to assess the separate vs. combined potentials of cAMP inducers and LPS to induce IL-10 expression. We show here that IL-10 is synergistically expressed by cAMP inducers (or a cell-permeable cAMP analog) and LPS in RAW264.7 macrophages. Likewise, PCERA-1 drives IL-10 production in synergism with a TLR2 agonist or with a TLR7 agonist. We demonstrate that neither cAMP elevation alone, nor TLR activation alone, can significantly induce IL-10 production. However, significant IL-10 production by LPS alone can be obtained under conditions where basal cAMP level is elevated. Our findings thus highlight the relevance of both basal and stimulated cAMP level to LPS-induced IL-10 production.

2. Materials and methods

2.1. Reagents and cell culture

Lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5), imiquimod, peptidoglycan, dibutyryl cAMP, H-89, rolipram, isoproterenol, PMSF and DMSO, were purchased from Sigma–Aldrich (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). Macrophage colony stimulating factor (M-CSF) was purchased from PeproTech (Rocky Hill, NJ). BSA was purchased from Amresco (Solon, OH). MAPK inhibitors (SB203580, SB202190, SP600125 and PD98059) were from A.G. Scientific (San Diego, CA). ELISA reagents sets for IL-10 were purchased from R&D Systems (Minneapolis, MN). Antibodies against doubly phosphorylated p38 and general p38 were from Sigma. Infrared dye-labeled secondary antibodies and blocking buffer were from Li-Cor Biosciences (Lincoln, NE). Immobilon-FL polyvinylidene fluoride (PVDF) membranes were from Millipore (Billerica, MA). Complete protease inhibitors mixture and HD-fugene transfection reagent were purchased from Roche (Mannheim, Germany). Endofree Plasmid Maxi Kit was from Qiagen (Hilden, Germany). Dual-luciferase reporter assay kit was from Promega (Madison, WI). DH10B bacteria were from Invitrogen (Carlsbad, CA). PCERA-1 was synthesized according to published procedures (Matsui et al., 2003, 2002b), dissolved in PBS and freshly diluted in culture media. Mouse RAW264.7 macrophage cells, obtained from American Type Culture Collection (ATCC, Rockville, MD), were grown to 80–90% confluence in DMEM 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. Isolation, culture and *ex vivo* activation of mouse bone marrow-derived macrophages (BMDM) was performed as previously described (Avni et al., 2009).

2.2. IL-10 production assay

RAW264.7 macrophages were maintained for 48 h prior to the experiment in 96-well plates, at 2×10^5 cells per well, in culture medium supplemented with 5% FBS, at 37 °C in a humidified incubator with 5% CO_2. The culture medium was replaced 2 h before treatment in order to avoid the artifact of medium replacement on signaling (Smith et al., 1997). The cells were stimulated with LPS (100 ng/ml) and/or PCERA-1 (1 μM) at 37 °C for 2 h (unless indicated otherwise). IL-10 secretion to the medium was measured with a commercially available ELISA reagents set, according to the manufacturer's instructions, using a microplate reader (Bio-Tek, Winooski, Vermont). The samples were stored at −80 °C until used. All experiments were repeated as least three times.

2.3. MAP kinase phosphorylation assay

RAW264.7 macrophages were maintained for 24 h prior to the experiment in 12-well plates, at 8×10^5 cells per well, in culture medium supplemented with 0.1% FBS. The cells were stimulated with LPS (100 ng/ml) and/or PCERA-1 (1 μM) at 37 °C for 15 min, unless indicated otherwise. The cells were washed twice with cold PBS and lysed for 1 h at 4 °C with buffer containing 1% Triton X-100, 50 mM Tris (pH 8.0), 100 mM NaCl, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 30% glycerol, 1 mM PMSF and a "complete" protease inhibitor mixture diluted according to the manufacturer instructions. Cell extracts were centrifuged (14,000 × *g*, 15 min at 4 °C) and the supernatants were stored at −80 °C.

2.4. Western blotting

Cell extracts (60 μg protein) were boiled for 5 min in SDS–PAGE buffer, subjected to 10% SDS–PAGE, and proteins were transferred to immobilon-FL polyvinylidene fluoride (PVDF) membrane. 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. Protein determination

Protein was determined by a modification of the Bradford procedure, which yields linear results, increased sensitivity, and reduced detergent interference, as previously described by Zor and Selinger (1996). BSA served as standard.

2.6. Transfection and reporter gene assay

A plasmid containing the full-length (−1538/+64) mouse IL-10 promoter upstream to the firefly luciferase gene was a kind gift from Dr. S. Smale (Brightbill et al., 2000). The plasmid was amplified using DH10B bacteria, and purified using Endofree Plasmid Maxi Kit. RAW264.7 macrophages were grown for 24 h in 6-well plates, at 8×10^5 cells per well, in culture medium supplemented with 10% FBS, at 37 °C in a humidified incubator with 5% CO_2. The cells were then transfected for 24 h with 1.5 μg of reporter plasmid and 0.5 μg of pRL-TK vector encoding renilla luciferase (for normalization), that were initially incubated with 6 μl HD-fugene transfection reagent in culture medium for 15 min at room temperature, prior to addition to the cells. Following transfection the cells were washed and stimulated with LPS (100 ng/ml) and/or PCERA-1 (1 μM) at 37 °C for 3 h, after which luciferase activity in cell extracts was determined following the manufacturer's (Promega) instructions. Data were expressed as a ratio of IL-10 promoter-driven luciferase activity divided by the renilla luciferase activity, relative to the equivalent ratio in unstimulated cells. The macrophages were also transfected with the empty reporter vector (pGL2B) as a control.

