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Role of CREB in modulation of TNFα and IL-10 expression in LPS-stimulated RAW264.7 macrophages

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ABSTRACT

The role of CREB in LPS signaling is controversial. The objective of this study was to evaluate the effect of LPS on phosphorylation and transcriptional activation of CREB, in comparison to isoproterenol, a β-adrenergic receptor agonist. We show here that LPS elevates intra-cellular cAMP level in RAW264.7 macrophages, with slower kinetics and lower magnitude than isoproterenol. The two agents stimulated CREB phosphorylation on Ser-133 to a similar extent, but with a different mechanism; rapid and mostly PKA-mediated for isoproterenol; slow and MSK1-mediated for LPS. Interestingly, LPS-stimulated phosphorylation of CREB did not result in transcriptional activation of a CRE-regulated luciferase reporter, in contrast to stimulation by isoproterenol. Furthermore, inhibitors of p38 and MSK1, but not PKA, completely blocked the production of IL-10 and TNFα in LPS-stimulated macrophages. Distinctively, the PKA inhibitor H89 blocked the suppressive effect of isoproterenol on TNFα production, as well as its stimulatory effect on IL-10 induction, in LPS-stimulated macrophages. Likewise, while over-expression of dominant negative CREB had no effect on LPS-stimulated TNFα production, it blocked the suppressive effect of isoproterenol on TNFα production in the LPS-stimulated macrophages. Our results thus indicate that PKA-mediated phosphorylation of CREB promotes TNFα suppression and IL-10 induction, whereas the same phosphorylation event initiated by LPS and mediated by MSK1 is non-functional for transcriptional modulation.

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1. Introduction

The pro-inflammatory cytokine tumor necrosis factor (TNF)α and the anti-inflammatory cytokine interleukin (IL)-10 are key players in inflammation, initiated, among other stimuli, upon toll-like receptor 4 (TLR4)-mediated detection of lipopolysaccharide (LPS), a molecular component of Gram-negative bacteria. Tight regulation of cytokine production is essential for the balance between proper immune reaction against the pathogen and prevention of excessive damage to the host (Beutler, 2004).

Activation of the cAMP pathway in macrophages by a multitude of G protein-coupled receptor (GPCR) ligands, such as agonists of the β-adrenergic receptor (β-AR), serves to down-regulate production of TNFα, as well as to up-regulate production of IL-10 (Kast, 2000; Szelenyi et al., 2000; Zidek, 1999). The canonical cAMP pathway propagates via protein kinase A (PKA)-mediated phosphorylation of CREB (Gonzalez and Montminy, 1989), a transcription factor which binds to the CRE sites, present at both TNFα (Kuprash et al., 1999) and IL-10 (Platzer et al., 1999) promoters.

Phosphorylation and activation of CREB following treatment of LPS-stimulated RAW264.7 macrophages with a cAMP inducer results in transcriptional activation at the IL-10 promoter (Avni et al., 2010; Goldsmith et al., 2009a). Consistently, mutations at CRE sites in the human IL-10 promoter result in loss of transcriptional activity (Platzer et al., 1999). These observations thus indicate that cAMP-activated CREB can positively regulate IL-10 transcription in LPS-stimulated macrophages.

The role of CREB in TNFα transcription is far less understood. On the one hand, cAMP inducers negatively regulate TNFα expression (Goldsmith et al., 2009b; Kast, 2000). On the other hand, mutations at the CRE site in the mouse TNFα promoter result in loss of transcriptional activity in LPS-stimulated (O'Donnell and Taffet, 2002) or mycobacteria-stimulated (Roach et al., 2005) mouse RAW264.7 macrophages. Thus, while cAMP is considered a negative regulator of TNFα transcription, the latter studies suggested that CREB may be a positive regulator of TNFα transcription.

In addition to the controversy regarding the role of CREB in TNFα transcription, it is unclear whether LPS itself activates CREB. First, some groups (Chen et al., 1999; Osawa et al., 2006), but not others

Abbreviations: PKA, protein kinase A; β-AR, β-adrenergic receptor; GPCR, G protein-coupled receptor; AC, adenylyl cyclase; PVDF, Immobilon-FL polyvinylidene fluoride; TLR, toll-like receptor; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin.

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(Avni et al., 2010; Greten et al., 1996), have been able to observe a cAMP increase in LPS-stimulated RAW264.7 macrophages. The apparent contradictory may be explained by the different incubation times; long and short, respectively. Second, LPS has been shown to stimulate phosphorylation of CREB on Ser-133 via MSK1, a kinase which is downstream to p38 (Caivano and Cohen, 2000; Eliopoulos et al., 2002). Yet, in these studies it was not directly demonstrated whether CREB was activated by this phosphorylation event.

Therefore, the goal of our research was to evaluate the role of CREB in transcriptional regulation of TNFα and IL-10, in LPS-stimulated macrophages. We show here that isoproterenol induced PKA-mediated phosphorylation of CREB as well as transcriptional activation of a cAMP-dependent luciferase reporter, whereas LPS induced MSK1-mediated phosphorylation of CREB, but failed to activate it. Furthermore, PKA and CREB activation was found to be required for transcriptional regulation of TNFα and IL-10 by isoproterenol, while it was found to be irrelevant for LPS stimulation of cytokine expression.

