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# MKP-1 regulates cytokine mRNA stability through selectively modulation subcellular translocation of AUF1

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ABSTRACT

MAPK phosphatase-1 (MKP-1)/dual specificity protein phosphatase-1 (DUSP-1) is a negative regulator of the host inflammatory response to infection. However, the mechanisms underlying the regulation of cytokine expression by MKP-1, especially at the post-transcriptional level, have not been fully delineated. In the current study, MKP-1 specifically dephosphorylated activated MAPK responses and attenuated LPS-induced IL-6, IL-10, and TNF-α expression. In addition, MKP-1 was important in destabilizing cytokine mRNAs. In LPS-stimulated rat macrophages with overexpressed MKP-1, half-lives of IL-6, IL-10 and TNF-α mRNAs were significantly reduced compared to controls. Conversely, half-lives of IL-6, IL-10, and TNF-α mRNAs were significantly increased in bone marrow macrophages derived from MKP-1 knock out (KO) mice compared with macrophages derived from MKP-1 wild type (WT) mice. Furthermore, MKP-1 promoted translocation of RNA-binding protein (RNA-BP) ARE/poly-(U) binding degradation factor 1 (AUF1) from the nucleus to the cytoplasm in response to LPS stimulation as evidenced by Western blot and immunofluorescent staining. Knockdown AUF1 mRNA expression by AUF1 siRNA in MKP-1 WT bone marrow macrophages significantly delayed degradation of IL-6, IL-10 and TNF- α mRNAs compared with controls. Finally, AUF1 was immunoprecipitated with the RNA complex in cellular lysates derived from bone marrow macrophages of MKP-1 KO vs. WT mice, which had increased AUF1-bound target mRNAs, including IL-6, IL-10, and TNF-α in WT macrophages compared with MKP-1 KO macrophages. Thus, this work provides new mechanistic insight of MKP-1 signaling and regulation of cytokine mRNA stability through RNA binding proteins in response to inflammatory stimuli.



## 1. Introduction

MAPK pathways are essential immune response signaling pathways in mammalian cells that respond to external stress stimuli, growth factors, or infectious microorganisms [1,2]. There are three well-known subfamilies of MAPKs, including ERK, JNK, and p38 MAPK [3]. Numerous studies have revealed that ERK is mainly activated by growth factors and hormones, whereas JNK and p38 MAPK are activated by stress stimuli; therefore, JNK and p38 MAPK are also referred to as stress-activated kinases (SAPKs) [1]. Activation of the MAPK cascade initiates phosphorylation of downstream proteins including numerous transcription factors such as AP-1, CREB, NF-κB, STAT, nuclear hormone receptors, and nucleosomal proteins [1,2]. Thus, activation of MAPKs leads to production of various pro-inflammatory/or anti-inflammatory cytokines in response to stress stimuli. MAPKs play an essential role in the regulation of many physiological processes, including cell proliferation, differentiation, development, immune response, stress responses, and apoptosis [4].

Although activation of the MAPKs pathways is critical for mounting an aggressive immune response to eliminate invading pathogens and neutralize external insults, an exaggerated MAPK response can be deleterious to the host. In fact, an exaggerated MAPK response is associated with the pathophysiology of various human diseases, such as septic shock [5], autoimmune diseases [6], bone resorption [7], neurodegenerative diseases, and cancer [8,9]. Therefore, regulation of both the duration and magnitude

*Abbreviations:* MKP-1, MAPK phosphatase 1; DUSP1, dual specificity protein phosphatase 1; ARE, adenine and uridine (AU)-rich elements; RNA-BP, RNA-binding protein; AUF1, ARE/poly-(U) binding degradation factor 1; TTP, tristetraprolin; SAPK, stress-activated kinase; MOI, multiplicity of infection; MK2, MAPK-activated protein kinase 2; RIP, RNA-binding protein immunoprecipitation.

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of MAPK activation is necessary to maintain immune homeostasis and prevent harmful effects associated with an exaggerated MAPK response.

In mammalian cells, MAPKs are primarily inactivated by a group of dual-specificity protein phosphatases (DUSPs) called MAPK phosphatases (MKPs), which dephosphorylate both the tyrosine and threonine residues of activated MAPKs [3]. MKP-1/DUSP1 is the first mammalian MKP identified and it is induced by various stress stimuli, such as bacterial infection, UV light, hypoxia, heat shock, and oxidants [3]. Studies in MKP-1 KO mice revealed that MKP-1 KO mice were susceptible to endotoxic shock; the mice had increased production of proinflammamtory cytokines [10,11] and increases in the incidence and severity of experimentally induced autoimmune arthritis [12]. Also, the mice were more sensitive to hydrogen-peroxide-induced apoptosis [13], and they remained susceptible to the development of glucose intolerance and hyperinsulinemia caused by high-fat feeding [14] compared with wild type (WT) animals. These results suggest a critical role for MKP-1 in negative regulation of innate immune response to bacterial infection and modulation of cellular growth, apoptosis, and metabolism. However, the mechanisms underlying how MKP-1 restrains immune responses have not been fully defined, especially at the post-transcriptional level.

