Cytokine 56 (2011) 245–255

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

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

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ARTICLE INFO

Article history:
Received 30 December 2010
Received in revised form 2 May 2011
Accepted 6 June 2011
Available online 5 July 2011

Keywords:
MKP-1
AUF1
TTP
Cytokine
mRNA

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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doi:10.1016/j.cyto.2011.06.006

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-

tions used as follows: 50 °C for 2 min, 95 °C for 10 min, and 50 cycles of 95 °C for 15 s, 60 °C for 1 min. Amplicon primers of rat IL-6 (Rn01410330_m_1), rat TNF-α (Rn99999017_m_1), rat IL-10 (Rn99999012_m_1), rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rn01775763_g_1), mouse IL-6 (Mm00446190_m_1), mouse TNF-α (Mm00443258_m_1), mouse IL-10 (Mm00 439615_g_1), mouse AUF1 (Mm01201314_m1), mouse beta-actin (Mm01205647_g1), and mouse GAPDH (Mm99999915_g_1) were obtained from Applied Biosystems. Amplicon concentration was determined using threshold cycle values compared with standard curves for each primer. IL-6, TNF-α, IL-10, and AUF1 mRNA levels were normalized to endogenous control beta-actin or GAPDH expression and expressed as fold change compared with control groups. The half-life ($t_{½}$) of mRNA was determined as previously described [23].

2.7. Immunofluorescence

Bone marrow derived macrophages were fixed with 2% paraformaldehyde for 10 min at room temperature (RT). After washing with PBS for three times, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at RT. After washing by PBS, the cells were blocked by 10% goat serum in PBS for 1 h at RT. After a second washing with PBS, the cells were incubated with anti-AUF1 (1:100, Millipore) with 1% BSA overnight at 4 °C. Cells were washed again with PBS, and incubated with an Alexa-488 labeled secondary antibody (1:500, Invitrogen) with 1% BSA for 1 h at RT. After another PBS wash, cells were incubated with Alexa phalloidin 568 (1:10, Invitrogen) for 1 h at RT. Cells were washed again with PBS and mounted on a slide with Prolong Gold anti-fade reagent with DAPI reagent (Invitrogen), covered with coverslip, and incubated at RT overnight. Digital images were recorded using confocal microscopy (Leica, Bannockburn, IL).

2.8. AUF1 RIP-RT-qPCR

RNA-BP immunoprecipitation (RIP) and mRNA isolation was previously described [24]. Briefly, protein A agarose beads (Sigma) were either coated with anti-AUF1 (15 μg) or an equal amount of control normal rabbit serum (Cell Signaling Technology) at 4 °C in a rotating device overnight. After serial washing, cell lysates (2 mg) from bone marrow macrophages were immunoprecipitated with protein A-coated agarose beads overnight at 4 °C in a rotating device. Ten percent cell lysates were reserved as input control. After serial washing, the IP products were digested with proteinase K, and RNA was isolated. RNA was extracted using phenol and chloroform and precipitated in the presence of glycogen. For analysis of individual mRNAs, the RNA isolated from the IP was subjected to reverse transcription (RT) using random hexamers and SuperScriptII reverse transcriptase (Invitrogen). Amplification and quantification of the PCR products were performed using the Applied Biosystems Stepone Plus system (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems). Input beta actin mRNA was used as a loading control. Each reaction was carried out in triplicate and three independent experiments were performed.

2.9. Statistical analysis

Data were analyzed with the paired or unpaired two-tailed Student's *t* test with Welch's correction for unequal variances. All statistical tests were performed using a GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). *P*-values of 0.05 or less were considered significant.

3. Results

3.1. Ad.MKP-1 specifically dephosphorylates activated MAPKs after LPS stimulation

To test the efficacy of exogenous over-expression of MKP-1 by Ad.MKP-1, rat macrophages (NR8383 cells) were transduced with Ad.MKP-1 or control vector, Ad.LacZ, at multiplicity of infection (MOI) from 100 to 900 for 24–72 h. Optimal MKP-1 protein expression was achieved at 300 MOI for 48 h (data not shown). The LacZ gene expression was confirmed by β-galactosidase staining in cells (data not shown). Next, to verify how MKP-1 affects immune signaling pathways, rat macrophages were transduced with either Ad.MKP-1 or Ad.LacZ (300 MOI), or treated with equal volume of HEPES buffered saline. Forty-eight hours after the adenoviral transduction, the cells were stimulated with LPS (1 μg/ml) for 10, 30, 60, and 120 min. Then, MKP-1 protein, p-MAPKs and p-NF-κB p65 were measured by Western blot. As shown in Fig. 1, when nitrocellulose membrane was incubated with a low concentration of anti-MKP-1 (1:1500), rat macrophages transduced with Ad.MKP-1 displayed modest MKP-1 protein expression without LPS stimulation. However, the MKP-1 protein was significantly induced in those transduced cells 60 and 120 min after LPS stimulation. In contrast, rat macrophages transduced with Ad.LacZ or treated with HEPES buffered saline exhibited undetectable MKP-1 protein levels before or after LPS stimulation. When the nitrocellulose membrane was incubated with a higher concentration of anti-MKP-1 (1:500), there was only weak MKP-1 protein expression 60 and 120 min after LPS stimulation in macrophages transduced with Ad.LacZ or treated with HEPES buffered saline (data not shown). Importantly, macrophages transduced with Ad.MKP-1 significantly reduced p-JNK, p-ERK, and p-p38 after LPS stimulation compared with cells transduced with Ad.LacZ or treated with HEPES buffered saline. The enhanced MKP-1 protein expression 60–120 min after LPS stimulation was correlated with significant dephosphorylation of those activated MAPKs in transduced cells. However, macrophages transduced with Ad.MKP-1 failed dephosphorylating activated NF-κB p65, which suggested that Ad.MKP-1 specifically dephosphorylated activated MAPKs, not activated NF-κB signaling pathway.

3.2. MKP-1 selectively attenuates IL-6, TNF-α and IL-10 cytokine production after LPS stimulation

After toll-like receptor (TLR) engagement, LPS triggers a cascade of signal transduction events that lead to the formation of the MyD88/TNF receptor-associated factor-6 (TRAF6) complex, and subsequently triggers activation of NF-κB and MAPKs [25]. Activation of NF-κB and MAPKs, in turn, leads to phosphorylation of transcriptional factors and/or RNA-BPs, which controls the magnitude and duration of many pro-inflammatory/or anti-inflammatory cytokine responses. To investigate the effects of MKP-1 on pro-inflammatory/or anti-inflammatory cytokine expression, rat macrophages were transduced with Ad.MKP-1 or control Ad.LacZ (300 MOI) for 48 h. The cells were subsequently stimulated with LPS (100 ng/ml) for 24 h. IL-6, IL-10, TNF-α, IL-1β, CXCL1, M-CSF, IFN-γ, and PGE_2 in cell culture supernatant were measured with ELISA.

