RNA-Binding Protein AUF1 Regulates Lipopolysaccharide-Induced *IL10* Expression by Activating I κ B Kinase Complex in Monocytes^{∇†}

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Exposure of monocytes and macrophages to endotoxin/lipopolysaccharide (LPS) from Gram-negative bacteria activates the NF- κ B signaling pathway. At early times, this leads to their production of proinflammatory cytokines, but subsequently, they produce anti-inflammatory interleukin-10 (IL-10) to quell the immune response. LPS-mediated induction of *IL10* gene expression requires the p40 isoform of the RNA-binding protein AUF1. As LPS exerts modest effects upon *IL10* mRNA stability, we hypothesized that AUF1 controls the expression of signaling proteins. Indeed, knockdown of AUF1 impairs LPS-mediated p38 mitogen-activated protein kinase (MAPK) and NF- κ B signaling, and the expression of an RNA interference-refractory p40^{AUF1} cDNA restores both signaling pathways. To define the molecular mechanisms by which p40^{AUF1} controls *IL10* expression, we focused on the NF- κ B pathway in search of AUF1-regulated targets. Here, we show that p40^{AUF1} serves to maintain proper levels of the kinase TAK1 (transforming growth factor- β -activated kinase), which phosphorylates the IKK β subunit within the I κ B kinase complex to activate NF- κ B-regulated genes. However, p40^{AUF1} does not control the *TAK1* mRNA levels but instead promotes the translation of the mRNA. Thus, p40^{AUF1} regulates a critical node within the NF- κ B signaling pathway to permit *IL10* induction for the anti-inflammatory arm of an innate immune response.

Mononuclear phagocytes constitute the first line of defense against invading pathogens. Upon exposure to pathogen-derived products, monocytes and macrophages secrete a host of inflammatory cytokines. These cytokines are an integral part of the host response to infection, but their overproduction can lead to autoimmune symptoms associated with rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis (1, 2). In order to regulate the overproduction of inflammatory cytokines in response to exposure to pathogens, macrophages/monocytes also produce the anti-inflammatory cytokine interleukin-10 (IL-10) (24). At first glance, one of the paradoxes of pro- and anti-inflammatory cytokine regulation is that the same stimuli can produce both types of cytokines. However, the success of an immune response depends on achieving a balance between pro- and anti-inflammatory cytokines. In this context, IL-10 is critically important due to its anti-inflammatory and immunosuppressive properties (24). Recent studies have indicated that IL-10 biosynthesis in response to various

stimuli depends upon the activation of transcription factors, as well as covalent modification of histones associated with the *IL10* promoter. Many transcription factors, including STAT3, Sp1, Sp3, c-Maf, AP-1, NF- κ B, C/EBP β , and C/EBP δ , promote lipopolysaccharide (LPS)-mediated *IL10* expression in monocytes/macrophages of both mouse and human origin (4, 6, 7, 20, 21, 42, 49).

Binding of LPS to TLR4 (Toll-like receptor 4) signals via the adaptor protein MyD88 (myeloid differentiation primary-response gene 88), leading to activation of the NF- κ B pathway. In addition, binding of LPS to TLR4 activates signaling via TRIF (Toll/IL-1R [TIR] domain-containing adaptor protein inducing beta interferon [IFN- β]). The MyD88-dependent pathway is involved in rapid induction of proinflammatory cytokines by monocytes/macrophages primarily through the activation of NF- κ B. LPS-TLR4-mediated activation of the MyD88-independent pathway leads to the production of type I IFNs via interferon regulatory factor 3 (IRF3). This activates the JAK-STAT pathway, leading to phosphorylation of STAT3 and the production of anti-inflammatory IL-10 (3, 8, 41, 44). As mentioned above, NF- κ B has also been linked to *IL10* expression during innate immune responses (7, 20).

In addition to transcriptional activation, *IL10* expression is regulated through posttranscriptional mechanisms (25, 29, 34, 38). We have shown previously that AUF1 binds the 3'-untranslated region (3'-UTR) of *IL10* mRNA, which contains AU-rich elements (AREs) (5, 33). The AUF1/hnRNP D family of ARE-binding proteins destabilizes various mRNAs encoding cytokines, chemokines, and cell cycle regulators (36, 37). AUF1 consists of four isoforms (37, 40, 42, and 45 kDa) gen-

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TABLE 1. Sequences of qRT-PCR primers

Gene	Forward primer	Reverse primer
hsa <i>IL10</i>	5'-ACCTGCCTAACATGCTTCGAG-3'	5'-CTGGGTCTTGGTTCTCAGCTT-3'
hsa β -actin	5'-CCATCATGAAGTGTGACGTGGA-3'	5'-TTCTGCATCCTGTCGGCAA-3'
hsa <i>IL1α</i>	5'-GCCAATGATCAGTACCTCACG-3'	5'-TGGGTATCTCAGGCATCTCCT-3'
hsa <i>IL1β</i>	5'-GAAGCTGATGGCCCTAAACAG-3'	5'-AGCATCTTCCTCAGCTTGTCC-3'
hsa <i>TNFα</i>	5'-GTGCTTGTTCCTCAGCCTCTT-3'	5'-ATGGGCTACAGGCTTGTCACT-3'
hsa <i>CXCL3</i>	5'-TGCAGGGAATTCACCTCAAG-3'	5'-GGTGCTCCCCTTGTTCAGTAT-3'
hsa <i>CCL20</i>	5'-AGTTTGCTCCTGGCTGCTTT-3'	5'-CCGTGTGAAGCCCAATAA-3'
hsa <i>CXCL10</i>	5'-TGGCATTCAAGGAGTACCTCTC-3'	5'-CTTGATGGCCTTCGATTCTG-3'
hsa <i>TAK1</i>	5'-CATTGAGAGCCTGATGACTCG-3'	5'-TGACTATGGTGGCAGAGTTGCT-3'

erated by alternate pre-mRNA splicing (12, 13, 46, 48). Depletion of AUF1 in THP-1 cells, a human promonocytic leukemia cell line, suppresses LPS-mediated induction of *IL10* mRNA and protein. Upon complementation with either the p37^{AUF1} or the p40^{AUF1} plasmid, only p40^{AUF1} restores the induction of *IL10* mRNA and protein to near-normal levels. These observations suggest that the p40^{AUF1} isoform selectively plays a critical, positive role in *IL10* expression; however, AUF1 exerts only modest effects on *IL10* mRNA stability in response to LPS (33). As such, the underlying mechanisms of AUF1-mediated regulation of *IL10* in monocytes remain poorly understood.

