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PTPε Represses LPS-Mediated TNF-α Induction in RAW264.7 Cells by Inducing Dephosphorylation of p38

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Protein phosphorylation is a critical event in signal transduction pathways that regulate fundamental cellular processes such as differentiation, cell proliferation, apoptosis, immunological signaling, and cytoskeletal function.[1] Protein phosphorylation is regulated by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), and provides an important means of regulating protein function. The regulated activity and expression of PTPs in different cells control mitogen-activated protein kinase (MAPK) intensity and duration to determine the type of physiological response. In the immune system, a dynamic equilibrium between PTPs and PTKs regulates responses in both positive and negative ways, and PTP-deficient mice have been used to identify individual PTPs as key regulators of immune responses. In lipopolysaccharide (LPS)-stimulated RAW264.7 cells, DUSP1 (MKP-1) induction is associated with inactivation of c-Jun N-terminal kinase (JNK) and p38. Furthermore, *Dusp1*$^{-/-}$ macrophages have elevated p38 and JNK activities but no change in extracellular signal-regulated kinase (ERK) activity.[2,3] Four isoforms of PTPε (also named PTPRE) are generated from a single gene: a transmembrane form (RPTPε) and three cytoplasmic isoforms (cyt-PTPε, p67, and p65).[4-9] *Ptpre*$^{-/-}$ mice exhibited enhanced FcepsilonRI-induced Ca^{2+} mobilization and JNK and p38 activation.[10] The extracellular signal-regulated kinases (ERK1/2) were shown to be inhibited in both kinase activity and their phosphorylation status after overexpression of PTPε. In addition, ERK phosphorylation is increased in mouse fibroblasts and in mammary tumor cells lacking PTPε.[11,12]

Tumor necrosis factor-α (TNF-α) is a major cytokine that induces inflammation and becomes a therapeutic target in inflammation diseases.[13] The regulation site for TNF-α expression is an adenosine/uridine-rich element (ARE) residing in the 3' untranslated region of the TNF-α mRNA that represses TNF-α expression post-transcriptionally.[14,15] MAP kinases, including p38, JNK, and ERK, have been shown to target this ARE to increase TNF-α biosynthesis in response to LPS stimulation.[16]

In the present study, we studied the effect of MAPK inhibition and PTPε overexpression on TNF-α induction. To examine the effect of PTPε on the production of proinflammatory cytokines, a mammalian vector expressing FLAG-tagged PTPε was transfected into RAW264.7 cells. The effect of increased PTPε expression on the production of TNF-α was examined by ELISA. Compared with cells transfected with an empty plasmid, FLAG-PTPε exhibited a profoundly attenuated response to LPS stimulation in a dose-dependent manner, as shown by the 90% reduction in TNF-α secretion (Fig. 1). This result suggests that PTPε expression in macrophages inhibits LPS-stimulated TNF-α production.

In LPS-stimulated RAW264.7 cells, DUSP1 protein expression increased dramatically, reaching its maximal level between 1 and 2 h, and then decreased slightly (Fig. 2A).[17] Previously, it has been reported that PTPε mRNA level in peritoneal macrophages was slightly increased by LPS.[8] PTPε mRNA from RAW264.7 cells treated with LPS was examined by RT-PCR to investigate whether gene expression of PTPε is induced by LPS. PTPε mRNA level was unchanged in RAW 264.7 cells treated with LPS (Fig. 2A). Likewise, PTPε protein level was also unchanged in RAW 264.7 cell lysates (Fig. 2B). These results indicate that PTPε expression is not significantly regulated by LPS.

To further understand the relationship between PTPε induction and MAP kinase inactivation, we assessed the endogenous levels of phospho-JNK, phospho-p38, and phospho-ERK1/2. RAW264.7 cells were transiently transfected with FLAG-tagged PTPε expression plasmids or empty plasmids. After LPS treatment, the levels of phospho-JNK, phospho-p38, and phospho-

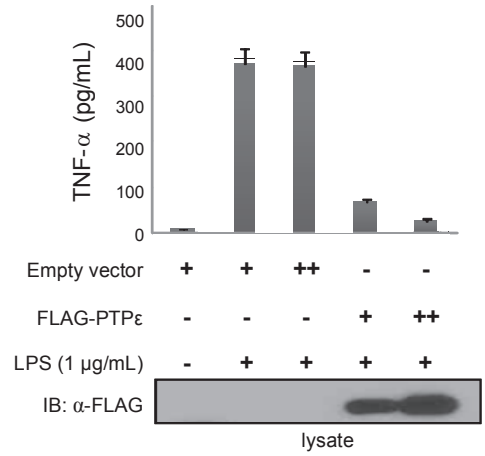

Figure 1. Overexpression of PTPε in macrophages inhibits LPS-stimulated TNF-α production. RAW264.7 cells were transfected with either the empty vector or the construct expressing FLAG-PTPε. After 16 h stimulation with LPS (1 μg/mL), supernatants were analyzed for TNF-α production using an ELISA assay as described in the Experimental Section. Cell lysates were subjected to immunoblotting using an anti-FLAG antibody for detection of PTPε. The results presented are representative of three independent experiments. Error bars indicate ±SEM.