As shown in Fig. 2a–c, rat macrophages transduced with Ad.MKP-1 significantly attenuated IL-6, TNF-α and IL-10 production 24 h after LPS stimulation compared with cells transduced with Ad.LacZ ($n = 5$, $^{*}p < 0.05$, $^{***}p < 0.001$). Compared with macrophages transduced with Ad.LacZ, macrophages transduced with Ad.MKP-1 had an approximately 2.0-fold decrease in IL-6; a 2.0-fold decrease in TNF-α, and a 4.0-fold decrease in IL-10 after LPS stimulation. However, there was no significant difference in

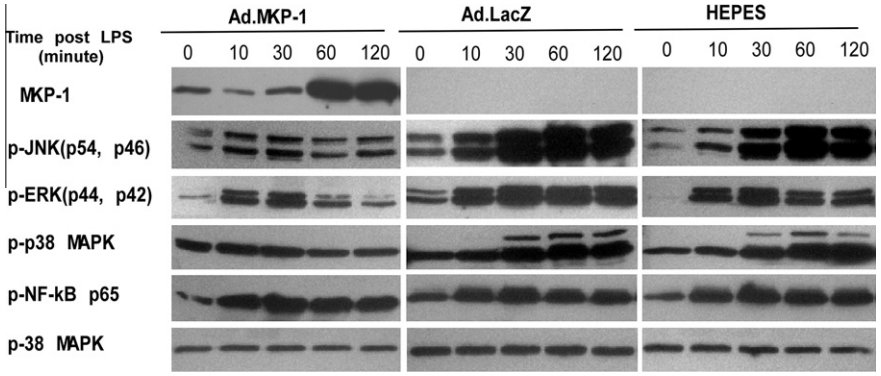

Fig. 1. Ad.MKP-1 specifically dephosphorylated MAPKs in rat macrophages after LPS stimulation. Rat macrophages were transduced with either Ad.MKP-1, or control Ad.LacZ (300 MOI), or treated with HEPES buffered saline for 48 h. Cells were either unstimulated or stimulated with 1 μg/ml of LPS (from *Escherichia coli* 0127:B8) for indicated time periods. MKP-1, p-JNK (p54, p46), p-p38, p-ERK (p44, p42), p-38 MAPK, and p-NF-κB p65 protein expressions were evaluated by Western blot assay. Total p-38 MAPK served as a loading control.

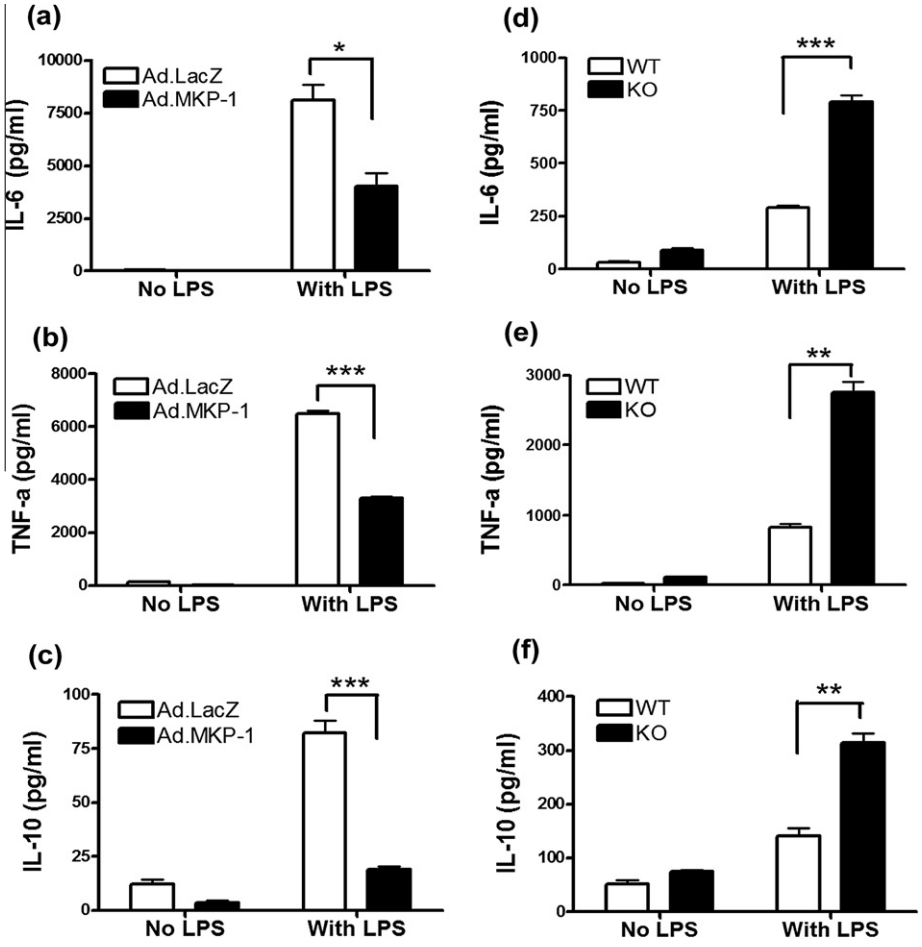

Fig. 2. MKP-1 specifically attenuated IL-6, TNF-α and IL-10 cytokine expression. Rat macrophages were transduced with Ad.MKP-1 or control Ad.LacZ (300 MOI) for 48 h. Primary bone marrow macrophages were either harvested from MKP-1 KO or WT mice. Rat macrophages and bone marrow macrophages from mice were either unstimulated or stimulated with 100 ng/ml of LPS (from *Escherichia coli* 0127:B8) for 24 h. (a) IL-6 expression in rat macrophages ($n = 5$, $^{*}p < 0.05$). (b) TNF-α expression in rat macrophages ($n = 5$, $^{***}p < 0.001$). (c) IL-10 expression in rat macrophages ($n = 5$, $^{***}p < 0.001$). (d) IL-6 expression in mouse macrophages ($n = 3$, $^{***}p < 0.001$). (e) TNF-α expression in mouse macrophages ($n = 3$, $^{**}p < 0.01$). (f) IL-10 expression in mouse macrophages ($n = 3$, $^{**}p < 0.01$). These data are representative of three separate experiments.

IL-1β and CXCL1 expression between these two groups of cells (data not shown). Other cytokines, such as M-CSF, PGE_2, and IFN-γ were below detection levels (data not shown).

To further confirm that MKP-1 regulates IL-6, TNF-α, and IL-10 production, primary bone marrow macrophages from MKP-1 KO or WT mice were either untreated or stimulated with LPS (100 ng/ml) for 24 h. As shown in Fig. 2d–f, bone marrow macrophages from MKP-1 KO mice had a 2.7-fold increase in IL-6; a 3.3-fold increase in TNF-α; and a 2.3-fold increase in IL-10 compared with WT mice ($n = 3$, $^{**}p < 0.01$, $^{***}p < 0.001$). Together, these results confirm that MKP-1 specifically down-regulates IL-6, TNF-α and IL-10 production.

3.3. MKP-1 inhibits IL-6, TNF-α, and IL-10 mRNA levels after LPS stimulation

Activated MAPKs phosphorylate numerous downstream target proteins, including transcriptional factors and RNA-BPs, which can regulate expression of multiple inflammatory genes at either the transcriptional or post-transcriptional level. To delineate the role of MKP-1 in steady state mRNA modulation, mRNA of IL-6, TNF-α, and IL-10 were quantified after LPS stimulation. Rat macrophages transduced by Ad.MKP-1 or control Ad.LacZ (300 MOI) for 48 h were either unstimulated or stimulated with LPS (100 ng/ml) for 4, and 8 h separately. As shown in Fig. 3a–c, mRNA of IL-6, TNF-α, and IL-10 was significantly reduced in macrophages transduced with Ad.MKP-1 compared with mRNA in cells transduced with Ad.LacZ ($n = 4$, $^{**}p < 0.01$, $^{***}p < 0.001$).