2. Materials and methods

2.1. Reagents, plasmids, and cell culture

Lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5), H89, rolipram, isoproterenol, propranolol, PMSF and DMSO, were purchased from Sigma–Aldrich (St. Louis, MO). Ro318220 was purchased from Calbiochem (Darmstadt, Germany). SB203580 was purchased from A.G. Scientific (San Diego, CA). L-Glutamine, penicillin and streptomycin were purchased from Biological Industries (Beit Haemek, Israel). DMEM and FBS were purchased from Gibco (Carlsbad, CA). BSA was purchased from Amresco (Solon, OH). ELISA reagents sets for TNFα and IL-10 were purchased from R&D Systems (Minneapolis, MN). The LANCE-cAMP kit was purchased from Perkin-Elmer (Waltham, MA). The antibodies against α-tubulin and general p38 were from Santa Cruz Biotechnology (Santa Cruz, CA). A CRE-containing EVX-1 promoter-luciferase reporter gene construct (hereafter CRE-luciferase) (Conkright et al., 2003), an antibody against phospho-Ser-133 CREB, and a dominant negative S133A CREB (Gonzalez and Montminy, 1989), were a kind gift from Dr. Marc Montminy (Salk Institute, La-Jolla, CA). The RSV control vector (for dominant negative CREB) was created by excision of the CREB cDNA fragment out of the construct with the restriction enzymes KpnI and BamHI (NEB, Ipswich, MA), blunting of the vector backbone overhang ends using Klenow DNA polymerase (NEB, Ipswich, MA), and ligation using T4 DNA ligase (Fermentas, Burlington, Canada). A luciferase reporter gene downstream of the full (−1167/+155) TNFα promoter (Chen et al., 2008) was a kind gift from Dr. Chundong Yu (Xiamen University, Xiamen, Fujian, China). The antibodies against phospho-Thr-581 MSK1 and doubly phosphorylated p38 were from Cell Signaling Technology (Danvers, MA). 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). 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 with 10% FBS. The cells were grown and maintained at 37 °C in a humidified incubator with 5% CO_2.

2.2. Macrophages activation assay and cytokine measurement

RAW264.7 macrophages were maintained for 48 h prior to the experiment in 96-well plates, at 1.5×10^5 cells per well, in culture medium supplemented with 5% FBS. 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) in the presence or absence of isoproterenol (1 μM) at 37 °C for 2 h. TNF-α and 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 (BioTek, Winooski, Vermont). The samples were stored at −80 °C until used.

2.3. Whole cell cAMP measurements

RAW264.7 macrophages were maintained for 48 h prior to the experiment in 96-well plates, at 1.5×10^5 cells per well, in culture medium supplemented with 5% FBS. The cells were incubated at 37 °C for 0–60 min with isoproterenol (1 μM) or LPS (100 ng/ml). The β-AR inverse agonist propranolol (1 μM) was added to LPS-treated cells and to control cells in order to prevent possible constitutive and basal β-AR activity. Lysis was then accomplished by incubation with HCl (0.1 M) in the presence of the phosphodiesterase 4 inhibitor rolipram (20 μM), for 20 min at 4 °C. The samples were diluted 2-fold with PBS/0.1% BSA and the intracellular cAMP level was measured by the LANCE-cAMP kit, in a 384-well plate suitable for time-resolved fluorescence (TRF) measurements, using a Synergy 2 multi-mode microplate reader (BioTek, Winooski, Vermont). The assay was performed according to the manufacturer's instructions, with the exception of antibody addition to the lysate rather than to the cells.

2.4. CREB, p38 and MSK1 phosphorylation assay

RAW264.7 macrophages were maintained for 24 h prior to the experiment in 12-well plates, at 5×10^5 cells per well, in culture medium supplemented with 0.1% FBS. The cells were stimulated with LPS (100 ng/ml) or isoproterenol (1 μM) at 37 °C for 0–60 min. The cells were then washed twice with cold PBS and lysed for 15 min at 4 °C with buffer containing Triton X-100 (1%), Tris buffer pH 8.0 (50 mM), NaCl (100 mM), β-glycerophosphate (50 mM), sodium orthovanadate (1 mM), EDTA (1 mM), EGTA (1 mM), glycerol (30%), PMSF (1 mM) 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.

2.5. Western blotting

Cell extracts (30 μg protein) were boiled for 5 min in SDS-PAGE buffer, subjected to 10% SDS-PAGE, and proteins were transferred to PVDF membrane. An antibody raised against phospho-Ser-133 CREB was used together with an antibody against α-tubulin, or separately from an antibody against CREB (total). Antibodies against doubly phosphorylated p38 and against phospho-Thr-581 MSK1 were used together with an antibody against p38 (total). Two-color imaging and quantitative analysis of western blots was performed using the Odyssey infrared imaging system (Li-Cor Biosciences), according to the manufacturer's instructions.

2.6. Protein determination

Protein was determined by a modification of the Bradford procedure, which yields linear and thus more accurate results,

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increased sensitivity, and reduced detergent interference, as previously described by Zor and Selinger (1996). BSA served as standard.

2.7. Transfection and reporter gene assay

RAW264.7 macrophages were transfected with either 1.5 μg of CRE-luciferase reporter plasmid or with 1.0 μg of TNFα promoter-luciferase reporter plasmid together with 1.0 μg of an expression plasmid for dominant negative S133A CREB, or with an empty RSV-SG vector. For normalization, 0.5 μg of pRL-TK vector, coding for renilla luciferase, was added. The details of plasmids amplification and transfection protocol were previously described (Avni et al., 2010). Following a 24 h transfection, the cells were washed and stimulated for 3 h at 37 °C with isoproterenol (1 μM) and/or LPS (100 ng/ml). Luciferase activity in cell extracts was determined following the manufacturer's (Promega) instructions. Data are expressed as a ratio of CRE-driven firefly luciferase activity divided by the renilla luciferase activity. Transfection with the empty reporter vector, performed as a control, yielded no detectable activity.

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 as least three times.