2.7. RT-PCR

The mRNA level of IL-10 in RAW264.7 cells was evaluated by RT-PCR. Hypoxanthin ribosyltransferase 1 (HPRT1), a house keeping gene, served as an internal reference for total mRNA. The cells were seeded in a 6-well culture plate at 1×10^6 cells per well and cultured for 48 h in culture medium supplemented with 5% FBS. The cells were then treated with LPS (100 ng/ml) and/or PCERA-1 (1 μM) for 1 h at 37 °C. Total RNA was then extracted using the EZ-RNA kit (Biological Industries, Israel) and an equal amount of RNA from each sample was reverse transcribed into cDNA using the oligo(dT) Maxime RT premix kit (iNtrRON Biotechnology, Korea). The cDNA was mixed with 10 pmol of a primer pair for the relevant transcript, and with Taq master mix (Lavora GmbH, Germany). PCR was preformed in a T-gradient Cycler (Biometra GmbH, Goettingen, Germany). The samples were initially heated to 94 °C for 5 min, then cycled 30 times between temperatures of 94 °C (30 s), 54 °C or 64 °C (30 s) for IL-10 and HPRT1, respectively, and 72 °C (45 s), and the amplification ended with a final extension step at 72 °C for 5 min. PCR products were detected by electrophoresis in a 1.5% agarose gel. The following primers were used: mouse IL-10: sense, 5′-CGGGAAGACAATAACTGC-3′ and antisense, 5′-TCCAAGGAGTTGTTTCCG-3′; mouse HPRT1: sense, 5′-GCGTCGTGATTAGCGATGATGAAC-3′ and antisense, 5′-CCTCCCATCTCCTTCATGACATCT-3′.

2.8. 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. Synergism between PCERA-1 and TLR agonists

We have previously shown that PCERA-1 enhances LPS-induced IL-10 production in cultured macrophages with an EC_{50} of 100 nM (Goldsmith et al., 2008). We have now sought to determine whether this enhancement is additive or synergistic. To this end, RAW264.7 macrophages were incubated with increasing concentrations of PCERA-1 in the presence or absence of a high LPS concentration for 2 h. Fig. 1A shows that IL-10 production was (a) minimal in the absence of either PCERA-1 or LPS, (b) dose-dependent on PCERA-1, and (c) synergistically induced by PCERA-1 and LPS. The maximal PCERA-1-induced fold-increase in IL-10 production (calculated as the ratio of LPS-induced IL-10 levels obtained in the presence and absence of PCERA-1) was 8.8. The synergistic factor (calculated as the ratio of (LPS + PCERA-1)-induced IL-10 and the sum of the separate LPS-induced and PCERA-1-induced IL-10 levels) was 3.5 at the maximal PCERA-1 concentration. Next we measured the effect of PCERA-1 on LPS-induced IL-10 production over time (Fig. 1B). We found that the effect was maximal (fold-increase of 8.4) at 2 h when LPS alone barely induced IL-10 production. At 4 h LPS-induced IL-10 was significantly higher, and PCERA-1 further elevated it 5.2-fold. LPS-induced IL-10 production reached a peak at 6 h and a plateau at longer incubations, with PCERA-1 further elevating it consistently 2.2-fold from 6 h and on (Fig. 1B).

As expected, synergistic IL-10 production was observed also when the macrophages were incubated with increasing concentrations of LPS in the presence or absence of a high PCERA-1 concentration (Fig. 2A). Maximal values of fold-increase and synergistic factor were 6.7 and 2.8, respectively. We have previously shown that IL-10 production was elevated by PCERA-1 in

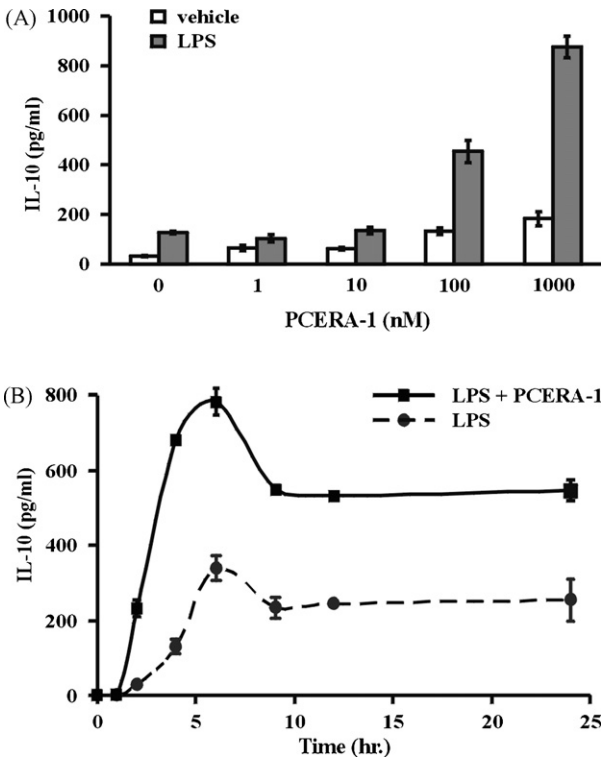

Fig. 1. LPS and PCERA-1 synergistically induce IL-10 production. (A) Mouse macrophage RAW264.7 cells were incubated at 37 °C for 2 h with the indicated concentrations of PCERA-1 in the presence (solid bars) or absence (open bars) of LPS (100 ng/ml). IL-10 release to the medium was measured by ELISA. Each data point represents the mean ± S.D. ($n = 6$). (B) RAW264.7 macrophages were incubated at 37 °C for the indicated time with LPS (100 ng/ml) in the presence (black squares, continuous line) or absence (gray circles, dashed line) of PCERA-1 (1 μM). IL-10 release to the medium was measured by ELISA. Each data point represents the mean ± S.D. ($n = 6$). IL-10 production by PCERA-1 alone was undetectable (<20 pg/ml).

macrophages stimulated with agonists of either TLR4 (LPS), TLR2 (peptidoglycan) or TLR7 (imiquimod) (Goldsmith et al., 2008). We have now sought to determine whether PCERA-1 synergizes with all TLR stimuli. To this end, RAW264.7 macrophages were incubated with increasing concentrations of peptidoglycan or imiquimod in the presence or absence of a high PCERA-1 concentration. Indeed, a synergistic production of IL-10 was observed in both cases (Fig. 2B and C). The maximal values of fold-increase and synergistic factor for PCERA-1 with peptidoglycan were 16.5 and 6.7, respectively. The maximal values of fold-increase and synergistic factor for PCERA-1 with imiquimod were 9.1 and 8.4, respectively. To rule out a possible contamination of LPS in the peptidoglycan and imiquimod samples, we added polymyxin B, a specific LPS blocker, and observed diminished cytokine production in the presence of LPS but not in the presence of the TLR2 or TLR7 agonist (data not shown). Together, these results indicate that synergistic IL-10 production is obtained by the combination of PCERA-1 with either of the tested TLR agonists. It is thus suggested that a signaling pathway which is practically required for IL-10 induction is activated by PCERA-1, but not by any of the tested TLR agonists.