To complement gain-of-function experiments, primary bone marrow macrophage cells from MKP-1 KO or WT mice were either untreated or stimulated with LPS (100 ng/ml) for 4 and 8 h separately. As shown in Fig. 3d–f, the mRNA levels of IL-6, TNF-α, and IL-10 from MKP-1 KO mice were significantly higher than mRNA from MKP-1 WT mice after LPS stimulation ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). These results support the idea that MKP-1 plays a negative regulatory role in mRNA expression of IL-6, TNF-α, and IL-10 after activation of MAPKs by LPS stimulation.

3.4. MKP-1 enhances the degradation of the mRNAs of IL-6, TNF-α, and IL-10 after LPS stimulation

Because cytokine mRNA stability is affected by the p38 MAPK signaling pathway, including MAPK-activated protein kinase 2 (MK2) [26], we examined the role of MKP-1 in regulation cytokine mRNA stability by performing actinomycin D chase experiments. Rat macrophages transduced by Ad.MKP-1 or Ad.LacZ (300 MOI) for 48 h were stimulated with LPS (100 ng/ml) for 4 h. Actinomycin D (10 μg/ml) was added to the cell culture to arrest RNA transcription, and total remaining cytoplasmic RNA was evaluated at various time points after addition of actinomycin D. As shown in Fig. 4a–c, cytokine mRNAs were rapidly degraded in macrophages transduced with Ad.MKP-1, with a $t_{1/2}$ of 2.9 h for IL-6 mRNA; 1.5 h for TNF-α mRNA; and 2.4 h for IL-10 mRNA. In contrast, the $t_{1/2}$ of mRNAs in macrophages treated with Ad.LacZ significantly increased 2.1-fold for IL-6 mRNA ($t_{½} = 6.0$ h) ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$); 1.8-fold for TNF-α mRNA ($t_{½} = 2.7$ h) ($n = 3$, $^{**}p < 0.01$, $^{***}p < 0.001$); and 1.7-fold for IL-10 mRNA ($t_{½} = 4.0$ h) ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$).

To further validate that MKP-1 signaling pathway plays a crucial role in controlling degradation of cytokine mRNA, we performed mRNA decay studies in primary bone marrow macrophages from MKP-1 KO and WT mice. As shown in Fig. 4d–f, bone

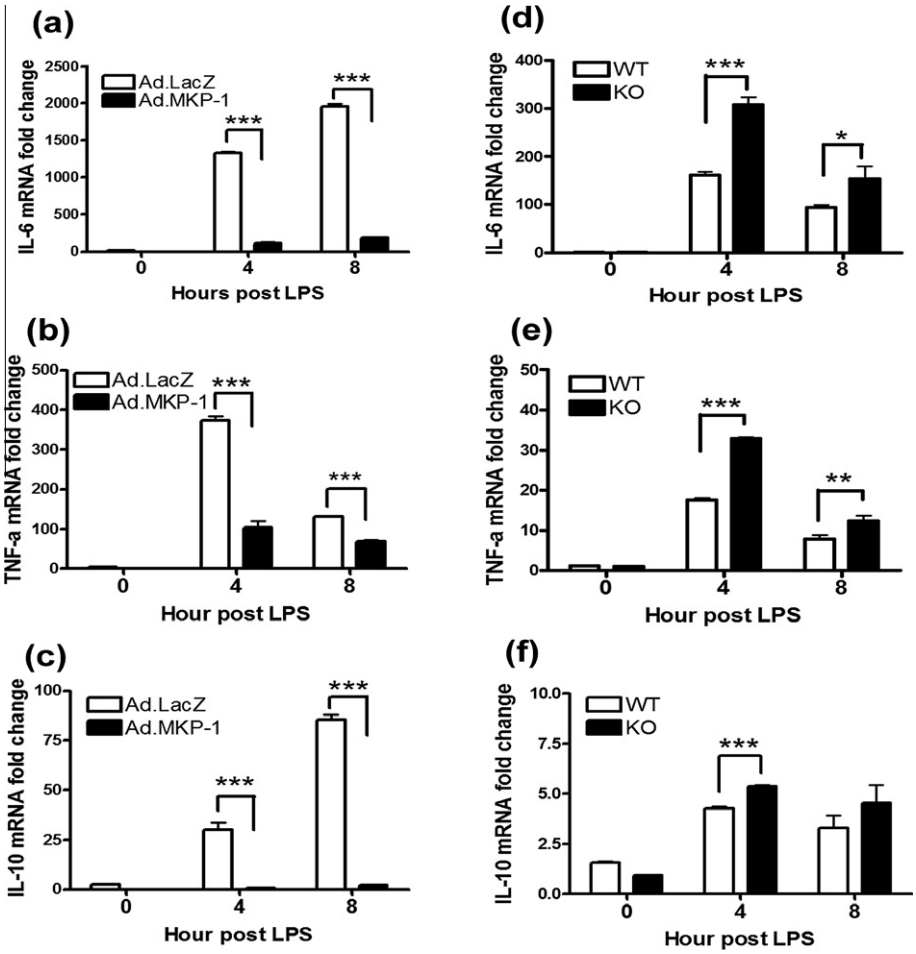

Fig. 3. MKP-1 inhibited IL-6, TNF-α, and IL-10 mRNA stimulated by LPS. Rat macrophages were transduced with Ad.MKP-1 or control Ad.LacZ (300 MOI) for 48 h. Primary bone marrow macrophages were either harvested from MKP-1 KO or WT mice. Cells were either unstimulated or stimulated with 100 ng/ml of LPS (from *Escherichia coli* 0127:B8) for 4 and 8 h. (a) IL-6 mRNA expression in rat macrophages ($n = 4$, $^{***}p < 0.001$). (b) TNF-α mRNA expression in rat macrophages ($n = 4$, $^{***}p < 0.001$). (c) IL-10 mRNA expression in rat macrophages ($n = 4$, $^{***}p < 0.001$). (d) IL-6 mRNA expression in mouse bone marrow macrophages ($n = 3$, $^{*}p < 0.05$, $^{***}p < 0.001$). (e) TNF-α mRNA expression in mouse bone marrow macrophages ($n = 3$, $^{**}p < 0.01$, $^{***}p < 0.001$). (f) IL-10 mRNA expression in mouse bone marrow macrophages ($n = 3$, $^{***}p < 0.001$). These data are representative of three separate experiments.