3. Results

3.1. LPS and isoproterenol differentially elevate intra-cellular cAMP level

In order to probe CREB activation by LPS, we initially measured the ability of LPS to elevate the intra-cellular level of cAMP in RAW264.7 macrophages. Fig. 1 shows that there was no apparent increase in cAMP level in response to LPS treatment at early time points of less than 30 min, in agreement with our previous results (Avni et al., 2010). Then however, a 1.8-fold increase in basal cAMP level was measured at 30–60 min of LPS treatment. In contrast to the modest and delayed effect of LPS, the β-AR agonist isoproterenol rapidly elicited a 4-fold increase in cAMP level within 15–30 min, and the response declined thereafter.

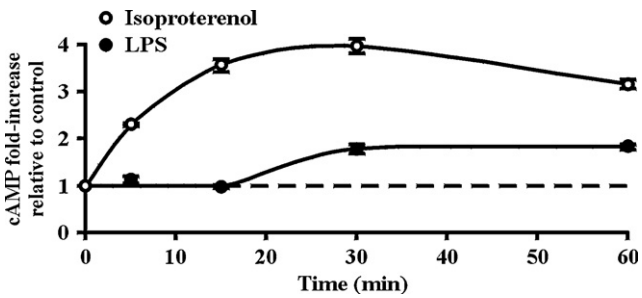

Fig. 1. LPS and isoproterenol elevate cAMP with different kinetics and magnitude. RAW264.7 macrophages were incubated with the β-AR agonist isoproterenol (1 μM, open circles) or with LPS (100 ng/ml, closed circles) for the indicated time at 37 °C. The β-AR antagonist propranolol (1 μM) was added to control cells, and to cells treated with LPS, in order to reduce the potential background of constitutive and basal β-AR activity. Each data point represents the mean ± S.D. ($n = 3$), relative to unstimulated control cells (represented by the dashed line).

3.2. LPS and isoproterenol stimulate CREB phosphorylation via distinct mechanisms

Phosphorylation of the transcription factor CREB on Ser-133 is commonly associated with its activation (Gonzalez and Montminy, 1989). We therefore examined the ability of LPS to stimulate CREB phosphorylation in the RAW264.7 macrophages. As shown in Fig. 2, both isoproterenol and LPS induced phosphorylation of CREB at Ser-133, up to 2-fold over the basal state. However, the kinetics of phosphorylation differed between the two stimuli. The phosphorylation induced by isoproterenol rapidly reached a peak at 15 min, whereas LPS-induced phosphorylation proceeded with a slower pace and reached a peak only at 30–60 min. Thus, the time-course of CREB phosphorylation by the two stimuli was consistent with the respective time-course of cAMP elevation (Fig. 1). However, the similar magnitude of CREB phosphorylation by the two stimuli was in apparent contrast with the respective magnitudes of cAMP elevation, suggesting a different mechanism.

Previous reports have shown that LPS-stimulated phosphorylation of CREB in macrophages is carried out by p38-activated MSK1 (Caivano and Cohen, 2000; Eliopoulos et al., 2002). Therefore, we sought to determine whether this pathway mediates the effect of LPS also in the RAW264.7 macrophages used in the current study. As expected, we found that LPS dramatically increased the phosphorylation level of both p38 (12-fold) and MSK1 (34-fold), with a peak at 30 min (Fig. 3). Thus, the time-course of p38 and MSK1 activation by LPS correlated with the time-course of CREB phosphorylation. Isoproterenol only modestly increased the phosphorylation level of p38 and MSK1 (data not shown), in agreement with our previous report (Goldsmith et al., 2009a).

To identify the kinases which mediate the phosphorylation of CREB in response to LPS and to isoproterenol, we have used the following inhibitors: H89, a PKA inhibitor which was demonstrated to inhibit also MSK1 (Caivano and Cohen, 2000); Ro318220, a specific MSK1 inhibitor; and SB203580, a specific p38 inhibitor. Fig. 4 shows that LPS-stimulated phosphorylation of CREB was strongly inhibited by the specific MSK1 inhibitor, and only modestly inhibited by H89. In contrast, isoproterenol-stimulated phosphorylation of CREB was strongly inhibited by H89, and only modestly

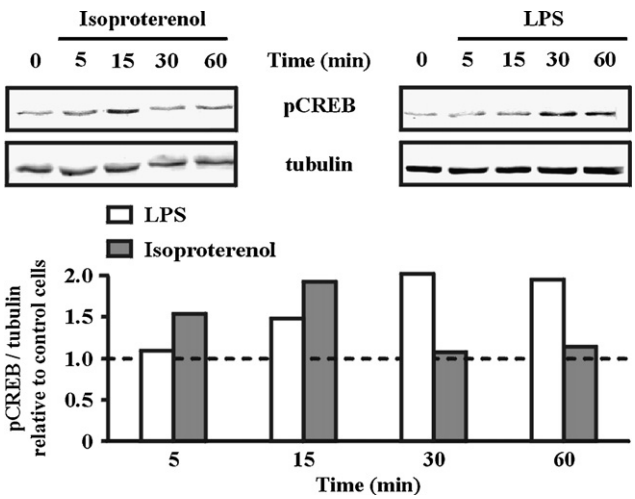

Fig. 2. LPS and isoproterenol stimulate CREB phosphorylation with different kinetics. RAW264.7 macrophages were stimulated with isoproterenol (1 μM) or with LPS (100 ng/ml) for the indicated time at 37 °C. Cell lysates (30 μg) were subjected to SDS-PAGE and transferred to a membrane which was simultaneously probed with antibodies against phospho-Ser-133 CREB, and against α-tubulin (for normalization). Quantitative western blot analysis is shown as the ratio of intensities of phospho-CREB and tubulin, relative to unstimulated control cells (represented by the dashed line). There was no effect on the total level of CREB (data not shown). The results are representative of 5 independent experiments.