To define the mechanisms by which AUF1 modulates the expression of *IL10*, we thus shifted our attention to LPS-mediated activation of the NF- κ B and mitogen-activated protein kinase (MAPK) pathways in THP-1 cells and in THP-1 cells upon AUF1 knockdown. Our results demonstrate that the levels of activation of IKK (I κ B kinase) and MAPKAPK-2 (mitogen-activated protein kinase-activated kinase 2), the latter of which is an immediate substrate for p38 MAPK, are significantly reduced in cells upon AUF1 knockdown compared to their activation in control cells. Both the NF- κ B and p38 MAPK pathways are essential for the proper regulation of *IL10* expression in monocytes exposed to LPS. We show that AUF1 knockdown impairs NF- κ B signaling and gene expression by reducing the abundance of kinase TAK1 (transforming growth factor- β [TGF- β]-activated kinase 1), a member of the MEK kinase family. TAK1, a key component of LPS-mediated signaling pathways, is essential for LPS-induced activation of the NF- κ B and MAPK pathways. Either enforced expression of TAK1 or ectopic expression of p40^{AUF1} in these cells is sufficient to restore NF- κ B binding and *IL10* gene expression in response to LPS. While AUF1 affects neither *TAK1* mRNA levels nor protein half-life, it acts to promote the translation of *TAK1* mRNA. Thus, AUF1 serves to maintain TAK1 at the requisite levels for activation of the NF- κ B and p38 MAPK pathways and *IL10* expression.

MATERIALS AND METHODS

Reagents. LPS (*Escherichia coli* O55:B5 and O127:B8 [Sigma]) was dissolved in complete medium, aliquoted, and stored at -20°C . Other reagents were obtained from the following sources: RPMI 1640 medium (CellGro), penicillin-streptomycin-L-glutamine (Invitrogen), fetal bovine serum (HyClone), anti- α -tubulin antibody (Sigma), anti-STAT3 antibody (Santa Cruz), anti-STAT1 antibody (Transduction Laboratories), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit and HRP-conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories). The following antibodies were purchased from Cell Signaling Technology: I κ B α (catalog no. 9242), phos-

pho-I κ B α (no. 9246), IKK α (no. 2682), IKK β (no. 2370), IKK γ (no. 2685), phospho-IKK α / β (no. 2078), TAK1 (no. 4505), phospho-TAK1^{Thr187} (no. 4536), TAB1 (no. 3225), TAB2 (no. 3744), TRAF6 (no. 4743), phospho-STAT^{Tyr701} (no. 9167), phospho-STAT3^{Tyr705} (no. 9138), phospho-STAT3^{Ser727} (no. 9134), phospho-p38 MAPK (no. 9216), phospho-MAPKAPK-2 (no. 3316), and MAPKAPK-2 (no. 3042).

Cell culture, plasmid construction, and transfection. THP-1, a human promonocytic leukemia cell line expressing short-hairpin RNAs (shRNAs) targeting *AUF1* mRNAs encoding all four isoforms of AUF1 (shAUF1) or expressing a random sequence (shCTRL) were constructed as described previously (33, 36). These cells were maintained in RPMI-1640, 10% fetal bovine serum, penicillin-streptomycin-L-glutamine, and 250 units/ml hygromycin B (Calbiochem) in a 5% CO₂ environment at 37°C. A TAK1 expression plasmid, pcDNA/TAK1, was constructed by cloning the *TAK1* open reading frame into plasmid pcDNA3 between the EcoRI and XbaI sites. THP-1/shAUF1 cells were transfected with the pcDNA/TAK1 plasmid with transfection reagent SuperFect (Qiagen) according to the manufacturer's protocol. Stably transfected cells were selected with Geneticin (500 $\mu\text{g}/\text{ml}$). THP-1/shAUF1/TAK1 cells and cells expressing both shAUF1 and shRNA-refractory p40^{AUF1} were maintained in 500 $\mu\text{g}/\text{ml}$ Geneticin (Gibco) and 250 units/ml hygromycin B.

RNA extraction and qRT-PCR. Total RNA was extracted from cells by using an RNeasy mini kit (Qiagen), and DNA was removed from RNA samples by RNase-free DNase treatment (Qiagen). Total RNA (250 to 1,000 ng) was reverse transcribed into cDNA with a TaqMan reverse transcription (RT) kit (Applied Biosystems) according to the manufacturer's protocol. The resulting cDNA was analyzed by PCR with Platinum SYBR green quantitative PCR (qPCR) Super-Mix UDG (Invitrogen) according to the manufacturer's protocol. Specific primer sets used for human *IL10*, *IL1 α* , *IL1 β* , *TNF α* , *CCL20*, *CXCL3*, *CXCL10*, *TAK1*, and β -actin mRNAs were designed with OligoPerfect Designer software (Invitrogen). Forward and reverse primers for each are listed in Table 1. The target gene expression levels were normalized to β -actin mRNA in all experiments. The levels of cDNA were calculated by the relative quantitation (change in cycle threshold [$\Delta\Delta C_T$]) method (27). Data were plotted relative to the results for the untreated control.

PCR arrays. PCR arrays were purchased from SuperArray Bioscience Corporation. The RT² profiler PCR array system (PAHS-11D) was used for comparing inflammatory cytokine and chemokine mRNAs in LPS-treated cells with or without AUF1 knockdown. LPS-mediated signal transduction pathways were examined by real-time PCR with the human signal transduction pathway RT² profiler PCR array (PAHS-14D).

Western blotting. Protein was extracted by boiling cells in 2.5 $\times$ SDS-PAGE sample buffer (1 $\times$ SDS-PAGE sample buffer contains 10% glycerol, 4% β -mercaptoethanol, 0.5 M Tris-HCl, pH 6.8, and 0.4% SDS) for 5 min. Samples were cooled on ice and cleared by centrifugation at 12,000 $\times g$ for 5 min. Proteins were fractionated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF; Bio-Rad) membrane, and incubated for 1 h in blocking solution (3% milk, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.05% Tween 20) at room temperature. Blots were incubated with specific antibodies overnight at 4°C. Anti- α -tubulin (1:5,000) was incubated for 1 h at room temperature. Blots were washed extensively in TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.05% Tween 20), incubated with HRP-conjugated secondary antibody for 1 h at room temperature, and visualized with the enhanced chemiluminescence reagent ECL Plus (PerkinElmer Life Sciences, Inc.).