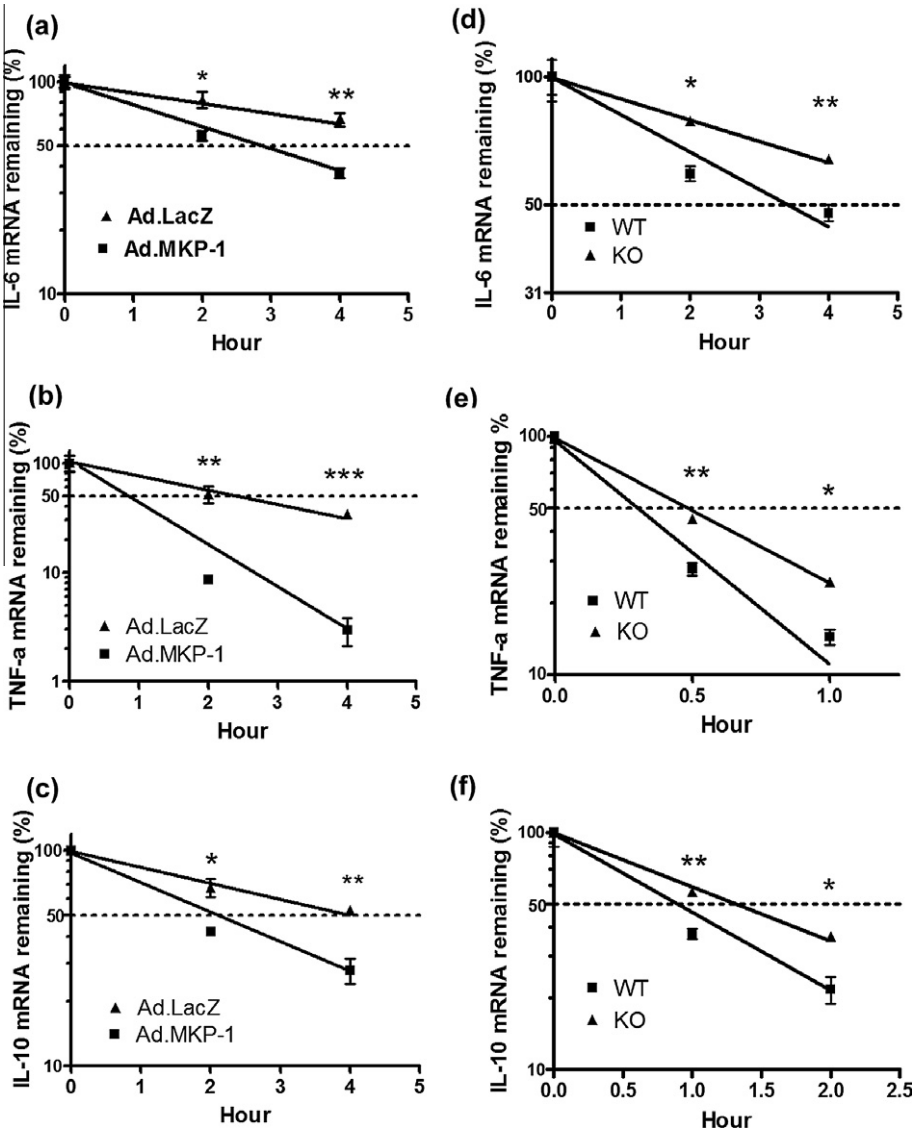

Fig. 4. MKP-1 increased the degradation of mRNAs of IL-6, TNF-α, and IL-10 stimulated by LPS. Rat macrophages were transduced with Ad.MKP-1 or control Ad.LacZ (300 MOI) for 48 h. Primary bone marrow macrophages were either harvested from MKP-1 KO or WT mice. Cells were stimulated with 100 ng/ml of LPS (from *Escherichia coli* 0127:B8) for 4 h. Actinomycin D (10 μg/ml) was added to the cells to stop mRNA transcription and the total RNAs were harvested at various time points indicated in the graphs after actimomycin D treatment. (a) IL-6 mRNA remaining percentage in rat macrophages ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). (b) TNF-α mRNA remaining percentage in rat macrophages ($n = 3$, $^{**}p < 0.01$, $^{***}p < 0.001$). (c) IL-10 mRNA remaining percentage in rat macrophages ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). (d) IL-6 mRNA remaining percentage in mouse bone marrow macrophages ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). (e) TNF-α mRNA remaining percentage in mouse bone marrow macrophages ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). (f) IL-10 mRNA remaining percentage in mouse bone marrow macrophages ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). These data are representative of three separate experiments.

marrow macrophages from MKP-1 KO mice exhibited slower degradation of IL-6, TNF-α and IL-10 mRNAs than macrophages from WT mice. Cytokine mRNA was rapidly degraded in macrophages derived from WT mice, with calculated $t_{1/2} = 3.5$ h for IL-6 mRNA, 0.3 h for TNF-α mRNA, and 0.9 h for IL-10 mRNA. In contrast, the $t_{1/2}$ of mRNA in macrophages derived from MKP-1 KO mice significantly increased 1.5-fold for IL-6 mRNA ($t_{½} = 5.4$ h) ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$); 2.0-fold for TNF-α mRNA ($t_{½} = 0.6$ h) ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$); and 1.6-fold for IL-10 mRNA ($t_{½} = 1.4$ h) ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). Together, these results suggest that signaling through MKP-1 dependent pathways is required for promoting cytokine mRNA degradation.

3.5. MKP-1 modulates subcellular translocalization of AUF1

RNA-BP AUF1 and TTP can bind AREs in the 3′-UTR of many inflammatory target gene mRNAs, facilitating mRNA degradation [26,27]. To define which RNA-BP contributes to cytokine mRNA destabilization related to MKP-1, we evaluated subcellular localization of MKP-1, p-MAPK, AUF1 and TTP in the cytoplasm vs. in nucleus. Rat macrophages were transduced with Ad.MKP-1 or Ad.LacZ (300 MOI), or treated with an equal volume of HEPES buffered saline for 48 h. Cells were then either left unstimulated or were stimulated with LPS (1 μg/ml) for 2 and 4 h. The cytoplasmic and nuclear proteins were extracted and analyzed using Western blot. As shown in Fig. 5a, MKP-1 protein was mainly induced in the cytoplasm at 2 and 4 h after LPS stimulation in cells transduced with Ad.MKP-1. MKP-1 was undetectable in cells either transduced with Ad.LacZ or treated with HEPES buffered saline both in the cytoplasm and in the nucleus. In cells transduced with Ad.MKP-1, with greater MKP-1 induction, there was a significant decrease of p-p38, p-JNK, and to a lesser extent, p-ERK expression, in the cytoplasm compared with cells transduced with Ad.LacZ or treated with HEPES buffered saline. p-p38 and p-JNK were mainly expressed in the cytoplasm. In contrast, p-ERK was distributed in both the nucleus and the cytoplasm.