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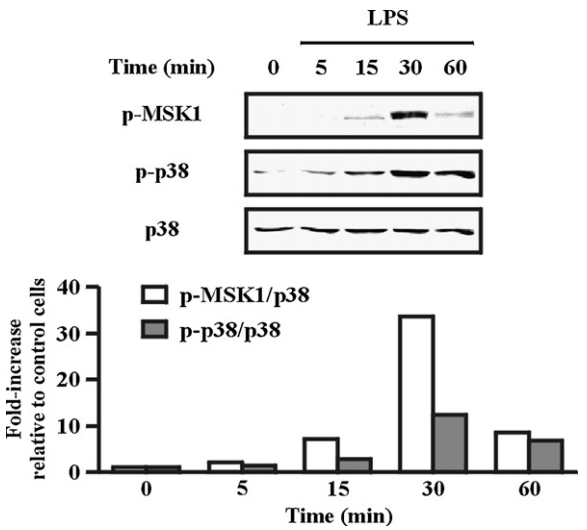

Fig. 3. LPS stimulates phosphorylation of p38 and MSK1. RAW264.7 macrophages were stimulated with LPS (100 ng/ml) for the indicated time at 37 °C. Cell lysates (30 μg) were subjected to SDS-PAGE and transferred to a membrane which was simultaneously probed with antibodies against doubly phosphorylated p38, phospho-Thr-581 MSK1, and p38 (for normalization). Quantitative western blot analysis is shown as the ratio of intensities of the phosphorylated kinase and general p38, relative to unstimulated control cells. The results are representative of 7 and 3 independent experiments, for p38 and for MSK1, respectively.

inhibited by the specific MSK1 inhibitor. The specific p38 inhibitor decreased phosphorylation of CREB in response to both LPS and isoproterenol, consistent with a previous report that LPS and isoproterenol can independently activate the p38 pathway (Goldsmith et al., 2009a). Taken together, these results indicate that phosphorylation of CREB on Ser-133 in RAW264.7 macrophages treated with LPS and isoproterenol, is carried out by distinct mechanisms. LPS elicited the phosphorylation via the p38–MSK1 pathway, whereas isoproterenol elicited the phosphorylation mostly via PKA and partially also via p38–MSK1.

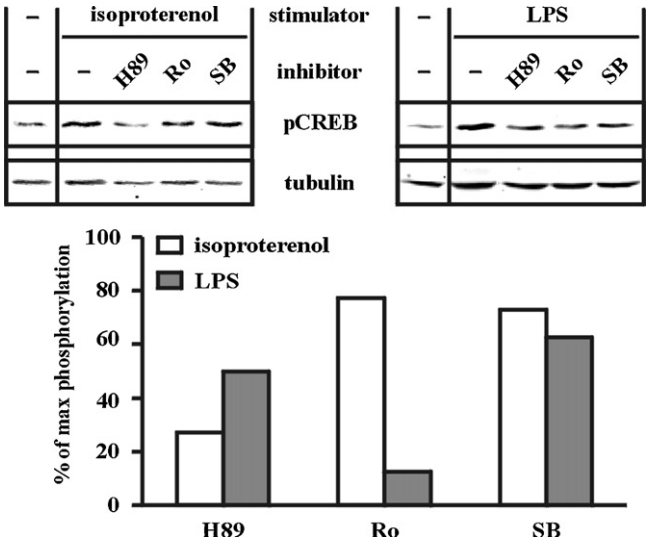

Fig. 4. LPS and isoproterenol stimulate phosphorylation of CREB via distinct signaling pathways. Pre-incubation of RAW264.7 macrophages for 30 min at 37 °C with either H89 (PKA and MSK1 inhibitor, 30 μM), Ro318220 ("Ro", MSK1 inhibitor, 5 μM), SB203580 ("SB", p38 inhibitor, 30 μM) or vehicle, was followed by addition of isoproterenol (1 μM) for an additional 15 min or LPS (100 ng/ml) for an additional 30 min. Cell lysates were analyzed as in Fig. 2. The results are representative of 6 independent experiments.

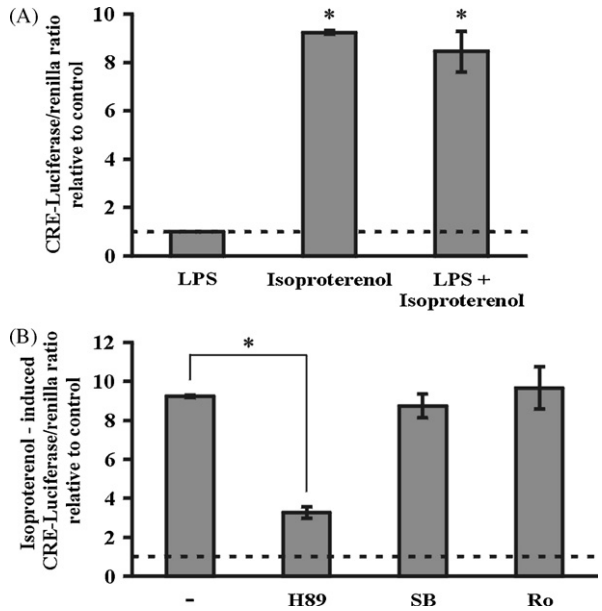

Fig. 5. Isoproterenol, but not LPS, stimulates CRE-luciferase activity. RAW264.7 macrophages were transiently transfected for 24 h at 37 °C with a reporter gene construct which codes for firefly luciferase under the regulation of CRE, and with a renilla luciferase construct for normalization. Luciferase activity assay was performed as described under Section 2. Each data point represents mean ± S.D. ($n = 6$) of values normalized against renilla luciferase activity, and relative to unstimulated control cells (represented by the dashed line). (A) The cells were washed and incubated with isoproterenol (1 μM) and/or LPS (100 ng/ml), for 3 h at 37 °C. $p < 0.0001$ (compared to control). (B) The cells were washed and pre-incubated for 30 min at 37 °C with H89 (PKA and MSK1 inhibitor, 30 μM), Ro318220 ("Ro", MSK1 inhibitor, 5 μM), SB203580 ("SB", p38 inhibitor, 30 μM) or vehicle, and then further incubated with isoproterenol (1 μM) for an additional 3 h. $p < 0.0004$.