EMSA. The activation of NF- κ B was measured by electrophoretic mobility shift assay (EMSA) as follows. Briefly, 2×10^7 cells were collected by centrifugation and washed in cold phosphate-buffered saline (PBS), resuspended in 100 μl hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1

FIG. 1. AUF1 regulates the expression of cytokine and chemokine genes. THP-1 cells expressing shAUF1 and shCTRL were exposed to LPS (1 μ g/ml) for 4 h or left untreated. Total RNA was extracted and assayed by qRT-PCR using primer sets from the RT² profiler PCR array system. Target gene expression levels were normalized to the β -actin mRNA level in this and all subsequent experiments. Fold change is the ratio of the mRNA levels in LPS-treated versus untreated cells. (A) mRNAs with ratios of >100. (B) mRNAs with ratios of <20. The PCR array data were obtained with a single array. However, the results were validated by real-time PCR with a different set of gene-specific primers, yielding comparable results (data not shown).

mM EGTA, 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], and 1 $\times$ protease inhibitor cocktail [Sigma], and incubated for 15 min. Cells were then lysed with 6.5 μ l of 10% NP-40. The nuclear pellet was isolated by centrifugation at 13,000 $\times$ g for 1 min and resuspended in 50 μ l ice-cold, high-salt buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 25% glycerol [vol/vol], 0.5 mM PMSF, and 1 $\times$ protease inhibitor cocktail). After 30 min of incubation with occasional vortexing, the extracts were centrifuged and the supernatants containing nuclear extract were collected. The protein concentration was measured, and 8 μ g of nuclear extract was incubated with ³²P-end-labeled NF- κ B consensus oligonucleotide DNA (sense strand, 5'-TCGACAGGGACTTCCGAGAG-3', and antisense strand, 5'-TCGACTCTCGGAAAGTCCCTG-3') for 20 min at room temperature. The resulting DNA-protein complex was resolved in a 6% native polyacrylamide gel run with 0.25 $\times$ Tris-borate-EDTA buffer. The gel was dried, and the radioactive bands were visualized and quantitated by phosphorimager. For competition assays, either unlabeled NF- κ B or IFN- γ activation sequence (GAS) oligonucleotides (sense strand, 5'-GATCGATTTCGCCGAAAT-3', and antisense strand, 5'-CATGATTTCGGGAAATC-3') were used as specific or non-specific competitor, respectively. For antibody supershift, nuclear extracts were incubated with specific antibody for 15 min prior to incubation with radiolabeled oligonucleotide probe DNAs.

Luciferase reporter assay. Cells were transfected with luciferase reporter plasmid pIL-6 containing three copies of the IL-6 NF- κ B motif (kindly provided by Celine Gelinus, CABM, UMDNJ) and Effectene reagent, following the manufacturer's protocol. Renilla luciferase vector pRK-Tk was used as an internal control. Twenty-four hours after transfection, the cells were exposed to LPS (1 μ g/ml) and incubated for 24 h. The cells were lysed and assayed with a dual luciferase kit following the manufacturer's protocol (Promega).

Determination of TAK1 protein half-lives. Cells were cultured with 10 μ g/ml cycloheximide (Sigma-Aldrich) to block translation. Cells were harvested at each time point and lysed in SDS sample buffer, and equal cell equivalents of proteins were resolved by 8% SDS-PAGE. Proteins were detected by Western blot analyses as described above. TAK1 levels were normalized to the level of α -tubulin and plotted as the percentage of the value at time zero (set at 100%). Data were analyzed by nonlinear regression, and protein half-life was determined from the first-order decay constant (k) by utilizing PRISM software.

mRNP immunoprecipitation. Endogenous RNA-protein complexes were immunoprecipitated as described previously (18, 19). Briefly, 5×10^7 cells were lysed in an equal volume of cold lysis buffer (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.0, 0.5% NP-40, 1 mM DTT) supplemented with RNase and protease inhibitors. Cell lysates were precleared by incubation with preimmune serum and protein A beads. Lysates were then incubated overnight with protein A beads precoated with either AUF1 antiserum or preimmune serum at 4°C.

After being washed in NT2 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, and 0.05% NP-40), the beads were treated with RNase-free DNase I and proteinase K. Immunoprecipitated RNA was isolated by phenol-chloroform-isoamyl alcohol extraction. The levels of *TAK1* and control *GAPDH* mRNAs in the immunoprecipitates were determined by qRT-PCR. The qRT-PCR products were visualized by fractionation in 2% agarose to ensure correct product size.

Polyribosome gradient analyses. Cell lysates were prepared from THP-1 cells expressing shCTRL or shAUF1 and fractionated on sucrose gradients as described previously (18). As a control, parallel lysates were prepared in EDTA-containing buffer and fractionated. The percentages of total *TAK1* and *GAPDH* mRNAs in each fraction were determined by qRT-PCR.

RESULTS

AUF1 modulates chemokines, cytokines, and their receptors. We have shown that AUF1 knockdown in THP-1 cells inhibits *IL10* gene expression induced by LPS exposure (33). To determine whether AUF1 regulates the expression of other cytokines and chemokines in monocytes exposed to LPS, AUF1 knockdown (shAUF1) and control cells (shCTRL) were exposed to LPS for 4 h or left untreated. RNA from these cells was extracted and examined by real-time PCR with primer sets from PCR arrays. In addition to *IL10*, knockdown of AUF1 reduced the levels of (GenBank accession numbers in parentheses) *CCL20* (NM_0045910), *CXCL3* (NM_002090), *TNF α* (NM_000594), *IL1 β* (NM_000576) (Fig. 1A), *IL1 α* (NM_000575), *IL1* family member 9 (NM_019618), *IL1* receptor (NM_000877), and *CCR7* (NM_001838) mRNAs (Fig. 1B) compared to their levels in cells expressing a control shRNA. However, LPS-induced levels of *IL8* (NM_000584) mRNA were higher upon AUF1 knockdown (Fig. 1A). The results of PCR arrays were validated by real-time RT-PCR with gene-specific primer sets (data not shown). Thus, LPS-induced cytokine and chemokine gene expression is severely compromised in AUF1-deficient cells. Earlier work demonstrated that AUF1 has a modest impact on *IL10* mRNA

FIG. 2. AUF1 regulates LPS-stimulated signaling pathways. (A and B) Total RNAs described in the legend to Fig. 1 were compared by qRT-PCR with primer sets from the human signal transduction pathway RT² profiler PCR array system. Fold change is the ratio of mRNA levels in LPS-treated versus untreated cells. The PCR array data were obtained with a single array. However, the results were validated by real-time PCR with a different set of gene-specific primers that yielded comparable results (data not shown).

stability in response to LPS (33). For this reason, we next considered that AUF1 knockdown may lead to impaired LPS-mediated signaling.