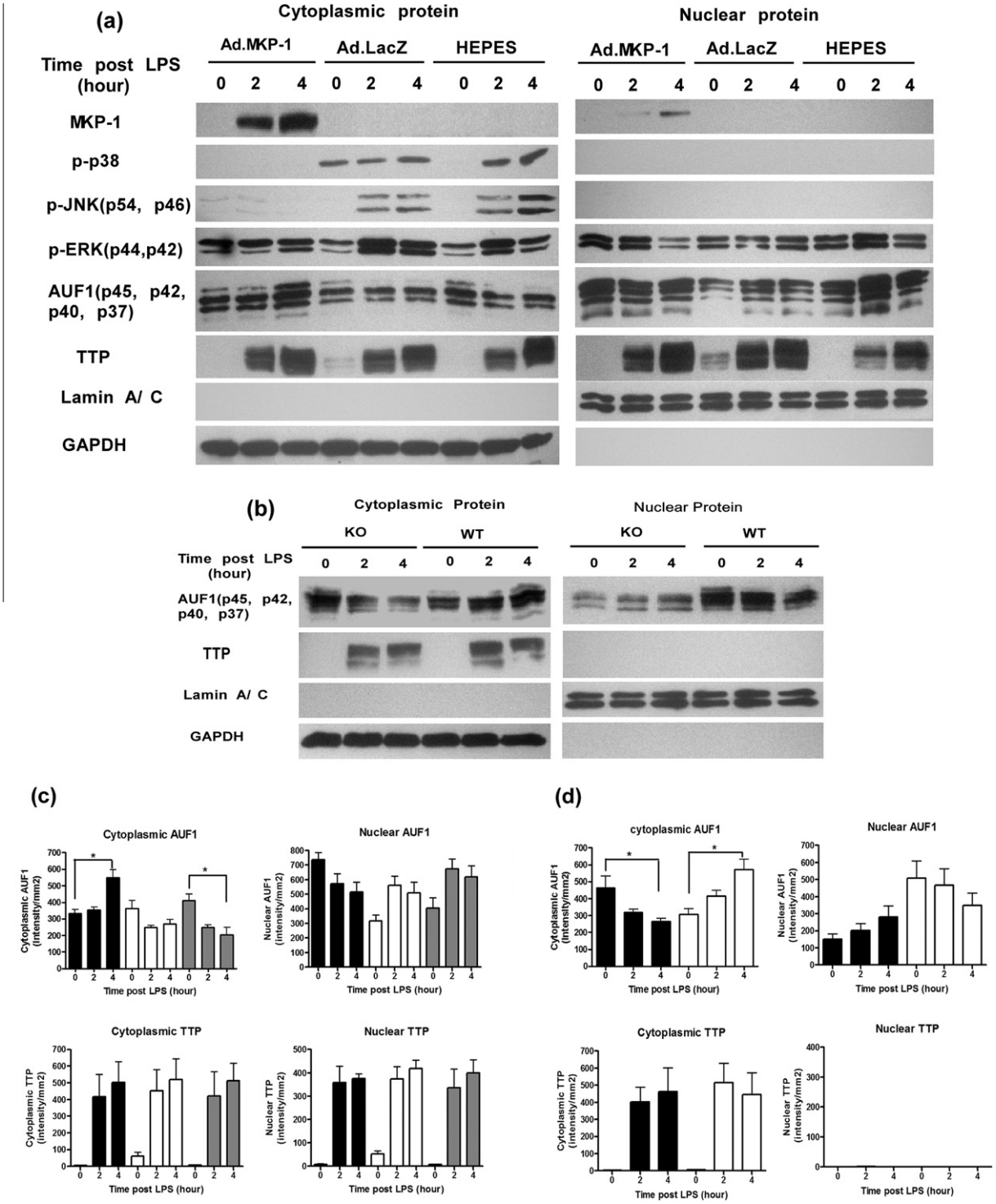

Fig. 5. MKP-1 modulates subcellular translocalization of AUF1. Rat macrophages were transduced with Ad.MKP-1 or control Ad.LacZ (300 MOI), or treated with HEPES buffered saline for 48 h. Primary bone marrow macrophages were either harvested from MKP-1 KO or WT mice. The macrophages were either unstimulated or stimulated with 1 μg/ml of LPS (from *Escherichia coli* 0127:B8) for 2 or 4 h. The nuclear and cytoplasmic proteins were extracted and analyzed by Western blot assay. Cytoplasmic protein (30 μg) and nuclear protein (15 μg) was loaded on the gel. (a) MKP-1, p-JNK (p54, p46), p-p38, p-ERK (p44, p42), AUF1 (p45, p42, p40, p37), and TTP proteins expression in the cytoplasm vs. in the nucleus of rat macrophages. (b) AUF1 (p45, p42, p40, p37), and TTP proteins expression in the cytoplasm vs. in the nucleus of mouse bone marrow macrophages. The GAPDH (cytoplasmic protein) and Lamin A/C (nuclear protein) are loading controls for cytoplasmic protein and nuclear protein separately. (c) AUF1 and TTP protein density in rat macrophages transduced with Ad.MKP-1 (black bars), or Ad.LacZ (white bars) or treated with HEPES buffered saline (gray bars) ($n = 3$, $^{*}p < 0.05$). (d) AUF1 and TTP protein density in bone marrow (BM) macrophages from MKP-1 KO mice (black bars) and WT mice (white bars) ($n = 3$, $^{*}p < 0.05$). The AUF1 and TTP protein density/mm^2 was analyzed by Quantity One software and normalized by cytoplasmic protein GAPDH or nuclear protein Lamin A/C, respectively. These data are representative of three separate experiments.

In macrophages transduced with Ad.MKP-1, there was a significant increase in AUF1 (p37^{AUF1}, p40^{AUF1}, p42^{AUF1} and p45^{AUF1}) in the cytoplasm and a slight decrease in AUF1 (p37^{AUF1}, p40^{AUF1}, p42^{AUF1} and p45^{AUF1}) in the nucleus 4 h after LPS stimulation (Fig. 5a–c, $n = 3$, $^{*}p < 0.05$), suggesting that AUF1 shuttles from the nucleus to the cytoplasm after LPS stimulation. In contrast, in

cells transduced with Ad.LacZ or treated with HEPES buffered saline, there was a modest decrease in AUF1 in the cytoplasm and a concomitant increase in nuclear AUF1 post-LPS stimulation. Cytoplasmic TTP expression was induced in all cells stimulated with LPS, and no treatment group was significantly different.

To confirm that AUF-1 subcellular translocation is related to MKP-1 status, bone marrow macrophage from MKP-1 KO or WT mice were either untreated or stimulated with LPS (1 μg/ml) for 2 and 4 h. The cytoplasmic and nuclear proteins were extracted and analyzed by Western blot. As shown in Fig. 5b–d, AUF1 ($p37^{AUF1}$, $p40^{AUF1}$, $p42^{AUF1}$ and $p45^{AUF1}$) was moderately increased in the cytoplasm and decreased in the nucleus in macrophages derived from WT mice 4 h after LPS stimulation ($n = 3$, $^{*}p < 0.05$). In contrast, AUF1 ($p37^{AUF1}$, $p40^{AUF1}$, $p42^{AUF1}$ and $p45^{AUF1}$) was moderately reduced in the cytoplasm and increased in the nucleus in macrophages derived from MKP-1 KO mice. TTP expression was only induced in the cytoplasm stimulated by LPS, and was not detectable in nuclear fractions of bone marrow macrophages. In addition, there was no significant difference in cytoplasmic expression of TTP observed in LPS stimulated WT and MKP-1 KO macrophages. Together these results demonstrate that signaling through MKP-1 specifically promotes translocation of AUF1 from the nucleus to the cytoplasm in response to LPS stimulation, but not TTP.