3.3. Isoproterenol, but not LPS, stimulates CRE-regulated transcriptional activity

To assess the effect of CREB phosphorylation on its activity as a transcription factor, RAW264.7 macrophages were transiently transfected with a reporter gene construct which codes for luciferase under the regulation of CRE. Fig. 5A shows that isoproterenol elevated CRE-dependent luciferase activity 9-fold. As expected, isoproterenol-stimulated CRE-luciferase activity was inhibited by the PKA inhibitor H89, but unexpectedly, it was not inhibited by the specific p38 and MSK1 inhibitors (Fig. 5B). Interestingly, in spite of its ability to increase phosphorylation of CREB, LPS failed to induce CRE-dependent luciferase activity or to intensify the effect of isoproterenol (Fig. 5A). Taken together, the observations, made with an exogenous reporter gene which is specifically regulated by CRE, thus suggest that isoproterenol activates CREB by PKA-mediated phosphorylation, whereas LPS-induced phosphorylation of CREB, which is mediated by the p38–MSK1 pathway, is not functional.

3.4. Isoproterenol-activated CREB regulates LPS-induced TNFα and IL-10 expression

To assess the role of CREB, p38 and MSK1 in LPS signaling, and in isoproterenol-initiated regulation of LPS signaling, we measured the effect of kinase inhibitors on induction of IL-10 and TNFα in RAW264.7 macrophages stimulated with LPS, in the presence or absence of isoproterenol. Fig. 6A shows that isoproterenol radically elevated (by 19-fold) LPS-induced expression of the key anti-inflammatory cytokine IL-10. Cells treated with isoproterenol alone (in the absence of LPS) produced only a negligible quantity of IL-10 (data not shown), indicating that isoproterenol and LPS

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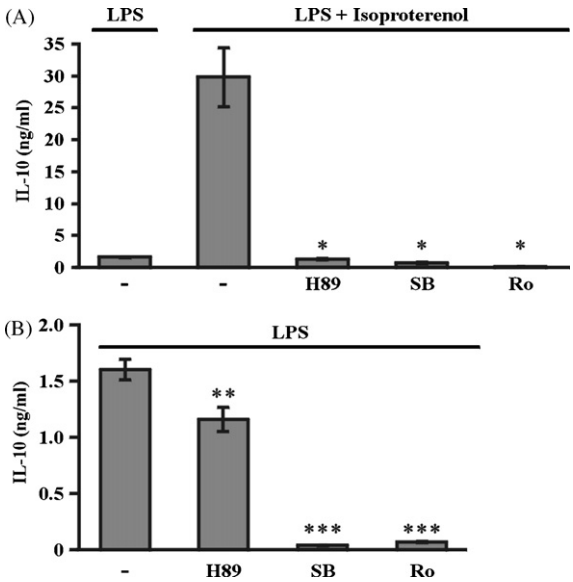

Fig. 6. LPS and isoproterenol modulate IL-10 production via distinct signaling pathways. RAW264.7 macrophages were pre-incubated for 30 min at 37 °C with H89 (PKA and MSK1 inhibitor, 30 μM), Ro318220 ("Ro", MSK1 inhibitor, 5 μM), SB203580 ("SB", p38 inhibitor, 30 μM) or vehicle, and then further incubated with LPS (100 ng/ml) in the presence (A) or absence (B) of isoproterenol (1 μM) for 4 h. IL-10 release to the medium was measured by ELISA. Background IL-10 level (in the absence of LPS) was negligible: 13 ± 4 pg/ml and 31 ± 5 pg/ml, in the absence or in the presence of isoproterenol, respectively. Each data point represents the mean ± S.D. ($n = 6$). *$p < 0.002$, **$p < 0.006$, ***$p < 0.00001$: for cells treated with vs. without a kinase inhibitor.