To provide further insight into the molecular mechanism underlying the PCERA-1-dependent up-regulation of IL-10 expression, the mRNA level of IL-10 in RAW264.7 cells stimulated with LPS and/or PCERA-1 was evaluated by RT-PCR. Fig. 3A shows that each stimulus alone modestly elevated IL-10 mRNA level, whereas a co-stimulus of LPS and PCERA-1 was significantly more effective. Analysis was performed at the peak of mRNA accumulation (1 h), which preceded the onset of significant protein expression (2 h). These results indicate that PCERA-1 is involved in regulation of

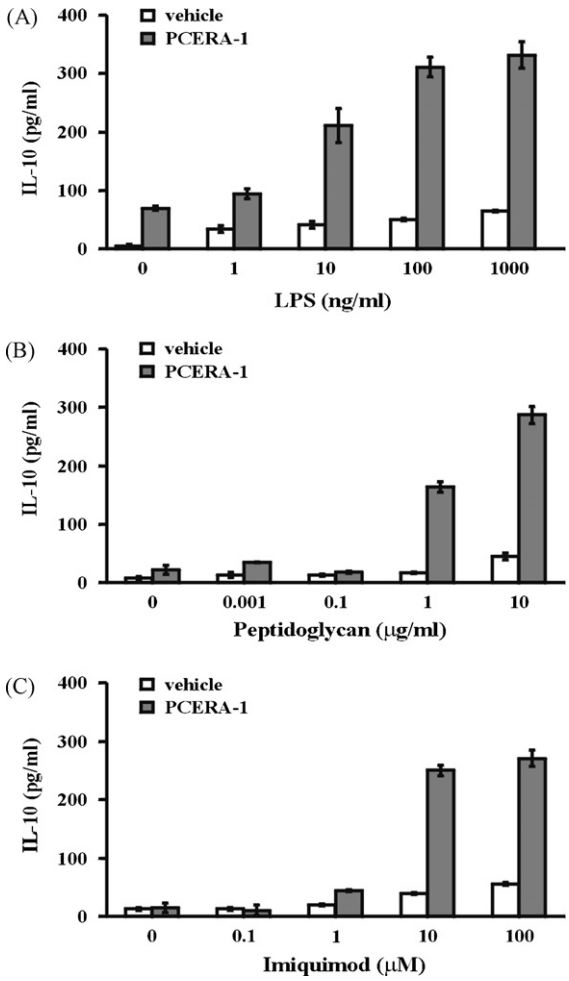

Fig. 2. Synergistic IL-10 production by PCERA-1 and various TLR agonists. Mouse macrophage RAW264.7 cells were incubated at 37 °C for 2 h with (solid bars) or without (open bars) PCERA-1 (1 μM) and with the indicated concentrations of either LPS (TLR4 agonist, panel A), peptidoglycan (TLR2 agonist, panel B), or imiquimod (TLR7 agonist, panel C). IL-10 release to the medium was measured by ELISA. Each data point represents the mean ± S.D. ($n = 6$).

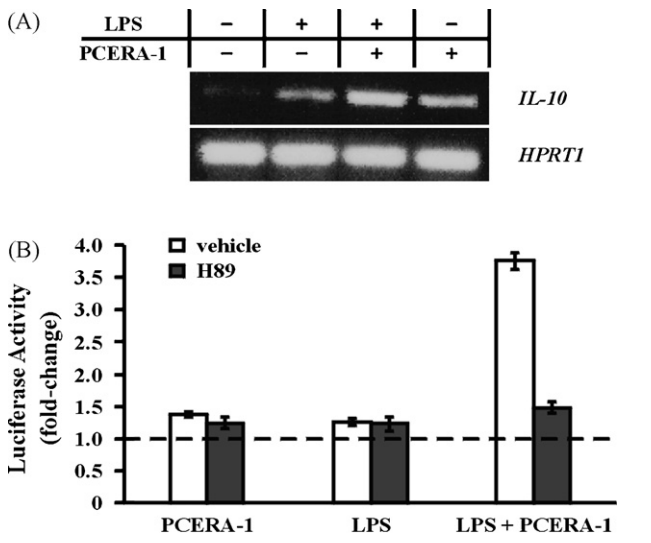

Fig. 3. LPS and PCERA-1 cooperatively modulate IL-10 transcription. (A) Mouse macrophage RAW264.7 cells were incubated with PCERA-1 (1 μM) and/or LPS (100 ng/ml) for 1 h at 37 °C. DMEM served as vehicle. Total RNA was isolated from the cells and IL-10 mRNA levels were assessed by RT-PCR, as described under Section 2. The results are representative of 3 independent experiments. The mRNA level of HPRT1 served for normalization. (B) Mouse macrophage RAW264.7 cells were transfected with a full-length mouse IL-10 promoter–luciferase construct. After 24 h at 37 °C the cells were washed and pre-incubated for 45 min with the specific PKA inhibitor H89 (30 μM, solid bars) or with vehicle (open bars). The cells were then stimulated with LPS (100 ng/ml) and/or PCERA-1 (1 μM), and further incubated for 3 h. Luciferase activity assay was performed as described under Section 2. Each data point represents the mean ± S.D. ($n = 6$) relative to unstimulated control cells (represented by the dashed line).

IL-10 mRNA level in RAW264.7 cells. As mRNA level is influenced both by transcription rate and by degradation rate, we further dissected the mechanism of synergistic IL-10 production using RAW264.7 macrophages transiently transfected with a luciferase reporter gene, for which transcription is dictated by the sequence of the full mouse IL-10 promoter, whereas mRNA stability is related to the luciferase sequence. Fig. 3B shows that LPS or PCERA-1 alone only modestly increased reporter gene activity, whereas a co-stimulus of LPS and PCERA-1 synergistically induced luciferase expression, with a synergistic factor value of 4.4. The effect of PCERA-1 on reporter transcription at the IL-10 promoter is thus comparable to its effect on IL-10 protein expression. Transfection with an empty control vector resulted in undetectable luciferase activity (data not shown). It is therefore suggested that synergistic IL-10 expression is accomplished by cooperative operation of LPS-activated and PCERA-1-activated transcription factors.