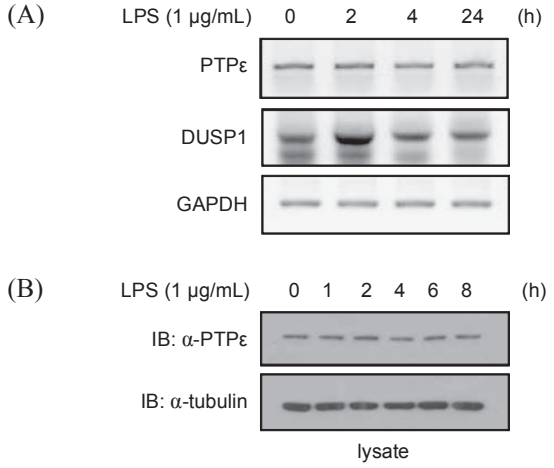

Figure 2. PTPε expression in RAW264.7 cells is not induced after treatment with LPS. (A) Expression levels of PTPε mRNA in RAW264.7 cells were determined at the indicated times after treatment of LPS (1 μg/mL) by RT-PCR. GAPDH transcripts were amplified using specific primers (forward 5'-ACCACCATGGAGAAGGC-3'; reverse 5'-CTCAGTGTAGCCCAGG ATGC-3') as a control for RNA integrity. DUSP1 transcripts were amplified and used as a control for LPS treatment. (B) Cells were treated with LPS (1 μg/mL) for the indicated periods and were harvested. PTPε protein was detected by immunoblotting analysis using an anti-PTPε antibody. Equal protein loading was evidenced by the anti-tubulin antibody.

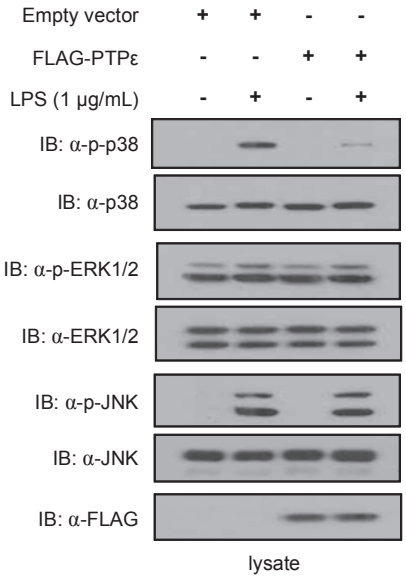

Figure 3. Effects of PTPε on LPS-mediated MAPK signal transduction. PTPε expression plasmid was transfected into RAW264.7 cells and then cells were stimulated with LPS (1 μg/mL) for 1 h. Immunoblottings were performed for total and phosphorylated proteins as indicated. Three independent experiments were performed with similar results.

ERK1/2 were determined by immunoblotting analysis. Levels of phospho-JNK, phospho-p38, and phospho-ERK1/2 were significantly enhanced by LPS. Of three MAPKs, only phospho-p38 was reduced in cells transfected with PTPε expression plasmid compared with cells transfected with empty plasmid, while the protein level of p38 was unchanged (Fig. 3). Taken together, these results suggest that PTPε induces dephosphorylation and thus inactivation of p38 but not JNK and ERK1/2.

In conclusion, the results of this study suggest that PTPε acts as a negative regulator in the inflammatory response of macrophages. PTPε inhibits TNF-α production in LPS-stimulated RAW264.7 cells *via* inactivation of p38.

Experimental Section

Cell culture and transfection. The murine macrophage RAW 264.7 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad California) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin/streptomycin in the presence of 5% CO_2. For transient transfection, 1.4×10^6 cells were plated in each 60 mm cell culture plate, grown overnight, and transfected with DNA using FuGENE HD transfection reagent (Roche, Basel, Switzerland).

Plasmid constructs. The N-terminal FLAG-tagged PTPε for expression in mammalian cells was constructed by polymerase chain reaction (PCR), followed by cloning into the pcDNA3.1/Zeo plasmid.

Reagents and antibodies. Polyclonal anti-JNK, anti-phospho-JNK (Thr-183/Tyr-185), anti-p38, anti-phospho-p38 (Thr-180/Tyr-182), anti-ERK1/2, and anti-phospho-ERK1/2 (Thr-202/Tyr-204) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-FLAG M2, anti-tubulin antibodies, and LPS were from Sigma-Aldrich (St. Louis, MO). Anti-PTPε antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

TNF-α ELISA. TNF-α protein concentrations were determined by standard sandwich ELISA using antibodies and standards obtained from BD Biosciences (San Diego, CA) and used according to manufacturer's instructions. Assays were performed on neat and diluted samples in duplicate on 96-well plates. Absorbance was measured by a microplate reader at 450 nm and concentrations were determined by comparison to a standard curve. All transfections were repeated at least three times.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNAs were prepared from cells by Trizol (Roche) and reverse transcription was performed using M-MLV Reverse Transcriptase (Invitrogen). PCR for mouse PTPε cDNA was carried out using the following primers: forward 5'-CATTG TGATCGATGCCATGATG-3' and reverse 5'-GTTGCCCGT CCTCATGTTCTC-3'.

Immunoblotting analysis. After RAW264.7 cells were transiently transfected with FLAG-tagged PTPε expression plasmids or empty plasmids for 48 h, cells were washed twice with phosphate buffered saline (PBS) buffer and lysed in PTP lysis buffer (0.5% NP-40, 0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL Aprotinin) for 30 min at 4 °C. Cleared cell lysates from centrifugation were resuspended with protein sample buffer, boiled at 100 °C for 5 min, subjected to SDS-PAGE, and subsequently transferred onto nitrocellulose membrane. Immunoblotting was carried out as previously described.[18]

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