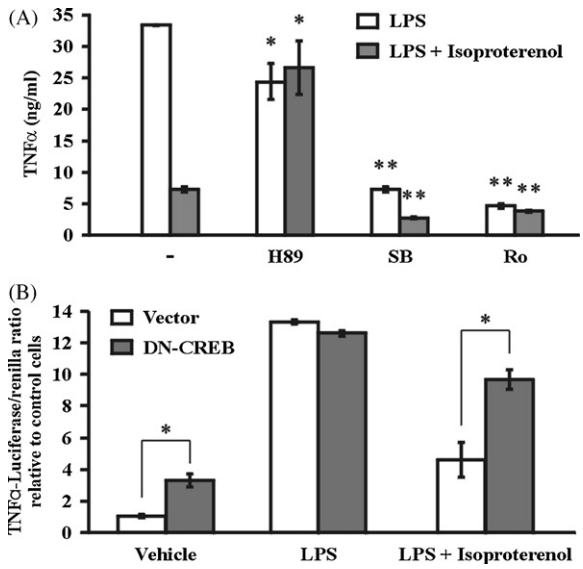

Fig. 7. The PKA–CREB pathway mediates the effect of isoproterenol, but not of LPS, on TNFα production. (A) RAW264.7 macrophages were treated as described in Fig. 6. TNFα was undetectable (lower than 10 pg/ml) in the absence of LPS. *$p < 0.006$, **$p < 0.0002$: for cells treated with vs. without a kinase inhibitor. (B) RAW264.7 macrophages were transiently transfected for 24 h at 37 °C with a reporter gene construct which codes for firefly luciferase under the regulation of the full TNFα promoter, a renilla luciferase construct for normalization, and either a dominant negative S133A CREB (DN-CREB, solid bars) or a control vector (open bars). The cells were washed and incubated with LPS (100 ng/ml), in the presence or absence of isoproterenol (1 μM), for 3 h at 37 °C. Luciferase activity assay was performed as described under Section 2. Each data point represents mean ± S.D. ($n = 2$) of values normalized against renilla luciferase activity, and relative to unstimulated vector-transfected control cells. *$p < 0.02$ for cells transfected with DN-CREB vs. control vector.

act synergistically to induce IL-10. Strikingly, inhibitors of either PKA, or p38 or MSK1 completely blocked the synergistic effect of isoproterenol on LPS-induced IL-10 expression (Fig. 6A). However, when the macrophages were stimulated with LPS alone (in the absence of isoproterenol), only the specific p38 and MSK1 inhibitors completely blocked IL-10 expression, whereas the PKA inhibitor H89 just modestly inhibited it (Fig. 6B). Taken together, the results shown in Fig. 6A and B strongly suggest that both PKA and p38–MSK1 pathways are required for IL-10 induction by the combination of LPS and isoproterenol, whereas only the p38–MSK1 is active in macrophages stimulated with LPS alone in the absence of isoproterenol. The small effect of H89 on LPS-stimulated IL-10 expression (28% inhibition) can be attributed to partial MSK1 inhibition (Caivano and Cohen, 2000).

Production of the key pro-inflammatory cytokine TNFα was also measured in the RAW264.7 macrophages which were pre-treated with kinase inhibitors and then stimulated with LPS, in the presence or absence of isoproterenol. We found that TNFα expression in macrophages stimulated with LPS alone (in the absence of isoproterenol), was severely impaired by the specific p38 and MSK1 inhibitors, but just modestly inhibited by the PKA inhibitor H89 (Fig. 7A). Thus, the results presented in Figs. 6 and 7A suggest that induction of both TNFα and IL-10 in LPS-stimulated cells (in the absence of isoproterenol) is mediated by the p38–MSK1 pathway and not by the PKA pathway. Treatment of the LPS-stimulated macrophages with isoproterenol suppressed 80% of TNFα production (Fig. 7A). In these cells, pre-incubation with the specific p38 and MSK1 inhibitors further inhibited TNFα production, but in contrast, pre-incubation with the PKA inhibitor H89 reversed the suppressive effect of isoproterenol on TNFα production (20% rather than 80% reduction, Fig. 7A). Importantly, in the presence of H89, LPS-induced TNFα production was not modulated by isoproterenol (Fig. 7A). Thus, these results suggest that PKA mediates the suppressive effect of isoproterenol on LPS-induced TNFα production.

To further examine the role of CREB in LPS-stimulated macrophages, we transiently transfected RAW264.7 cells with an expression vector for dominant negative (S133A) CREB, together with a reporter gene construct which codes for luciferase under the regulation of the full TNFα promoter. The dominant negative CREB was quantitatively expressed in the cells that were actually transfected with the luciferase construct, as judged by the complete blocking of isoproterenol-induced CRE-luciferase activity (data not shown). Fig. 7B shows that the dominant negative CREB partially reversed the suppressive effect of isoproterenol on LPS-induced TNFα production, but it had no significant effect on the activity of LPS in the absence of isoproterenol. Thus, the dominant negative CREB data reinforce the conclusion drawn from the kinase inhibitors study, namely that CREB mediates the suppressive effect of isoproterenol on LPS-induced TNFα production, whereas it is inactive in LPS-stimulated macrophages in the absence of isoproterenol.

4. Discussion

The role of CREB in LPS signaling has been poorly defined. The results presented here indicate that LPS stimulates MSK1-mediated phosphorylation, but not activation, of CREB. Accordingly, CREB appears not to be involved in TNFα and IL-10 production in LPS-stimulated macrophages. Yet, cAMP inducers such as β-AR agonists suppress TNFα production and elevate IL-10 production via PKA and CREB.

We have demonstrated here that isoproterenol sequentially activates the signaling components of the cAMP pathway, as it elevates cAMP level, induces CREB phosphorylation and up-regulates CRE-dependent reporter transcription. In addition, its effect on TNFα reporter activity is sensitive to pharmacologic

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(PKA inhibitor) and molecular (dominant negative CREB expression) inhibition of the pathway. In contrast, stimulation of the RAW264.7 macrophages with LPS yields apparently conflicting consequences regarding activation of signaling components along the cAMP pathway. We found that LPS: (i) modestly elevates cAMP level with a delay (compared to isoproterenol), (ii) induces CREB phosphorylation, and (iii) does not effect CRE-dependent reporter transcription. In addition, pharmacologic and molecular inhibition of the PKA–CREB pathway does not affect LPS-induced TNFα reporter activity.