It is well-known that many short-lived mRNAs, including mRNA encoding cytokines, proto-oncogenes, growth factors, transcriptional activators, cell cycle regulatory proteins, and various inflammatory mediators carry adenine and uridine (AU)-rich elements (AREs) in the 3′-untranslated region (3′-UTR) [15–19]. Some RNA-binding proteins (RNA-BPs) specifically bind with those ARE regions of mRNAs. RNA-BPs shuttle between the cytoplasm and the nucleus, contributing to the regulation of RNA splicing, export, surveillance, decay, and translation [20]. Two RNA-BPs that specifically bind to the defined ARE region of mRNAs to mediate their degradation are AUF1 and tristetraprolin (TTP) [21,22].

Understanding the molecular mechanisms of MKP-1 regulation of cytokine response is critical for understanding innate immunity and mechanisms of immune tolerance. Using gain-or loss-of-function strategies, we evaluated the role of MKP-1 in regulation of MAPK protein and cytokine expression, cytokine mRNA and cytokine mRNA stability, and its relationship with RNA-BPs. The present study provides new evidence that MKP-1 regulates cytokine expression post-transcriptionally by altering nuclear export of AUF1.

## 2. Materials and methods

### 2.1. Cells and reagents

Rat macrophage NR8383 cells and mouse fibroblast L929 cells were obtained from American Type Cell Collection (ATCC, Manassas, VA). NR8383 cells were cultured with Ham's F12K medium (ATCC), supplemented with 15% heat inactivated FBS, 100 unit/ml penicillin, and 100 μg/ml streptomycin in a 37 °C incubator with 5% $CO_2$. The human embryonic kidney (HEK) 293A cell line was purchased from Invitrogen (Carlsbad, CA). L929 and 293A cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 37 °C incubator with 5% $CO_2$. L929 supernatant, a source of M-CSF, was harvested from L929 cells grown for 7 days after confluency. LPS from *Escherichia coli* 0127:B8 (L4516) was purchased from Sigma Aldrich (St. Louis, MO) and diluted in PBS. Actinomycin D was obtained from CalBiochem (San Diego, CA). Mouse Accell SMARTpool AUF1 siRNA (E-042940-00) and control Accell Non-targeting Pool siRNA (D-001910-10) were obtained from Dharmacon (Lafayette, CO).

### 2.2. Animals and primary cells

MKP-1 KO and WT mice were obtained through a Material Transfer Agreement from Bristol-Myers Squibb (NY) and bred at the Medical University of South Carolina (MUSC). Animal genotype was confirmed by animal DNA genotype PCR assays. All animal-related work was performed in accordance with NIH guidelines, and protocols were approved by the MUSC Institutional Animal Care and Use Committee. Bone marrow cells were obtained by flushing the femur and tibia marrow cavities of 6–8-week-old mice. Isolated bone marrow cells were cultured in DMEM with 10% FBS, 30% L929 supernatant (a supplement of M-CSF), and antibiotics for 7 days to induce the formation of bone marrow macrophages.

### 2.3. Adenovirus

Adenovirus serotype 5 (Ad5)-CMV-MKP-1, which expresses the full-length of MKP-1 gene, and control Ad5-CMV-LacZ were obtained from Seven Hills Bioreagents (Cincinnati, OH). Adenovirus were propagated in HEK 293A cells, purified by cesium chloride density gradient ultracentrifugation method, and desalted by PD-10 column (GE Healthcare, Pittsburgh, PA) in HEPES buffered saline.

### 2.4. Western blot assays

Total proteins were extracted by RIPA buffer (Cell Signaling Technology, Beverly, MA) supplemented with proteinase inhibitor cocktail and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The nuclear and cytoplasmic proteins were extracted by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instruction. Proteins were loaded on 10% or 12% Tris–HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA) and electrotransferred to nitrocellulose membranes, blocked in 5% milk, and then incubated overnight at 4 °C with primary antibodies. Anti-MKP-1 (M-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to p-p38 MAPK; p-SAPK/JNK; p-p44/42 MAPK (Erk1/2); p38 MAPK, p-NF-κB p65, Lamin A/C, and GAPDH were purchased from Cell Signaling Technology. Anti-AUF1 was obtained from (Millipore, Billerica, MA). The anti-TTP was obtained from Abcam (Cambridge, MA). The presence of the primary antibodies was detected on radiographic film by using HRP-conjugated secondary antibodies (Cell Signaling Technology) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL). Digitalized images of the radiographic films were obtained in a gel documentation system (Bio-Rad Laboratories).

### 2.5. ELISA

IL-6, IL-10, TNF-α, IL-1β, CXCL1, M-CSF, IFN-γ and a $PGE_2$ parameter assay ELISA kits were purchased from R&D systems (Minneapolis, MN). Protein concentration in cell lysates was determined by DC protein Assay Kit (Bio-Rad Laboratories). The concentration of cytokines in cell culture supernatant was normalized by protein concentrations in cell lysates.

### 2.6. RT-qPCR

Total RNA was isolated from cells using TRIZOL (Invitrogen) according to the manufacturer's instructions and quantified by a spectrophotometer. Complementary DNA was synthesized by a TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA) using 500 ng of total RNA. Real time PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems) condi-