To confirm that MKP-1 promotes AUF1 cytoplasmic trafficking in response to LPS stimulation, we immunofluorescently stained bone marrow cells derived from MKP-1 KO vs. WT mice. Cells were treated with LPS for 4 h or left untreated. As shown in Fig. 6, AUF1 was primarily present in the nucleus in both MKP-1 KO and WT cells prior to LPS stimulation. Post-LPS stimulation, AUF1 remained in the nucleus in KO mice macrophages, whereas AUF1 was preferentially localized to the cytoplasm or localized to both the cytoplasm and the nucleus in WT macrophages. These results support the previous observation that MKP-1 signaling promotes AUF1 nuclear–cytoplasmic translocation after LPS stimulation.

To conclude that MKP-1-mediated mRNA decay is via AUF1, siRNA, experiments were performed in MKP-1 WT bone marrow cells. Bone marrow macrophages from MKP-1 WT mice were treated with either Accell SMARTpool AUF1 siRNA or control Accell Non-targeting Pool siRNA (500 nM) for 72 h. Then the cells were stimulated with LPS (100 ng/ml) for 4 h. RNA decay assay was performed after addition of actinomycin D. As shown in Fig. 7a, macrophages treated with AUF1 siRNA significantly reduced AUF1 mRNA expression compared with cells treated with control scrambled (Scr) siRNA ($n = 3$, $^{***}p < 0.001$). Cytokine mRNAs were degraded slower in macrophages treated with AUF1 siRNA compared with controls (Fig. 7b–d). The $t_{1/2}$ of mRNA in macrophages treated with control scrambled siRNA was calculated 1.8 h for IL-6 mRNA, 0.4 h for TNF-α mRNA, and 0.8 h for IL-10 mRNA. In contrast, the $t_{1/2}$ of mRNA in macrophages treated with AUF1 siRNA significantly increased 1.6-fold for IL-6 mRNA ($t_{½} = 2.9$ h) ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$); 2.0-fold for TNF-α mRNA ($t_{½} = 0.8$ h) ($n = 3$, $^{*}p < 0.05$); and 1.4-fold for IL-10 mRNA ($t_{½} = 1.1$ h) ($n = 3$, $^{*}p < 0.05$). These results confirm that AUF1 plays an important role in mediating IL-6, TNF-α, and IL-10 mRNA decay. Together with previous results, we further conclude that MKP-1-mediated mRNA decay is via AUF1.

To confirm that AUF1 cytoplasmic trafficking correlated with the conjugated amount of cytokine mRNA, a RNA-BP Immunoprecipitation (RIP)-RT-qPCR experiments were performed in which protein A agarose beads coated with anti-AUF1 (Millipore) or normal rabbit serum (Cell signaling) were immunoprecipitated with protein lysates from MKP-1 WT vs. KO cells with or without LPS stimulation. mRNA was extracted from RIP products, and cytokine

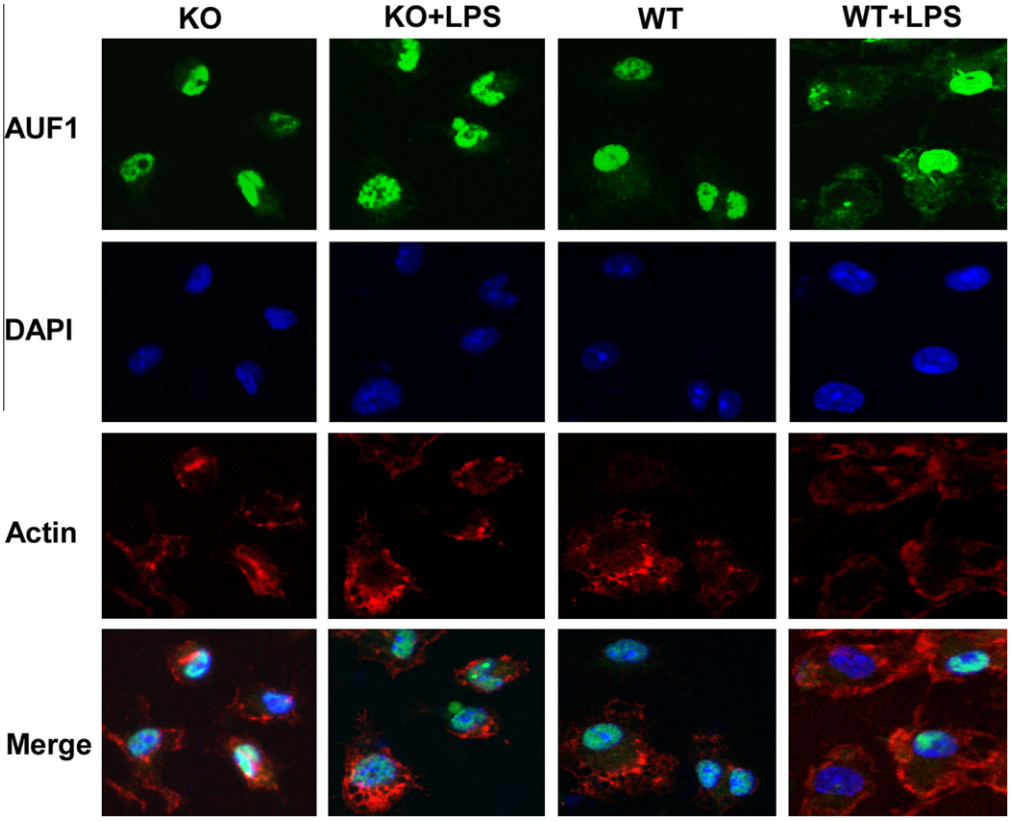

Fig. 6. MKP-1 modulates subcellular translocalization of AUF1. Primary bone marrow macrophages were either harvested from MKP-1 KO or WT mice. Cells were either unstimulated or stimulated with 1 μg/ml of LPS (from *Escherichia coli* 0127:B8) for 4 h. The cells were stained AUF1 by immunofluorescence staining. The nucleus was stained with DAPI. The cytoplasm was stained with Alexa fluor phalloidin 568-labeled actin. For each experiment, each sample was duplicated. Digital images were recorded using confocal microscopy (Leica, Bannockburn, IL). Five fields (about 100 cells) from each sample were counted. Results are representative of three separate experiments.