LPS treatment of RAW264.7 macrophages leads to a modest but sustained increase in intra-cellular cAMP level, evident only from 30 min stimulation. This is in agreement with previous reports that were unable to detect an LPS effect on cAMP production during a short time scale normally used for stimulation by GPCR ligands (Avni et al., 2010; Greten et al., 1996), as well as with other reports that have observed a cAMP increase in RAW264.7 macrophages, following very long periods (4 or 6 h) of LPS stimulation (Chen et al., 1999; Osawa et al., 2006). The cAMP increase was attributed in one case to LPS-induced sensitization of AC to an external cAMP elevating agent (Osawa et al., 2006), or in the second case to an autocrine activity of PGE2 which was produced and released following several hours of LPS stimulation (Chen et al., 1999). Inversely, macrophages pre-exposed to LPS for 1 h produced less cAMP in response to PGE2 (compared to macrophages that have not been pre-treated with LPS), due to LPS-induced up-regulation of phosphodiesterase activity (Okonogi et al., 1991). Thus, LPS can modulate long-term cAMP production in macrophages in either a positive or negative manner, possibly depending on the absence or presence of a GPCR ligand as a co-stimulus, but it does not affect short-term cAMP formation.

The ability of LPS to elevate cAMP following 30 min incubation is in apparent conflict with the inability of LPS to activate the PKA–CREB pathway, as realized from analysis of the CRE-reporter and cytokine measurements. However, the pattern of cAMP formation in response to LPS can be described as a modest elevation of basal cAMP level, rather than a sharp peak of the type observed in response to GPCR ligands (Fig. 1). Thus, these findings insinuate that CREB activation via PKA cannot be achieved by a low and sustained cAMP increase. Alternatively, the timing of the cAMP increase may be critical for transcriptional activity of CREB. Accordingly, an immediate cAMP peak, as elicited by GPCR ligands, but not by LPS, may be essential for CREB activation.

cAMP-independent phosphorylation of CREB by LPS can be accomplished by LPS-induced activation of a CREB kinase other than PKA, as previously demonstrated in macrophages (Bradley et al., 2003; Caivano and Cohen, 2000; Eliopoulos et al., 2002; Gorgoni et al., 2002) to occur via a p38-activated kinase (Bradley et al., 2003; Caivano and Cohen, 2000; Eliopoulos et al., 2002), such as MSK1 (Caivano and Cohen, 2000; Eliopoulos et al., 2002). Indeed we found that LPS dramatically activated p38 and MSK1, and that specific p38 and MSK1 inhibitors blocked LPS-induced phosphorylation of CREB. In contrast, when stimulated by isoproterenol, the same phosphorylation event was most efficiently blocked by H89, while the specific p38 and MSK1 inhibitors had a modest effect, consistent with the ability of isoproterenol to modestly activate the p38 pathway, in addition to the PKA pathway (Goldsmith et al., 2009a). In particular, the effect of the specific MSK1 inhibitor on CREB phosphorylation by LPS and isoproterenol sharply distinguishes between the mechanisms (Fig. 4). These findings demonstrate that although H89 can inhibit the activity of both PKA and MSK1, the usage in parallel of H89 and a specific MSK1 inhibitor, enabled the assignment of CREB kinase activity to MSK1 in LPS-stimulated cells, and to PKA (at large) in isoproterenol-stimulated cells.

While in both stimuli CREB is phosphorylated on Ser-133, the lack of effect of LPS on CRE-dependent reporter gene activation contrasts with the clear effect of isoproterenol, indicating that phosphorylation of CREB on Ser-133 is not sufficient for its activation, which further depends on the identity of the kinase. These results are supported by the elegant experiments of Montminy and coworkers, demonstrating stimulus-specific activity of CREB in HEK293, PC12 and D5 cells (Mayr et al., 2001; Wagner et al., 2000). It was shown that both PKA and PKC phosphorylate CREB on Ser-133 to a similar extent; yet only PKA activity results in interaction of CREB with CREB binding protein (CBP) and subsequent CRE-dependent transcription (Mayr et al., 2001; Wagner et al., 2000). Thus, it was proposed that in addition to phosphorylation of CREB on Ser-133, a second PKA-mediated event is required to allow CREB to activate transcription (Johannessen et al., 2004; Mayr et al., 2001). We infer that the differential outcome of CREB Ser-133 phosphorylation by MSK1-activated LPS and PKA-activated isoproterenol may be similarly rationalized.

We found that IL-10 was synergistically induced by LPS and isoproterenol (Fig. 6A), consistently with our previous report showing that activation of CREB by a cAMP inducer, or by a cell-permeable cAMP analog, in the background of LPS stimulation, leads to synergistic transcriptional activation of the IL-10 promoter (Goldsmith et al., 2009a). The usage of PKA, p38 and MSK1 inhibitors showed that the synergistic effect on IL-10 production was PKA-dependent as well as p38–MSK1-dependent (Fig. 6A). Interestingly, in the absence of isoproterenol, LPS-induced IL-10 expression was fully blocked by the specific p38 and MSK1 inhibitors, but was only modestly inhibited by the PKA and MSK1 inhibitor H89 (Fig. 6B). The differential effect of H89 in the presence and absence of isoproterenol suggests that when IL-10 is induced by LPS alone, the partial effect of H89 is attributed to MSK1 inhibition. The critical dependency of LPS-induced IL-10 expression on the p38 pathway has been previously suggested to be mediated by the transcription factor Sp1 (Brightbill et al., 2000; Ma et al., 2001). Our findings further suggest that activation of the p38–MSK1 pathway by LPS and activation of the PKA–CREB pathway by a cAMP inducer (e.g. the β-AR agonist isoproterenol) are minimally effective for IL-10 expression unless simultaneously occurring. The analysis of IL-10 induction, taken together with the data regarding CREB phosphorylation and activation discussed above, strongly suggests that CREB is inactive at the IL-10 promoter in the presence of LPS alone, that MSK1 is required in a CREB-independent manner for the minimal LPS-induced IL-10 expression, and that activation of CREB via the cAMP–PKA pathway (by a cAMP inducer such as a β-AR agonist) is essential for efficient IL-10 induction in LPS-stimulated macrophages.