3.2. The cAMP pathway is required for IL-10 production

We have previously reported that PCERA-1 activates the cAMP pathway (Goldsmith et al., 2008). We therefore measured the effect of PKA inhibition on the IL-10 promoter-dependent reporter gene activity. Fig. 3B clearly shows that H89, a specific PKA inhibitor, efficiently blocked the synergistic induction of luciferase expression. Importantly, in the presence of H89, the low residual activities of LPS and PCERA-1 were additive rather than synergistic (Fig. 3B). As expected, H89 also efficiently blocked expression of the endogenous IL-10 protein (data not shown).

The synergism displayed between LPS and PCERA-1 raised the question whether other cAMP inducers can also synergize with LPS in IL-10 induction. To answer that question, RAW264.7 macrophages were incubated with LPS and/or either of the following extra-cellular agents: PCERA-1, PGE2, and isoproterenol, a β-AR agonist. Fig. 4A shows that while neither of the agents was capable of significantly inducing IL-10 production in the absence of LPS, they all considerably augmented the activity of LPS. The fold-increase of LPS-induced IL-10 level was 8.3, 5.0 and 13.1, for PCERA-1, PGE2, and isoproterenol, respectively.

As these results suggest that cAMP is the common intra-cellular mediator of synergism with LPS, we now turned to determine whether the cell-permeable dibutyryl cAMP can mimic the activity of PCERA-1. To this end, RAW264.7 macrophages were incubated with increasing concentrations of dibutyryl cAMP in the presence or absence of a high LPS concentration. Fig. 4B shows indeed that IL-10 formation was synergistically driven by LPS and dibutyryl cAMP, in a dose-dependent manner. The maximal synergistic factor was calculated to be 6.2. In order to determine whether dibutyryl cAMP fully mimicked the IL-10 induction activity of PCERA-1, we also measured the levels of IL-10 induced by the combination of PCERA-1, LPS, and increasing concentrations of dibutyryl cAMP. PCERA-1 alone did not induce IL-10 production over the background. Yet, as shown in Fig. 4B, PCERA-1 had a marked positive effect on IL-10 production in the presence of LPS and dibutyryl cAMP at 50 μM and 100 μM. The addition of PCERA-1 resulted in a fold-increase of 5.3, 4.2, and 2.2 in LPS-induced IL-10 production in the presence of 0, 50 μM and 100 μM dibutyryl cAMP, respectively. PCERA-1

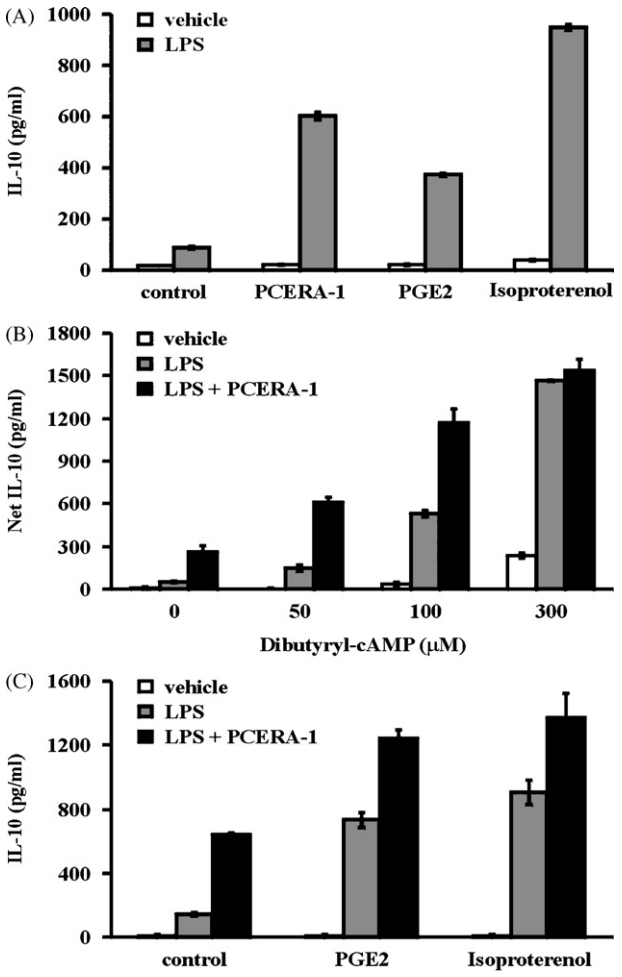

Fig. 4. LPS-induced IL-10 production is up-regulated by cAMP. (A) Mouse macrophage RAW264.7 cells were incubated at 37 °C for 2 h with (solid bars) or without (open bars) LPS (100 ng/ml) in the presence of either PCERA-1 (1 μM), PGE2 (100 nM), or the β-AR agonist isoproterenol (1 μM). IL-10 release to the medium was measured by ELISA. Each data point represents the mean ± S.D. (n = 6). (B) RAW264.7 cells were pre-incubated at 37 °C for 30 min with the indicated concentrations of dibutyryl cAMP, followed by the addition of either 100 ng/ml LPS (gray bars), 100 ng/ml LPS + 1 μM PCERA-1 (black bars), or vehicle (open bars), for an additional 2 h. IL-10 release to the medium was measured by ELISA. Each data point represents the mean ± S.D. (n = 6) of net IL-10 production, following reduction of background IL-10 level in unstimulated cells (136 ± 8 pg/ml). IL-10 level in cells treated with PCERA-1 (1 μM) alone, was identical to the background in unstimulated cells. (C) RAW264.7 cells were incubated at 37 °C for 2 h with either PGE2 (100 nM), or the β-AR agonist isoproterenol (1 μM) in the presence of either 100 ng/ml LPS (gray bars), 100 ng/ml LPS + 1 μM PCERA-1 (black bars), or vehicle (open bars). IL-10 release to the medium was measured by ELISA. Each data point represents the mean ± S.D. (n = 6). IL-10 level in cells treated with PCERA-1 (1 μM) alone, was identical to the background in unstimulated cells.

did not further elevate IL-10 production induced by the combination of LPS and dibutyryl cAMP at the highest concentration used, 300 μM. Similar patterns of synergistic IL-10 production were observed with the combination of LPS, dibutyryl cAMP and other cAMP inducers, either PGE2 or isoproterenol (data not shown). It should be noted that each cAMP inducer was added at a concentration which yields maximal response. Interestingly, the addition of PCERA-1 to LPS-stimulated macrophages, which were already co-treated with either PGE2 or isoproterenol also gave rise to synergistic IL-10 production (Fig. 4C). Taken together, these results point out at PCERA-1-derived cAMP as a major mediator of synergism in IL-10 production by LPS-stimulated macrophages, and suggest that additional PCERA-1-activated pathways may participate in the synergistic IL-10 production.