LPS-mediated expression of inflammatory mediators involves multiple signaling pathways. The binding of LPS to TLR4 activates multiple signaling pathways, leading to the activation of many transcription factors. To determine the signaling pathways involved in AUF1-mediated modulation of inflammatory cytokines, we profiled the expression of 84 key genes representative of 18 different signal transduction pathways. As shown in Fig. 2A, upon LPS exposure, there were differences in the induction of 9 mRNAs out of 84 tested in cells, with reduced AUF1 abundance compared to the level of induction in cells expressing a control shRNA. LPS-mediated expression of all but one of these genes was reduced in AUF1-deficient cells. Of these, the NF- κ B pathway regulates *CCL20*, *ICAM1* (GenBank accession no. NM_000201), *TNF α* , *IL1 α* , *IL8*, and *TANK1* (GenBank accession no. NM_004180). By contrast, only three genes represent other pathways, such as the Jak-Stat pathway (*BCL2L1*, GenBank accession no. NM_138578), phospholipase C pathway (*PTGS2*, GenBank accession no. NM_000963), and low-density lipoprotein (LDL) pathway (*CCL2*, GenBank accession no. NM_002982). Therefore, activation of the NF- κ B pathway by LPS seems to be impaired upon AUF1 knockdown.

LPS also activates the p38 MAPK pathway. Analysis of the PCR array data revealed that LPS-mediated expression of the *CXCL3*, *TNF α* , and *IL1 β* genes was reduced by AUF1 knockdown (Fig. 2B). It is noteworthy that inhibition of the p38 MAPK pathway blocks the expression of *TNF α* , *IL1 β* , and *CXCL3* at both the mRNA and the protein level (9). These data indicate that, in addition to the NF- κ B pathway, the p38 MAPK pathway is defective upon AUF1 knockdown.

MAPK p38 and NF- κ B pathways regulate LPS-induced *IL10* expression in monocytes. The three major MAPKs of mammalian cells are extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (9).

The ERK pathway is activated by mitogenic stimuli, whereas the JNK and p38 MAPK pathways are activated by proinflammatory stimuli. We examined the effects of MAPK inhibitors on *IL10* mRNA levels in cells with or without AUF1 knockdown. Cells were exposed to LPS in the presence or absence of MAPK inhibitors. The level of *IL10* mRNA was measured by qRT-PCR (Fig. 3A). Inhibition of the p38 MAPK pathway by SB203580 reduced *IL10* expression in control cells. The pyridinyl imidazole compound SB203580 inhibits p38 MAPK by blocking the access of ATP to the catalytic site of the kinase (9, 11). Inhibition of the JNK and ERK pathways had little effect on *IL10* expression. The low level of *IL10* expression upon AUF1 knockdown was slightly further reduced in the presence of all MAPK inhibitors. Thus, LPS-mediated *IL10* expression is dependent upon the p38 MAPK pathway. It is noteworthy that p38 MAPK is involved in the activation of many transcription factors, including STAT3, Sp1, and AP-1 (23, 26, 45). All of these transcription factors have been implicated in LPS-mediated *IL10* expression in monocytes/macrophages (4, 23). In addition, p38-mediated modification of histones in the promoters of many genes induced by inflammatory stimuli is required for NF- κ B recruitment (31, 32).

LPS exposure leads to phosphorylation of I κ B proteins and their subsequent polyubiquitination and proteasome-mediated degradation. This, in turn, leads to the activation and nuclear translocation of NF- κ B. To determine whether activation of NF- κ B is involved in the LPS-mediated induction of *IL10*, we used NF- κ B inhibitor BAY 11-7082 to block this pathway. Bay 11-7082 selectively inhibits the functions of the I κ B kinase IKK β , thus preventing the activation and translocation of free NF- κ B to the nucleus (28). Cells expressing shAUF1 and shCTRL were exposed to LPS in the presence or absence of BAY 11-7082. RNA was extracted from cells, and the levels of *IL10* mRNA were measured by qRT-PCR. Treatment with the NF- κ B inhibitor nearly abolished *IL10* expression in cells expressing shCTRL, as well as in those expressing shAUF1 (Fig. 3B). Therefore, LPS-mediated *IL10* expression involves acti-

FIG. 3. Inhibitors of p38 MAPK and NF- κ B pathways block LPS-mediated *IL10* expression. shCTRL- and shAUF1-expressing cells were left untreated or exposed to LPS (1 μ g/ml) with or without p38 MAPK inhibitor (2 μ M SB203580 [SB]), JNK kinase inhibitor (20 μ M SP6000125 [SP]), ERK pathway inhibitor (20 μ M PD98059 [PD]) (A) or NF- κ B pathway inhibitor (3.75 μ M BAY 11-7082 [BAY]) (B). Total RNA was extracted and analyzed by qRT-PCR. Fold change in *IL10* mRNA level is the ratio of mRNAs in LPS-treated versus untreated cells. Data from a representative experiment are shown.

vation of the NF- κ B pathway. The roles of NF- κ B in the regulation of *IL10* expression in monocytes/macrophages have been described (7, 20, 32). Thus, we conclude that activation of both the p38 MAPK and the NF- κ B pathway is essential for LPS-induced *IL10* expression in monocytes.