The role of CREB in transcription of TNFα has been poorly defined. On the one hand, cAMP inducers negatively regulate TNFα expression (Kast, 2000), suggesting that cAMP-activated CREB is also a negative regulator of the process. Several reports suggested that stimulation of mouse RAW264.7 macrophages (Delgado et al., 1998; Leceta et al., 2000; Pozo et al., 2000) or human THP-1 monocytes (Yao et al., 1997) with LPS leads to the displacement of CREB by activated c-Jun at the TNFα CRE site. Additionally in these studies (Delgado et al., 1998; Leceta et al., 2000; Pozo et al., 2000), cAMP inducers were reported to suppress TNFα transcription by displacement of LPS-activated c-Jun with cAMP-activated CREB. Finally, deletion of CRE at the TNFα promoter resulted in an increase of basal TNFα reporter activity, insinuating that CREB has a repressive role in unstimulated cells (Diaz and Lopez-Berestein, 2000). On the other hand, substitution or deletion mutations of the TNFα CRE site have a negative impact on reporter activity in stimulated mouse RAW264.7 macrophages (O'Donnell and Taffet, 2002; Roach et al., 2005) or human THP-1 monocytes (Diaz and Lopez-Berestein, 2000), suggestive of a possible positive role played by CREB in TNFα transcription.

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The evidence shown here regarding the activating vs. non-functional phosphorylation of CREB by isoproterenol and LPS, respectively, suggest that isoproterenol-activated CREB negatively regulates TNFα production, while CREB is inactive during LPS stimulation. This conclusion is also supported by the finding that PKA inhibition by H89 reversed the negative effect of isoproterenol on TNFα production, but distinctively did not have such a positive impact on LPS-induced TNFα production in the absence of isoproterenol (Fig. 7A). LPS-induced TNFα expression critically depended on the p38–MSK1 pathway, but was only modestly compromised by H89, probably due to the ability of H89 to inhibit MSK1 to some extent. Moreover, similar TNFα production was observed in LPS-stimulated cells pre-incubated with H89, whether isoproterenol was present or absent. This finding supports the conclusion that isoproterenol-induced activation of CREB is completely blocked by H89, whereas CREB is inactive in the presence of LPS alone. Finally, over-expression of a dominant negative CREB mutant had no measurable effect on LPS-induced TNFα reporter activity in the absence of isoproterenol, but it profoundly blocked the suppressive effect of isoproterenol (Fig. 7B). Collectively, our findings strongly suggest that the p38–MSK1 pathway is essential for LPS-induced TNFα expression, whereas the PKA–CREB pathway, only when activated by an appropriate GPCR ligand, is a negative regulator of this process. Interestingly, the dominant negative CREB mutant significantly elevated TNFα reporter expression in unstimulated cells (Fig. 7B). This finding is consistent with a suppressive role for CREB (with only basal activation) in unstimulated cells, as inferred also from deletion of CRE at the TNFα promoter (Diaz and Lopez-Berestein, 2000). The disappearance of the positive effect of dominant negative CREB upon LPS stimulation (Fig. 7B) is consistent with the reports of Delgado et al. (1998), Leceta et al. (2000) and Pozo et al. (2000) that used the CRE sequence from the TNFα promoter in EMSA studies to demonstrate binding of CREB to the DNA in unstimulated RAW264.7 macrophages and its replacement by c-Jun upon LPS stimulation. Together with our findings the inferred conclusion is that CREB has no role in TNFα expression in LPS-stimulated cells in the absence of an agonist which activates the cAMP pathway.

Phosphorylation of CREB by MSK1 in LPS-stimulated RAW264.7 and bone marrow-derived macrophages was found to be correlated with transcriptional activation of the COX-2 promoter which includes a CRE critical for transcription (Caivano and Cohen, 2000; Eliopoulos et al., 2002). However, the activity of CREB was not measured in these reports and it was therefore noted that other transcription factors may be accountable for the CRE-mediated effects. Indeed, Wadleigh et al. (2000) showed that c-Jun contributed to LPS-induced COX-2 expression in RAW264.7 macrophages whereas over-expression of CREB actually blocked COX-2 reporter activity in these cells. As for the TNFα CRE site (Delgado et al., 1998; Leceta et al., 2000; Pozo et al., 2000), competition on enhancer binding was suggested in this case as well, based on the ability of c-Jun to bind the CRE site in the COX-2 promoter (Xie and Herschman, 1995). These reports are thus in agreement with our direct observation which reveals that phosphorylation of endogenous CREB in LPS-stimulated RAW264.7 macrophages is not correlated with its activity as a transcription modulator. Specifically, phosphorylation of CREB by a cAMP-mediated stimulus leads to its activation, whereas LPS-induced phosphorylation via MSK1 is not functional.

5. Concluding note

We have demonstrated here that the outcome of CREB phosphorylation on Ser-133 is context-dependent, as isoproterenol both phosphorylated and activated CREB in a cAMP-dependent manner, whereas LPS phosphorylated CREB in a cAMP-independent manner, which bears no transcriptional activity. The p38–MSK1 pathway mediates high expression of TNFα and only minimal expression of IL-10 in LPS-stimulated RAW264.7 macrophages, in a CREB-independent manner. Agonists of the β-AR activate the cAMP–PKA–CREB pathway, and divert LPS-stimulated macrophages to an anti-inflammatory direction, by reciprocal regulation of TNFα and IL-10 expression.

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