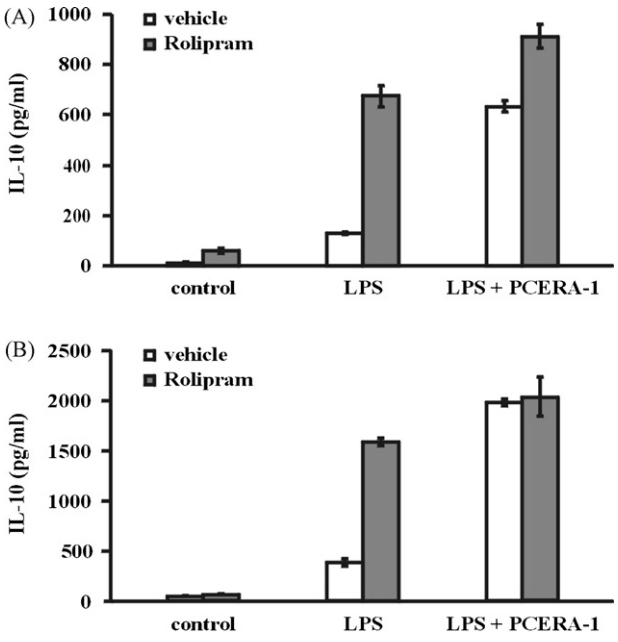

Fig. 5. PDE4 activity preserves low LPS-induced IL-10 production in the absence of a cAMP inducer. Mouse macrophage RAW264.7 cells were pre-incubated at 37 °C for 20 min with the specific PDE-4 inhibitor rolipram (20 μM, solid bars) or with vehicle (open bars), before LPS (100 ng/ml) with or without PCERA-1 (1 μM) was added for an additional 2 h (A) or 4 h (B). IL-10 release to the medium was measured by ELISA. Each data point represents the mean ± S.D. (n = 6).

As we found that the low LPS-induced IL-10 production is greatly elevated by cAMP producers or by dibutyryl cAMP, we next decided to evaluate the effect of the specific phosphodiesterase-4 (PDE-4) inhibitor rolipram. This strategy was chosen since cAMP degradation in macrophages is predominantly carried out by the PDE-4 isoform (Burnouf and Pruniaux, 2002). Fig. 5A shows that LPS-induced IL-10 production at 2 h was elevated by the PDE-4 inhibitor, to the same extent that it was elevated by PCERA-1 (fold-increases of 5.7). Moreover, the addition of rolipram to cells co-stimulated with both LPS and PCERA-1 resulted in a further modest but significant increase in IL-10 production (total fold-increase of 7.7) over LPS-stimulated cells. These results imply that basal cAMP production in RAW264.7 macrophages is counter-balanced by high PDE-4 activity, which dictates extremely low induction of IL-10 by LPS within the 2 h time frame. Furthermore, even at 4 h rolipram considerably elevated IL-10 level (4.2-fold), comparably to PCERA-1 (5.3-fold), implying that cAMP is a limiting factor for LPS-induced IL-10 production also at longer incubations when LPS alone causes significant IL-10 expression (Fig. 5B).

3.3. The p38 pathway, cooperatively activated by LPS and PCERA-1, is required for IL-10 production

In order to study whether MAP kinases are involved in the synergistic induction of IL-10 by LPS and PCERA-1, RAW264.7 macrophages were co-stimulated with these two agents, in the presence of specific MAP kinase inhibitors. Fig. 6A shows that SB203580, a specific p38 inhibitor, blocked 80% of the production of IL-10 by LPS and PCERA-1. Similar suppression was achieved also with a different p38 inhibitor, SB202190 (data not shown). The low LPS-induced IL-10 production in the absence of PCERA-1 was also blocked by the p38 inhibitors (data not shown). In contrast, specific inhibitors of ERK1/2 and JNK did not affect IL-10 level in the same experiment (Fig. 6A).

As p38 was demonstrated to be required for the induction of IL-10 by LPS and PCERA-1, we decided to follow the kinetics of