AUF1 is required for activation of both the NF- κ B and p38 MAPK pathway but not the type I IFN pathway. The NF- κ B and MAPK pathways are required for the expression and activation of various transcription factors involved in LPS-mediated gene expression in monocytes. To test the possibility that AUF1 regulates LPS-mediated *IL10* expression by modulating these pathways, cells with or without AUF1 knockdown were exposed to LPS for various times. Protein extracts were prepared from these cells and analyzed by Western blotting to measure the levels of the indicated phosphorylated and total proteins (Fig. 4). As mentioned earlier, exposure of monocytes to LPS leads to the activation and translocation of NF- κ B following the phosphorylation, ubiquitination, and degradation of I κ B α (14). The activation of the I κ B α protein was measured with an antibody specific for I κ B α phosphorylated at serines 32

and 36. As expected, phosphorylation of I κ B α occurred between 2 and 6 h of exposure in control cells (Fig. 4A, phospho-I κ B α [p-I κ B α], lanes 3 to 5), and degradation of I κ B α was evident between 0 and 2 h (Fig. 4A, I κ B α , lanes 1 to 3). I κ B α returned to the time zero level between 4 and 6 h of exposure due to its resynthesis in control cells (Fig. 4A, I κ B α , compare lanes 4 and 5 with lane 1). This induction of I κ B α , a canonical NF- κ B-responsive gene, is indicative of rapid recruitment and activation of NF- κ B by LPS (30). By contrast, the Western blots of extracts from cells with reduced AUF1 abundance showed several differences in comparison to the results for control cells, as follows: (i) I κ B α was only weakly phosphorylated in cells with AUF1 knockdown that were exposed to LPS for 2 to 6 h (Fig. 4A, p-I κ B α , compare lanes 9 to 11 with lanes 3 to 5); (ii) this may be due in part to reduced levels of total I κ B α protein in cells with AUF1 knockdown relative to the levels in control cells (Fig. 4A, I κ B α , compare lanes 7 to 12 with lanes 1 to 6); and (iii) degradation of I κ B α during the first 2 h of LPS exposure was less pronounced in cells with reduced AUF1 abundance than in control cells (Fig. 4A, I κ B α , com-

FIG. 4. LPS-mediated activation of NF- κ B and p38 MAPK pathways is impaired following AUF1 knockdown. (A) shCTRL- and shAUF1-expressing cells were exposed to LPS (1 μ g/ml) for the indicated times or left untreated, and protein extracts were analyzed by Western blotting to detect the proteins listed on the left. (B) Cells were exposed to LPS in the presence and absence of p38 MAPK inhibitor SB203580 or NF- κ B inhibitor BAY 11-7082 for 2 h or left untreated, and protein extracts were analyzed by Western blotting with antibodies to detect proteins indicated on the left. α -Tubulin served as an internal control. The results of a representative experiment are shown. In this and subsequent figures, Western blots with anti-phospho (p)-protein antibodies indicate activation of pertinent signaling pathways.

FIG. 5. Type I IFN pathway is unaffected by AUF1 knockdown. Cells expressing shAUF1 or shCTRL were left untreated or exposed to LPS (1 μ g/ml) for the indicated times. Cells were harvested for preparation of protein extracts and RNA. (A) Western blot analyses were performed using antibodies to detect proteins listed on the right. (B) Results of qRT-PCR analyses of *CXCL10* mRNA. Forward and reverse primers are shown in Table 1. Fold change is the ratio of the mRNA levels in LPS-treated versus untreated cells. Values are the means of two independent experiments, with standard deviations shown as error bars.

pare lanes 7 to 9 with lanes 1 to 3). Taken together, these results suggest that AUF1 knockdown impairs NF- κ B activation by reducing the appropriate levels of p-I κ B α . But why does this occur?

Phosphorylation of I κ B α is catalyzed by an enzyme complex consisting of catalytic subunits IKK α and IKK β and a regulatory subunit, IKK γ , also known as NF- κ B essential modulator (NEMO). The activation of IKK α / β by phosphorylation is essential for the induction of NF- κ B by most proinflammatory stimuli (15). Stimulation of cells expressing shCTRL with LPS led to IKK α / β phosphorylation that peaked at 2 h of LPS exposure and then declined gradually, as expected (Fig. 4A, p-IKK α / β , lanes 2 to 6). In contrast, IKK α / β phosphorylation was markedly reduced in cells expressing shAUF1 (Fig. 4A, p-IKK α / β , lanes 8 to 12). This result is consistent with the observation that phosphorylation of I κ B α is reduced upon AUF1 knockdown.

As shown above (Fig. 3), inhibition of the p38 MAPK pathway by a specific inhibitor, SB203580, blocked the induction of *IL10* mRNA in monocytes exposed to LPS. SB203580 prevents the activation of MAPKAPK-2 by p38 MAPK. To examine the hypothesis that the p38 MAPK pathway is also impaired upon AUF1 knockdown, we compared the level of MAPKAPK-2 activation in cells with or without AUF1 knockdown by Western blot analyses with antibody specific for phospho-MAPKAPK-2. In cells expressing shCTRL, LPS-induced phosphorylation of MAPKAPK-2 peaked at 2 h (Fig. 4A, p-MAPKAPK-2, lane 3) and declined thereafter. Knockdown of AUF1 led to reduced phosphorylation of MAPKAPK-2 (Fig. 4A, p-MAPKAPK-2, lanes 9 to 12). Therefore, we conclude that LPS-mediated activation of both the NF- κ B and p38 MAPK pathways is impaired in the absence of AUF1.

To examine whether the activation of IKK is dependent upon the activation of p38 or vice versa, we exposed cells, with or without AUF1 knockdown, to LPS in the presence or absence of either p38 inhibitor SB203580 or IKK β inhibitor BAY 11-7082. Protein extracts were prepared 2 h following treatment and analyzed by Western blotting. Treatment with SB203580 abolished phosphorylation of MAPKAPK-2 in both

cell lines, as expected (Fig. 4B, p-MAPKAPK-2, compare lane 2 with lane 3 and lane 7 with lane 8), while LPS-mediated phosphorylation of IKK remained constant (Fig. 4B, p-IKK α / β , compare lane 2 with lane 3 and lane 7 with lane 8). Similarly, treatment with BAY 11-7082 abolished LPS-mediated phosphorylation of IKK α / β in both cell lines, as expected (Fig. 4B, p-IKK α / β , compare lane 2 with lane 4 and lane 7 with lane 9). However, treatment with BAY 11-7082 did not abolish LPS-mediated phosphorylation of MAPKAPK-2 (Fig. 4B, p-MAPKAPK-2, lanes 4 and 9). These data indicate that phosphorylation of IKK and MAPKAPK-2 occurs independently. Thus, the observation that AUF1 knockdown impairs both the NF- κ B and the p38 MAPK pathway could be due to effects of AUF1 on (i) individual genes within both signaling pathways or (ii) common upstream modulator(s) that activate these genes. The observation that blocking the NF- κ B pathway with BAY11-7082 increases the activation of MAPKAPK-2 (Fig. 4B) supports the latter conclusion.