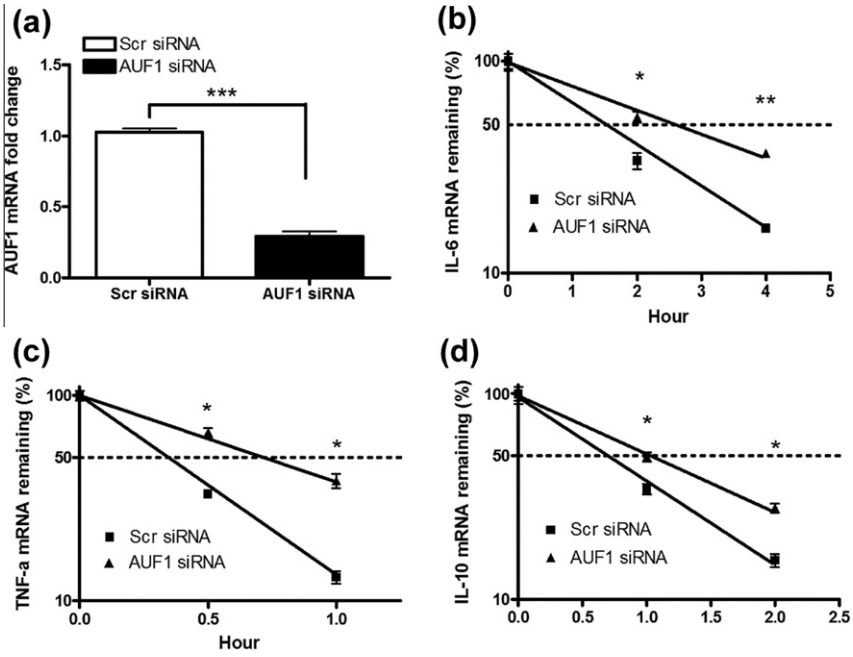

Fig. 7. AUF1 was associated with degradation of IL-6, TNF-α, and IL-10 mRNA. Bone marrow cells from MKP-1 WT mice were treated with either Accell SMARTpool AUF1 siRNA or control Accell Non-targeting Pool siRNA (500 nM) for 72 h. Cells were then stimulated with LPS (from *Escherichia coli* 0127:B8, 100 ng/ml) for 4 h. RNA decay assay was performed after addition of actinomycin D. (a) AUF1 mRNA expression ($n = 3$, $^{***}p < 0.001$). (b) IL-6 mRNA remaining percentage ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). (c) TNF-α mRNA remaining percentage ($n = 3$, $^{*}p < 0.05$). (d) IL-10 mRNA remaining percentage ($n = 3$, $^{*}p < 0.05$). These data are representative of three separate experiments.

mRNA was measured using real time PCR. As shown in Fig. 8a–c, more AUF1-conjugated IL-6, IL-10 and TNF-α mRNA were observed in WT cells compared with KO cells post-LPS stimulation ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). There was a 1.6-fold increase in IL-6 mRNA, a 2.3-fold increase in TNF-α mRNA, and a 2.0-fold increase in IL-10 mRNA in WT cells post-LPS stimulation compared with mRNA in KO cells. No target cytokine expression was detected in cells without LPS stimulation, or in control groups of protein A agarose beads coated with normal rabbit serum. These results demonstrate that AUF1 cytoplasmic trafficking in MKP-1 WT cells is correlated with increases in AUF1-conjugated IL-6, IL-10, TNF-α mRNAs, and further degradation of those cytokine mRNAs.

4. Discussion

Understanding the molecular mechanisms underlying cytokine regulation by MKP-1 is critical for comprehension of immune cytokine restraint and immune homeostasis after inflammatory stimuli. The current study confirms an essential role of MKP-1 in modulating cytokine production in response to inflammatory stimuli and provides new insights regarding how MKP-1 controls subcellular localization of RNA-BP, and subsequently affects mRNA stability. Ectopic expression of MKP-1 specifically dephosphorylated exaggerated? MAPK activity after LPS stimulation, but not the NF-κB signaling pathway. Over-expression of MKP-1 selectively attenuated pro-inflammatory cytokines IL-6, TNF-α, and anti-inflammatory cytokine IL-10 production and increased the degradation of those cytokine mRNAs. This increased degradation of specific cytokine mRNAs was associated with the increased AUF1 shuttling from the nucleus to the cytoplasm, and enhanced AUF1-conjugated IL-6, TNF-α, IL-10 mRNA.

Of note, exogenous expression of MKP-1 by Ad.MKP-1 did not induce increased MKP-1 protein expression in unstimulated cells. Increased MKP-1 protein expression was only observed 60 and 120 min after LPS stimulation. Without any stimulus, macrophages transduced with Ad.MKP-1 did not suppress cytokine responses. MKP-1 only attenuated the cytokine responses after the LPS stimulus. Interestingly, over-expression of exogenous MKP-1 not only attenuated the pro-inflammatory cytokine (IL-6, TNF-α) response, but also decreased anti-inflammatory cytokines (IL-10) after LPS stimulation. This demonstrates that MKP-1 gene activation is dependent on stress stimuli and activation of MAPKs. MKP-1 activation is essential for attenuation exaggerated MAPKs activities and maintaining immune homeostasis.

In this study, over-expression of MKP-1 failed to dephosphorylate activated NF-κB, suggesting that MKP-1 specifically dephosphorylates MAPK, not the NF-κB signaling pathway. With exogenous over-expression of MKP-1, IL-1β and CXCL1 were not significantly different between cells transduced with Ad.MKP-1 and cells transduced with Ad.LacZ, which indicates that IL-1β and CXCL1 might be controlled by other signal pathways, such as the NF-κB pathway. Although other investigators [27] have reported that MKP-1 inhibited NF-κB activity, they suggested that MKP-1 did not regulate the phosphorylation of IκB, and, instead MKP-1 might regulate NF-κB activity at the translational level.

Phosphorylation of MAPKs modulates downstream coregulatory proteins, and nucleosomal proteins, which are implicated in the regulation of cellular localization of various transcriptional factors, and affect mRNA export, localization, stability and translation [1]. In a cDNA array study [28] using probes for 950 ARE-containing mRNA, 45 AU-rich mRNA decayed at least 1.5 times—and up to 6.6 times—faster in the presence of p38 inhibitor. The cDNA array study agrees with our observations: MKP-1 not only inhibits mRNA transcription of inflammatory cytokines (IL-6, TNF-α), anti-inflammatory cytokine (IL-10), but also affects the degradation of those cytokine mRNAs. Cells transduced with Ad.MKP-1 had significantly increased rates of mRNA degradation compared with cells transduced with Ad.LacZ. Conversely, macrophages from MKP-1 KO mice had delayed cytokine mRNA degradation compared with WT macrophages.

It is well known that MKP-1 is a nuclear protein and it is immediately induced in response to activated MAPKs. In the current study, we show that ectopically expressed MKP-1 is mainly in-