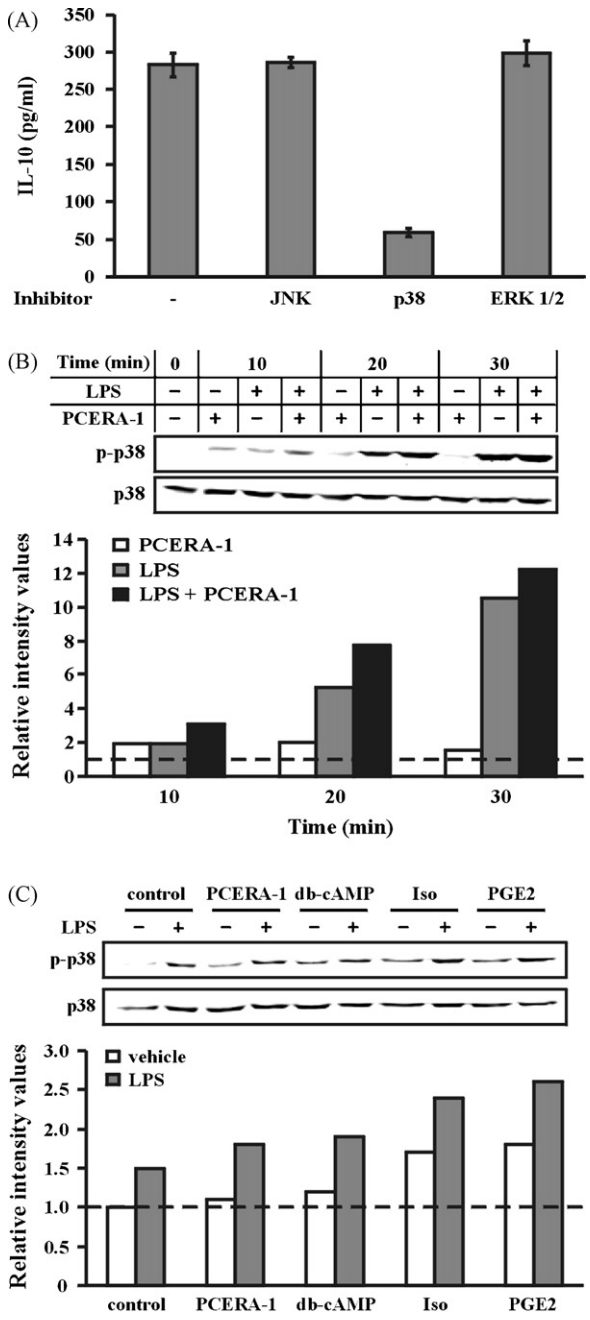

Fig. 6. The p38 pathway is co-activated by LPS and cAMP inducers and is required for IL-10 induction. (A) Mouse macrophage RAW264.7 cells were incubated at 37 °C for 2 h with LPS (100 ng/ml) and PCERA-1 (1 μM) in the presence of either SB203580 (p38 inhibitor, 25 μM), SP600125 (JNK inhibitor, 10 μM), PD98059 (ERK1/2 inhibitor, 50 μM), or vehicle. IL-10 release to the medium was measured by ELISA. Each data point represents the mean ± S.D. ($n = 6$). (B) Mouse macrophage RAW264.7 cells were stimulated with LPS (100 ng/ml) and/or PCERA-1 (1 μM), for the indicated time at 37 °C. DMEM/0.1% FBS served as vehicle. Cell lysates (60 μg protein) were subjected to SDS–PAGE, followed by transfer to a membrane which was simultaneously probed with an antibody against the doubly phosphorylated form of p38, and with a general antibody against p38 for normalization (two-color imaging, using the Odyssey infrared imaging system). The results are representative of 5 independent experiments. Bottom panel: quantitative western blot analysis is shown as the ratio of intensities of phospho-p38 and total p38, relative to unstimulated cells (represented by the dashed line). (C) Mouse macrophage RAW264.7 cells were incubated with either PCERA-1 (1 μM), PGE2 (100 nM), the β-AR agonist isoproterenol (Iso, 1 μM), the cell-permeable dibutyryl cAMP (db-cAMP, 100 μM), or control buffer (DMEM/0.1% FBS) in the presence or absence of LPS (100 ng/ml) for 15 min at 37 °C. Cell lysates were analyzed as in (B). The results are representative of 3 independent experiments. Bottom panel: quantitative western blot analysis is shown as the ratio of intensities of phospho-p38 and total p38, relative to unstimulated cells (represented by the dashed line).

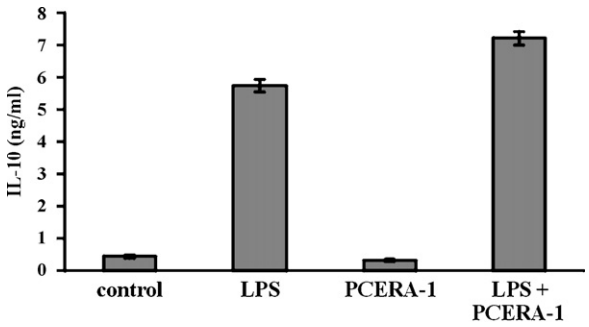

Fig. 7. LPS and PCERA-1 synergistically induce IL-10 production in primary macrophages. Bone marrow-derived macrophages (BMDM) were incubated at 37 °C for 24 h with PCERA-1 (1 μM) and/or LPS (1 μg/ml). IL-10 release to the medium was measured by ELISA. Each data point represents the mean ± S.D. ($n = 6$). $p < 0.001$ for cells incubated with LPS + PCERA-1, compared to cells incubated with LPS alone.

phosphorylation, and hence activation, of p38 in RAW264.7 cells stimulated with LPS alone or in combination with PCERA-1. Fig. 6B shows that at 10 min LPS or PCERA-1 alone increased p38 phosphorylation 1.9-fold, whereas in the presence of both LPS and PCERA-1, a 3.1-fold increase in p38 phosphorylation was detected. At 20 min, up-regulation of p38 phosphorylation was 5.2-fold, 2.0-fold and 7.8-fold, for LPS alone, PCERA-1 alone and for LPS + PCERA-1, respectively. At 30 min, activation of p38 by PCERA-1 alone was lower (1.6-fold), and the phosphorylation caused by LPS alone (10.5-fold) was almost equal to that by LPS + PCERA-1 (12.2-fold), in agreement with our previous findings (Goldsmith et al., 2008). Overall, PCERA-1 alone had modest activity in this assay, and the effect of a co-stimulus of LPS and PCERA-1 on p38 activation was more than additive (Fig. 6C). Although the level of cooperativity is modest, this result was confirmed in several experiments. Interestingly, the cAMP inducers PGE2 and isoproterenol, as well as the cell-permeable dibutyryl cAMP, also induced modest p38 phosphorylation in the absence of LPS, and elevated LPS-induced activation of p38, in a manner which was more than additive (Fig. 6C). Taken together, the above findings suggest that LPS and cAMP inducers, such as PCERA-1, cooperatively activate the p38 pathway, thereby attributing to their synergistic roles in IL-10 induction.

Finally, as the above findings were observed in a macrophage cell line (RAW264.7), the effect of PCERA-1 on LPS-induced IL-10 production was also examined in BMDM, which are primary macrophages. Fig. 7 shows that incubation of these cells with PCERA-1 for 24 h elevated LPS-induced IL-10 production by 1.3-fold, whereas PCERA-1 alone had no measurable effect over the background. Thus, the level of synergism displayed by LPS and PCERA-1 at long incubations in BMDM (Fig. 7) and in the RAW264.7 macrophages (Fig. 1B) was found to be close, confirming that the observed activities of PCERA-1 are relevant to primary macrophages, rather than influenced by the identity of the chosen cell line.