In addition to MyD88-dependent activation of NF- κ B and MAPK signaling, LPS binding to TLR4 triggers MyD88-independent signaling pathways via TRIF (3). This pathway signals activation of IRF3, leading to the production of type I IFNs that subsequently activate STAT3 by phosphorylation of Ser727 and Tyr705 residues on STAT3. Activation of STAT3 has been implicated in the LPS-mediated expression of *IL10* (4). As well, the importance of type I IFNs in LPS-induced *IL-10* synthesis is well documented (8). We therefore examined whether the inhibition of LPS-induced *IL10* expression upon AUF1 knockdown is due to failure to activate this pathway. We first compared STAT3 phosphorylation in shAUF1- and shCTRL-expressing cells exposed to LPS. There was no significant difference in serine or tyrosine phosphorylation of STAT3 in the two cell lines after LPS exposure (Fig. 5A, p-STAT3, compare lanes 4 to 6 with lanes 10 to 12). The phosphorylation of STAT1 was also comparable in shAUF1- and shCTRL-expressing cells (Fig. 5A, p-STAT1^{Tyr701}, compare lanes 4 to 6 with lanes 10 to 12). In addition, the induction of *CXCL10* (IFN-inducible gene CXC-chemokine ligand 10, also known as IP10) mRNA was comparable for shCTRL- and shAUF1-ex-

FIG. 6. AUF1 knockdown reduces NF- κ B activity. (A) Cells expressing the indicated shRNAs were exposed to LPS (1 μ g/ml) for the indicated times, and nuclear extracts were prepared. NF- κ B activity was analyzed by EMSA. Lane 1 is a reaction mixture without nuclear extract. (B) Oligonucleotides containing NF- κ B (lanes 5, 6) or IFN- γ activation sequence (GAS) (lanes 3, 4) were used as specific and nonspecific competitors, respectively. Numbers represent molar excess over 32 P-labeled oligonucleotide. Lane 1 is a reaction mixture without nuclear extract. (C) Antibody supershift was performed with anti-p65, anti-p50, and control antibodies (Ab; normal mouse and rabbit IgG). Lane 1 is a reaction mixture without nuclear extract. Arrow shows the supershifted band in lane 4. (D) Cells expressing shCTRL or shAUF1 were transfected with pIL-6 luciferase reporter plasmid together with *Renilla* luciferase plasmid pRL-Tk. Twenty-four hours after transfection, cells were exposed to LPS (1 μ g/ml) for 24 h, and cell lysates were analyzed for luciferase activities. Results are expressed as the ratio of firefly and *Renilla* luciferase activities. Values are the means of two independent experiments, with standard deviations shown as error bars.

pressing cells upon LPS exposure (Fig. 5B). The activation of STAT1 and the expression of *CXCL10* are strictly dependent on endogenous type I IFN signaling via IFNAR1 (type I IFN receptor). It is worth noting that studies with mice deficient in IFN- α/β receptor showed that the expression of *CXCL10* in response to LPS is a secondary consequence of IFN- β production (16, 43). These results indicate that the type I IFN pathway is unaffected by AUF1 knockdown. Thus, reduced expression of *IL10* upon AUF1 knockdown is not due to failure to activate the type I IFN pathway.

To determine whether the reduced phosphorylation of IKK (Fig. 4) correlates with reduced nuclear translocation and DNA binding by NF- κ B, we performed electrophoretic mobility shift assays (EMSA). The LPS-mediated DNA-binding activity was reduced by 10- and 3.3-fold in shAUF1-expressing cells at 1 and 2 h, respectively, compared to the levels in control cells (Fig. 6A, compare lanes 8 to 10 with lanes 3 to 5). The specificity of DNA binding was demonstrated by comparing competition with specific (Fig. 6B, lanes 5 to 6) and with nonspecific oligonucleotide competitor (Fig. 6B, lanes 3 to 4). The results of antibody supershift experiments indicate that p50 (NF- κ B1), a member of the NF- κ B family, interacts with the NF- κ B binding sequence in these cells (Fig. 6C, lane 4,

arrow); supershift with anti-p65 antibody was negative (Fig. 6C, lane 6). The role of p50 in the LPS-mediated expression of *IL10* has been demonstrated in murine macrophages (7).

Transactivation assays were also performed to compare with the DNA-binding data. A NF- κ B-responsive luciferase reporter plasmid was transfected into shAUF1-expressing and control cells (39). Following LPS exposure, a significant increase in luciferase activity was observed in control cells. By contrast, the LPS-mediated activation of luciferase activity was greatly reduced in shAUF1-expressing cells (Fig. 6D). Taken together, these results indicate that AUF1 modulates the LPS-mediated activation of NF- κ B and gene expression.

AUF1 controls an upstream mediator of IKK. Activation of the IKK complex is essential for NF- κ B activation by various inflammatory stimuli (14). We showed above that phosphorylation of IKK is reduced upon AUF1 knockdown following exposure to LPS (Fig. 4), consistent with reduced activation (i.e., phosphorylation) of I κ B α in these cells. To examine whether the reduced level of activated IKK α/β upon AUF1 knockdown and LPS exposure is due to reduced levels of IKK subunits, we compared the levels of the three subunits by Western analyses with specific antibodies. No significant differences in the levels of IKK α , IKK β , or IKK γ /NEMO were

FIG. 7. IKK and TAK1 activation is reduced upon AUF1 knockdown. (A and B) shCTRL- and shAUF1-expressing cells were exposed to LPS (1 μ g/ml) and collected at the indicated times, and lysates were analyzed by Western blotting with antibodies to detect proteins listed at the right of each panel. Asterisk shows a nonspecific band.

observed between shAUF1- and shCTRL-expressing cells (Fig. 7A, IKK α , IKK β , and NEMO). These results led to the hypothesis that upstream mediators that participate in the phosphorylation of IKK subunits are impaired upon AUF1 knockdown.