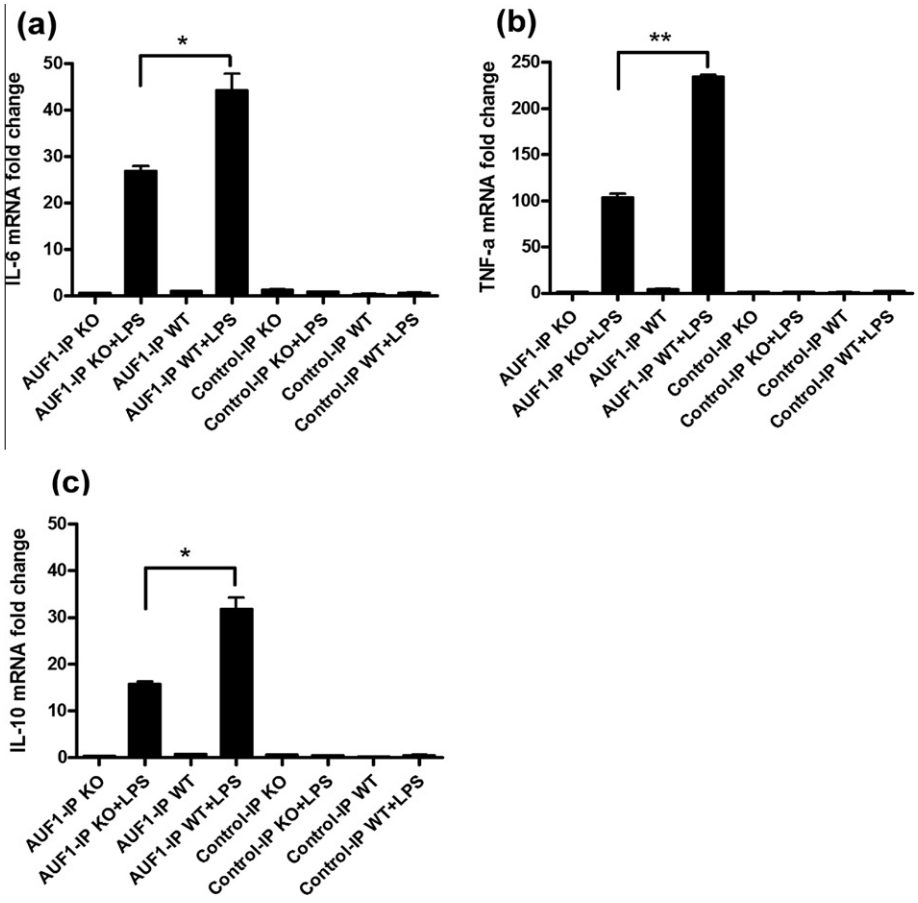

Fig. 8. AUF1 subcellular translocation post LPS stimulation in MKP-1 WT cells was associated with enhanced binding with IL-6, TNF-α, and IL-10 mRNAs. Primary bone marrow macrophages were either harvested from MKP-1 KO or WT mice. Cells were either unstimulated or stimulated with 1 μg/ml of LPS (from *Escherichia coli* 0127:B8) for 4 h. Protein A agarose beads coated with either anti-AUF1 or control normal rabbit serum were immunoprecipitated (IP) with cell lysates from bone marrow cells with or without LPS stimulation. RNA was extracted from IP products and RT-qPCR was performed. (a) IL-6 mRNA expression ($n = 3$, $^{*}p < 0.05$) (b) TNF-α mRNA expression ($n = 3$, $^{**}p < 0.01$). (c) IL-10 mRNA expression. ($n = 3$, $^{*}p < 0.05$). IL-6, TNF-α, and IL-10 mRNA were normalized to 10% input RNA endogenous beta-actin expression. The displayed result is representative of three separate experiments.

duced in the cytoplasm after LPS stimulation. Also, p-p38 and p-JNK are mainly induced in the cytoplasm in stimulated cells. These phenomena suggest that Ad.MKP-1 is preferentially retained in the cytoplasm or translocated from the nucleus to the cytoplasm in response to LPS stimulation, where MKP-1 dephosphorylates activated p-MAPKs. In contrast to p-p38 and p-JNK, p-ERK was present in both the nucleus and the cytoplasm. Although there was increased MKP-1 protein expression in cells transduced with Ad.MKP-1 at 2 and 4 h after LPS stimulation, p-ERK was less affected by MKP-1 compared with p-p38 and p-JNK. The results are consistent with findings by Chen et al. [29], supporting the idea that MKP-1 plays a major role in dephosphorylation of activated JNK and p38 MAPK, but it only has minor effects on activated ERK. Because ERK is primarily activated by hormone and growth factors, it may have a minor role in stress stimuli.

AUF1 is well characterized as a factor priming ARE-dependent mRNA decay [30–33]. Functionally, AUF1 differential splicing of AUF1 transcripts yields different mRNA, encoding four different isoforms: $p37^{AUF1}$, $p40^{AUF1}$, $p42^{AUF1}$ and $p45^{AUF1}$. AUF1-deficient mice exhibit symptoms of severe endotoxic shock, including vascular hemorrhage, intravascular coagulation, and high mortality after LPS stimulation as a result of decreased degradation of mRNA of TNF-α and IL-1β [34]. AUF1-deficient mice also develop chronic dermatitis with age that is characterized by pruritus and excoriations, which are associated with increased IL-2, TNF-α, and IL-1β [35]. Previous studies indicated that AUF1 could be phosphorylated at multiple sites, including serine and threonine [36–38]. Early studies suggest AUF1 can also form a complex with other proteins, including phosphoproteins [32]. Because MKP-1 can dephosphorylate serine/threonine residues, MKP-1 could act directly by dephosphorylation of AUF1 or indirectly through dephosphorylation of other novel polypeptides associated with AUF1. Ongoing studies are focused on understanding the molecular nature of phosphorylated forms of AUF1 and physical interactions with ARE-containing cytokine mRNAs.

Critical to the present studies, other investigators have shown that phosphorylation of AUF1 influences the ARE-binding affinity capacity and the global structure of the AUF1–ARE ribonucleoprotein complexes [37]. Sirenko et al. [39] revealed that ARE-binding activity and transcript stability were inversely regulated by phosphorylation. They reported that both a tyrosine kinase inhibitor genistein and a p38 MAPK inhibitor SK&F 86002 promoted assembly of AUF1–ARE-dependent mRNA complex, and the degradation of those ARE-dependent mRNAs in human monocytes.

In this study, we show that AUF1 intracellular translocation is correlated with MKP-1 expression, thus cells with over-expression of MKP-1 demonstrated increased cytoplasmic accumulation of AUF1 $p37^{AUF1}$, $p40^{AUF1}$, $p42^{AUF1}$ and $p45^{AUF1}$ 4 h after LPS stimulation compared with control groups of cells. Correspondingly, macrophages from MKP-1 WT mice displayed enhanced accumulation of AUF1 in the cytoplasm compared with cells from MKP-1 KO mice post LPS stimulus. The increased amount of cytoplasmic

AUF1 in WT macrophages was associated with enhanced AUF1-associated ARE-dependent mRNAs, and increased degradation of those targeted cytokine mRNAs.

In a previous study by Brook et al. [40], dephosphorylation of p38 MAPK by a p38 MAPK inhibitor SB202190 or a combined MEK1/ERK pathway inhibitor U0126 with p38 MAPK inhibitor SB202190 caused a translocation of TTP from the cytoplasm to the nucleus after LPS stimulation. However, in this study, TTP translocation from the cytoplasm to the nucleus was not observed in cells transduced with Ad.MKP-1, or in MKP-1 WT/KO mice stimulated with LPS. This discrepancy may be attributed to the non-specific p38 MAPK inhibitor SB202190, which might affect other signal pathways other than p38 MAPK pathway.

5. Conclusions

In summary, our results demonstrate that MKP-1 is activated in response to stress stimuli, which specifically restrains the exaggerated MAPKs responses. MKP-1 specifically attenuates IL-6, TNF-α, IL-10 cytokine expressions and plays an essential role in maintaining immune homeostasis. At the post-transcriptional level, MKP-1 specifically promotes AUF1 translocation from the nucleus to the cytoplasm in response to LPS stimulation, which facilitates the decay of mRNAs of IL-6, TNF-α, and IL-10.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgment

This study is supported by grants from National Institutes of Health (NIH): 1R01DE018290 (KL.K), 2P20 RR017696 (KL.K), R00DE018165 (V.P) and T32 DE017551 (H.Y).

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