4. Discussion

Among the complex array of enhancer sites at the IL-10 promoter, the CRE appears essential for efficient IL-10 transcription in response to various stimuli (Gee et al., 2006; Hu et al., 2006; Platzer et al., 1999). Yet, PCERA-1 only modestly (Figs. 1A, 2A and 3), if at all (Figs. 1B, 4 and 5), promotes IL-10 production in the absence of LPS. These relatively minor differences may be due to a slight variability in the reactivity of the cell culture in each experiment, and/or due to the relatively large error inherent to the IL-10 assay below 100 pg/ml. In any case, the inability of PCERA-1 to appreciably induce IL-10 expression by itself stands in apparent contrast to its ability to elevate intracellular cAMP (Goldsmith et al., 2008).

Likewise, the cell-permeable cAMP analog, dibutyryl cAMP, induces IL-10 expression in unstimulated cells only when present at very high concentrations (Fig. 4B). These findings indicate that physiological cAMP levels are probably insufficient to induce expression of the IL-10 gene. However, the pronounced amplification of LPS-induced IL-10 production by cAMP-elevating agents suggests that cAMP-activated transcription factors which bind at CRE act in coordination with other transcription factors regulated by LPS, to achieve maximal transcriptional activity at the IL-10 promoter. These findings parallel with a recent report by Hickey et al. (2008) showing that the adenylate cyclase toxin of *Bordetella pertussis* induces IL-10 production by dendritic cells in synergism with LPS, but not alone. Similarly, a PKA-specific cAMP analog was able to significantly induce IL-10 production by alveolar macrophages only in the presence of LPS (Aronoff et al., 2006).

Indeed we have found that p38 activity is also required for the production of IL-10 by LPS and PCERA-1 (Fig. 6A). Notably, LPS-induced IL-10 transcription was previously reported to be mediated by p38-dependent activation of the transcription factor Sp1 (Brightbill et al., 2000; Ma et al., 2001). Although an effect of LPS and/or PCERA-1 on mRNA stability cannot be excluded, it should be noted that mutations at the IL-10 promoter sites which respond to cAMP (Platzer et al., 1999) or p38 activation (Brightbill et al., 2000; Ma et al., 2001) have been reported to abolish IL-10 transcription. Consistently, we found that LPS and PCERA-1 synergistically induced transcription of an IL-10 promoter reporter construct, pinpointing transcription regulation rather than mRNA stability as the basis for the observed synergism.

We have found that the synergistic activation of transcription at the IL-10 promoter is mediated by PKA (Fig. 3B). The synergism displayed by the combination of PCERA-1 and dibutyryl cAMP, in the presence of LPS (Fig. 4B), can be explained by cooperative enzymatic activity of PKA due to cAMP coming from two sources: exogenous (dibutyryl cAMP) and endogenous (elevated by PCERA-1). This would imply that the PCERA-1-induced cAMP level does not suffice for full activation of PKA and its downstream targets. In support of this possibility, the β-AR agonist isoproterenol displays a higher level of synergism with LPS in IL-10 production, relative to PCERA-1 (Fig. 4A). This finding is consistent with a high expression of β-AR in macrophages (Abrass et al., 1985), and with a higher level of cAMP produced by RAW264.7 macrophages in response to isoproterenol, relative to PCERA-1 (data not shown). The evidence for synergistic IL-10 production by a combination of two cAMP inducers in the presence of LPS (Fig. 4C) further supports this conclusion. Alternatively, PCERA-1, which was suggested to act via a cell-surface receptor (Goldsmith et al., 2008), may activate multiple intracellular pathways leading to IL-10 induction, cAMP being one of them. Indeed we have found that synergistic p38 activation by LPS and PCERA-1 is required for IL-10 production (Fig. 6). However, phosphorylation of p38 was similarly elevated by dibutyryl cAMP and by PCERA-1 (Fig. 6C), and therefore the hyper-activation of the p38 pathway by PCERA-1 cannot account for the synergism displayed by PCERA-1 and dibutyryl cAMP in LPS-induced IL-10 production. We cannot exclude the possibility that another pathway, distinct from cAMP and p38, may be involved in the PCERA-1 effect on IL-10 level. Both alternative mechanisms are consistent with the synergistic IL-10 production displayed by the combination of either PGE2 or isoproterenol with either dibutyryl cAMP (data not shown) or PCERA-1 (Fig. 4C).

We show here that PCERA-1-induced IL-10 expression in synergism with various TLR agonists, whereas neither PCERA-1 nor any of the TLR agonists was able by itself to substantially increase IL-10 levels over background levels (Fig. 2). Of relevance, *in vivo* administration of dibutyryl cAMP (Arai et al., 1995), or of a cAMP-elevating agent, such as PCERA-1 (Avni et al., 2009), β-AR agonist (Szelenyi et al., 2000), or PGE2 (Takano et al., 1998), has been shown to amplify LPS-induced IL-10 production in mice. Taken together, these observations suggest that cAMP-elevating agents are required to limit the pro-inflammatory response, and to initiate an anti-inflammatory response. Indeed, several groups have reported that administration of the β-AR antagonist propranolol to LPS-challenged mice enhanced production of the pro-inflammatory cytokine TNFα (Boost et al., 2007; Giebelen et al., 2008; Szelenyi et al., 2000) and suppressed production of the anti-inflammatory cytokine IL-10 (Boost et al., 2007; Suberville et al., 1996; Szelenyi et al., 2000), indicating that endogenous circulating catecholamines exert a tonic suppression on the pro-inflammatory response, and play a permissive role for LPS effect on production of the anti-inflammatory IL-10. Interestingly, LPS administration is associated with the depletion of epinephrine and norepinephrine contents in the rat adrenal gland (Wang et al., 2000). Moreover, both PGE2 (Lee et al., 1992) and catecholamines (Spengler et al., 1994) are released by LPS-stimulated macrophages, and then act in an autocrine fashion to deactivate macrophages function. The results presented here support the notion that the mechanism of this negative feedback loop involves in part synergistic LPS-induced IL-10 production. The inability of the cAMP-elevating agents to significantly induce IL-10 production in the absence of LPS (Fig. 4A), predicts that the *in vivo* anti-inflammatory activity of cAMP-elevating agents will be restricted to local immune cells which are actively involved in responding to antigens.