The engagement of IL-1R/TLR leads to activation of IKK and JNK/p38 MAPK signaling via activation of the upstream kinase TAK1 (TGF- β -activated kinase 1) (40). TAK1, a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family also known as MAP3K7, was originally identified as a major regulator of MAPK activation by TGF- β (47). TAK1 is recruited into the IKK complex through a polyubiquitin chain and activates transcription factor NF- κ B. Additionally, two adaptor proteins, TAB1 and TAB2, associate with TAK1. TAB1 interacts with TAK1 constitutively, while TAB2 translocates from the cell membrane to the cytoplasm upon stimulation of IL-1R/TLR and links TAK1 to TRAF6. TAK1 is critical for regulated activation of multiple protein kinases during LPS signaling (17). To determine whether TAK1 and its adaptor proteins are involved in modulation of IKK activation by AUF1, we exposed cells expressing shAUF1 and shCTRL to LPS for various times and examined protein extracts by Western blotting. The active form of TAK1 was detected with a phospho-Thr¹⁸⁷-specific TAK1 antibody. LPS exposure reduced the levels of phosphorylated TAK1 upon AUF1 knockdown compared to the levels in control cells (Fig. 7B, p-TAK1, compare lanes 7 to 8 with lanes 3 to 4). This is likely due to reduced levels of total TAK1 protein upon AUF1 knockdown (Fig. 7B, TAK1, compare lanes 5 to 8 with lanes 1 to 4). By contrast, reduced AUF1 had little effect upon cellular levels of TAB1, TAB2, or TRAF6 (Fig. 7B). These results suggest that reduced levels of total (and phosphorylated) TAK1 upon AUF1 knockdown lead to reduced phosphorylation of IKK α / β and, consequently, inhibition of IkB α phosphorylation.

p40^{AUF1} restores TAK1 levels, the signaling cascade, and IL10 expression. We have shown that the expression of shAUF1-resistant p40^{AUF1} cDNA (p40^R) in cells with AUF1 knockdown restores LPS-induced IL10 expression (33). To determine whether rescue of IL10 expression in p40^R cells correlates with restoration of signaling pathways involved in LPS-mediated IL10 expression, we compared the levels of phosphorylated IKK α / β and MAPKAPK-2 in cells expressing shCTRL, shAUF1, or shAUF1 plus p40^R. The expression of p40^R in cells with AUF1 knockdown restored the

phosphorylation of IKK α / β to levels comparable with the levels in cells expressing shCTRL (Fig. 8A, p-IKK α / β , compare lanes 8 and 9 with lanes 2 and 3). In addition, the phosphorylation of MAPKAPK-2, a downstream substrate of p38 MAPK, was also restored in cells complemented with p40^R (Fig. 8A, p-MAPKAPK-2, compare lanes 7 to 9 with lanes 1 to 3). We then examined whether p40^R expression restores the activation of TAK1, which lies upstream of IKK and p38 MAPK. The phosphorylation of TAK1 was comparable in cells complemented with p40^R and cells expressing shCTRL, indicating that p40^{AUF1} restores TAK1 activation (Fig. 8B, p-TAK1, compare lanes 10 to 12 with lanes 2 to 4). In addition, p40^R increased the level of TAK1 protein (Fig. 8B, TAK1, compare lanes 9 to 12 with lanes 1 to 4). Thus, restoration of TAK1 abundance and activation in cells expressing p40^R correlated with the restoration of nuclear translocation and DNA binding of NF- κ B (Fig. 8C), as well as IL10 expression (Fig. 8D). These results demonstrate that p40^{AUF1} regulates TAK1 gene expression in monocytes to permit NF- κ B and MAPK signaling. Likewise, p40^{AUF1} expression promotes LPS-mediated induction of a number of NF- κ B- and p38 MAPK-dependent genes, including IL1 α , IL1 β , TNF α , and chemokines CCL20 and CXCL3, to various degrees (Fig. 8E). As such, p40^{AUF1} promotes the expression of a host of NF- κ B target genes.

TAK1 is sufficient to restore NF- κ B binding and gene expression upon AUF1 knockdown. Complementation with p40^{AUF1} in AUF1 knockdown cells restores TAK1 abundance and phosphorylation and, consequently, IL10 expression upon LPS exposure (Fig. 8B and D). We asked whether elevating TAK1 abundance in shAUF1-expressing cells would restore NF- κ B activation and gene expression. We therefore transfected shAUF1-expressing cells with a TAK1 expression vector. The results of Western blotting demonstrated that the abundance of TAK1 was 2- to 3-fold higher than in cells expressing shAUF1 alone (Fig. 9A). The activation of NF- κ B by LPS was analyzed by EMSA in cells expressing shCTRL, shAUF1, and shAUF1 plus TAK1. As before (Fig. 6), in cells expressing shCTRL, LPS induced NF- κ B binding which peaked at 2 h and diminished thereafter (Fig. 9B, lanes 2 to 6); as expected, knockdown of AUF1 reduced NF- κ B binding, but binding was sustained even to 8 h (Fig. 9B, lanes 7 to 11; see Discussion). By contrast, elevating the abundance of TAK1 in cells expressing shAUF1 increased NF- κ B binding to levels even higher than those in cells expressing shCTRL (Fig. 9B,

FIG. 8. Complementation with shAUF1-refractory p40^{AUF1} restores LPS-mediated signaling pathways and cytokine expression. Cells expressing shCTRL, shAUF1, or shAUF1 plus shRNA-refractory p40^{AUF1} (p40^R) were exposed to LPS (1 μ g/ml) for the indicated times, and RNA and protein were extracted. (A and B) Proteins were analyzed by Western blotting with antibodies to proteins shown on the right of each panel. (C) Cells expressing shCTRL, shAUF1, or shAUF1 plus shRNA-refractory p40^{AUF1} (p40^R) were exposed to LPS (1 μ g/ml) for the indicated times, and nuclear extracts were prepared. NF- κ B activity was analyzed by EMSA. Lane 1 is a reaction mixture without nuclear extract. NF- κ B DNA-binding activity was compared in shCTRL- (lanes 2 to 6), shAUF1- (lanes 7 to 11), and shAUF1-plus-p40^R-expressing cells (lanes 12 to 16). (D) qRT-PCR for *IL10* mRNA. Numbers represent percent mRNA compared to the amount in cells expressing shCTRL, which was set to 100%. (E) LPS-mediated levels of *IL1 α* , *CCL20*, *TNF α* , *IL1 β* , and *CXCL3* mRNAs at 4 h of LPS exposure determined by qRT-PCR. Levels are in comparison to levels in cells expressing shCTRL, which were set to 1 for each gene. Values are the means of two independent experiments, with standard deviations shown as error bars.

lanes 13 to 16). This binding was also sustained even to 8 h (see Discussion). Consistent with increased NF- κ B binding in these cells compared to the rate of binding in cells expressing shAUF1 only, the abundance of *IL10*, *IL1 β* , and chemokine *CCL20* mRNAs was also 3- to 4-fold higher (Fig. 9C). Together, the results shown in Fig. 8 and 9 are consistent with AUF1 control of TAK1 expression being a central regulatory point for NF- κ B signaling. We next considered how AUF1 might regulate TAK1 abundance.