In addition to cAMP inducers, the PDE-4 inhibitor rolipram also synergized with LPS in IL-10 production, consistent with previous reports (Eigler et al., 1998). This finding is indicative of the crucial role of PDE-4 in keeping cAMP levels in macrophages low enough to ensure that the anti-inflammatory IL-10 would be considerably induced only in the presence of a cAMP inducer in addition to LPS. Moreover, the PDE-4 inhibitor further elevated the synergistic production of IL-10 by LPS and PCERA-1. This finding supports the conclusion stated above regarding the inability of receptor-mediated cAMP increase to maximally drive IL-10 production. Notably, LPS-induced sensitization of adenylyl cyclase, demonstrated in RAW264.7 macrophages (Osawa et al., 2006), is expected to intensify the basal as well as stimulated activity of G_s-coupled receptors, and thus to contribute to increased intracellular cAMP level, and subsequently to IL-10 expression. Thus, our results suggest that the cAMP intracellular level, governed by the balance of activities of G_s-coupled receptors and phosphodiesterases, critically dictates the outcome of LPS stimulation of macrophages, regarding IL-10 production.

Activation of p38 MAP kinase was demonstrated to be crucial for IL-10 production in macrophages co-stimulated by LPS and PCERA-1 (Fig. 6A), consistent with other reports for LPS-stimulated macrophages (Brightbill et al., 2000; Ma et al., 2001). In contrast, the finding that the ERK1/2 and JNK pathways are not required in our system (Fig. 6A) stands in apparent contradiction to some other reports (Chanteux et al., 2007; Hickey et al., 2008; Liu et al., 2006). While PCERA-1 may possibly confer different requirements for LPS-induced IL-10 production, a more likely explanation lies in the time frame of IL-10 production. In order to show maximal synergism between LPS and PCERA-1 we measured IL-10 production at a relatively short time frame of 2 h, while the protocols yielding dependency on ERK1/2 and/or JNK routinely involve much longer (6–24 h) stimulation by LPS (Chanteux et al., 2007; Hickey et al., 2008; Liu et al., 2006). Thus, contribution of ERK1/2 and JNK to enhanced IL-10 production may occur via protein expression of new transcription factors, and thus will only be apparent at long incubations. In contrast, we show that p38, but not other MAP kinases, is essential for short-term IL-10 production, and thus we propose that it is required for activation of existing transcription factors.

We found that p38 phosphorylation, representing its activation, was synergistically induced by the co-stimulus of LPS and

PCERA-1 (Fig. 6B). The modest fold-increase of p38 phosphorylation in our study (~2-fold) is comparable to those observed and found to be functional in multiple other studies (Communal et al., 2000; Gong et al., 2008; Yamauchi et al., 1997; Zheng et al., 2000), implying physiological relevance. Furthermore, similar enhancement of LPS-induced p38 activation was observed also for other cAMP inducers, PGE2 and the β-AR agonist isoproterenol, and for the cell-permeable dibutyryl cAMP (Fig. 6C). Consistently, a modest elevation of LPS-induced p38 phosphorylation, which was mandatory for IL-10 induction, was found to be triggered by the adenylate cyclase toxin of *Bordetella pertussis* (Hickey et al., 2008). It is thus suggested that elevation of the intracellular cAMP level by PCERA-1 (Goldsmith et al., 2008) leads to synergistic IL-10 expression in LPS-stimulated cells, via PKA-activated CREB (Hagiwara et al., 1993), as well as the enhanced activation of additional transcription factors, downstream of p38. The mechanism of cAMP-mediated stimulation of p38 activity is cell-specific, and is probably also influenced by subtle variations in cell lines used by different groups (Communal et al., 2000; Zheng et al., 2000). Both PKA-dependent (Delghandi et al., 2005; Kobayashi et al., 2005; Zheng et al., 2000) and PKA-independent (Tan et al., 2007; Yin et al., 2006) pathways have been reported. Of particular relevance are two studies performed in RAW264.7 macrophages. Kobayashi et al. (2005) have found that PGE2 enhanced LPS-induced p38 activation via PKA-mediated phosphorylation and activation of TAK1 which serves as a MAPK kinase in the p38 pathway. Conversely, Tan et al. (2007) have found that activation of the β-AR in the same cell line led to p38 phosphorylation by a PKA-independent mechanism, and that the activity of the receptor was mimicked by a cAMP analog that is unable to activate PKA but does specifically activate a recently identified cAMP effector known as the exchange protein directly activated by cAMP (EPAC). This protein functions as a guanine nucleotide exchange factor for Rap1 (Bos, 2006), a small GTPase that regulates p38 activation (Huang et al., 2004; Kanda and Watanabe, 2007; Sawada et al., 2001). The mechanism by which PCERA-1 augments LPS-induced IL-10 production remains to be explored.

To conclude, we have shown here that PCERA-1 synergizes with various TLR agonists in the expression of IL-10 by RAW264.7 macrophages at the mRNA and protein levels. This activity is proposed to be mediated at least in part via cAMP, as PCERA-1 was previously shown to stimulate cAMP production, since other cAMP inducers or a cell-permeable cAMP analog can mimic it, and since PKA activity was found to be essential for it. Moreover, basal cAMP level in RAW264.7 macrophages was found to be insufficient for significant LPS-induced IL-10 production, due to PDE4 activity. In addition to the putative activation of the cAMP response element present on the IL-10 promoter, we show here that PCERA-1, as well as other cAMP inducers/analog, cooperates with LPS in activation of the p38 pathway, which is also essential for IL-10 induction. The findings presented here shed light on the mechanism of activity of PCERA-1, a phospholipid-like molecule which represents a novel class of anti-inflammatory drug candidates, and highlight the critical role of cAMP in LPS-induced IL-10 production.

Acknowledgments

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