Posttranscriptional control of TAK1 gene expression by AUF1. To address possible mechanisms by which AUF1 might regulate TAK1, we examined whether the reduced abundance of TAK1 is due to reduced accumulation of *TAK1* mRNA

upon AUF1 knockdown. However, the *TAK1* mRNA levels in shCTRL-, shAUF1-, and shAUF1-plus-p40^R-expressing cells were comparable in all three cell lines, even in cells exposed to LPS for 2 and 4 h (Fig. 10A). In addition, the levels of cytoplasmic *TAK1* mRNA were comparable in these cell lines (data not shown). Thus, differences in mRNA levels cannot account for the observed differences in TAK1 protein abundance in the cell lines. Another possibility was a significant reduction in the half-life of TAK1 protein upon AUF1 knockdown. However, protein stability assays revealed this not to be the case, as AUF1 knockdown had little effect on protein half-life compared to that in cells expressing shCTRL (10 h versus 9 h, respectively) (Fig. 10B).

FIG. 9. TAK1 expression restores NF- κ B DNA-binding activity. (A) shAUF1-expressing cells were transfected with *TAK1* expression plasmid. After selection for stably transfected cells, TAK1 abundance was examined by Western blotting. (B) NF- κ B DNA-binding activity was compared in shCTRL- (lanes 2 to 6), shAUF1- (lanes 7 to 11), and shAUF1+TAK1-expressing cells (lanes 12 to 16) by EMSA. (C) LPS-mediated levels of *IL10*, *CCL20*, and *IL1 β* mRNAs in shCTRL-, shAUF1-, and shAUF1+TAK1-expressing cells at 4 h of LPS exposure were determined by qRT-PCR. Levels are in comparison to levels in cells expressing shCTRL, which were set to 1 for each gene. Values are the means of three independent experiments, with standard deviations shown as error bars.

Nonetheless, mRNP immunoprecipitation with AUF1 antibody and microarray analyses of the purified mRNAs revealed that *TAK1/MAP3K7* mRNA is among a large number of AUF1-associated targets (see Fig. S1 in the supplemental material). Indeed, visual inspection of the *TAK1* 3'-UTR reveals a candidate class I ARE (i.e., AUUUA motifs within a U-rich sequence; GenBank accession no. NM_145331). To confirm that *TAK1* mRNA is a binding target of AUF1, mRNP immunoprecipitation was again performed, followed by qRT-PCR with cytoplasmic lysates of THP-1 cells. AUF1 antiserum precipitated >4-fold more *TAK1* mRNA than was immunoprecipitated with preimmune serum from the cytoplasmic lysates of THP-1 cells (Fig. 10C). No difference was observed in the amounts of *GAPDH* mRNA precipitated with anti-AUF1 and preimmune serum. Taken together, these observations are consistent with a mechanism whereby AUF1 knockdown de-

creases the translation of *TAK1* mRNA, as we have observed for *MYC* mRNA (18).

To address this question, we performed polyribosome profile experiments with cytoplasmic lysates of THP-1 cells expressing shCTRL or shAUF1. In shCTRL-expressing cells, ~75% of *TAK1* mRNA cosedimented with one to three ribosomes (Fig. 11C, fractions 7 to 9, solid bars). By contrast, upon AUF1 knockdown, ~50% of *TAK1* mRNA cosedimented with one to three ribosomes (Fig. 11C, fractions 7 to 9, open bars). AUF1 knockdown also increased the cosedimentation of *TAK1* mRNA with 40S/60S/80S subunits to 40% of total mRNA, compared to 15% for control cells (Fig. 11C, fractions 4 to 6, compare open bars with closed bars). AUF1 knockdown had no discernible effects on either the distribution of *GAPDH* mRNA (Fig. 11D) or the bulk distribution of polyribosomes (compare Fig. 11A and B). Thus, the effects of AUF1 knockdown on the distribution of *TAK1* mRNA are specific. As expected, the preparation and fractionation of extracts in the presence of EDTA disrupted polyribosomes and increased the abundance of free 40S and 60S ribosomal subunits (compare Fig. 11E and F with A and B). Also as expected, EDTA shifted the bulk of both *TAK1* and *GAPDH* mRNA to the lighter gradient fractions 1 to 3 (Fig. 11G and H, respectively). Together, these results indicate that AUF1 promotes the initiation step(s) of *TAK1* translation.

DISCUSSION

IL-10 plays a pivotal regulatory role in inflammation. Many chronic diseases, such as psoriasis, rheumatoid arthritis, inflammatory bowel diseases, multiple sclerosis, and asthma are linked to improper regulation of inflammatory responses. Furthermore, a frequent correlation between chronic inflammation and tumor development has been observed (10). Thus, the regulation of IL-10 is of clinical interest due to its anti-inflammatory and immunosuppressive properties. We showed previously that depletion of AUF1 in THP-1 monocyte cells suppresses the LPS-mediated induction of *IL10* without significantly affecting the degradation of *IL10* mRNA and that ectopic expression of the p40 AUF1 isoform selectively rescues LPS-mediated *IL10* expression (33). This led to the (correct) hypothesis that AUF1 regulates *IL10* expression by modulating signaling pathways that regulate the transcription of *IL10* during inflammatory responses.

Activation of both the NF- κ B and p38 MAPK pathways is essential for the induction of inflammatory genes in response to LPS exposure. Indeed, blocking either pathway with small-molecule inhibitors blocks the induction of *IL10* expression (Fig. 3). Also, knockdown of AUF1 impairs both these pathways (Fig. 4), consistent with the aforementioned hypothesis. As the importance of the NF- κ B pathway for *IL10* expression is well documented (7, 20, 32), we focused in this study on the mechanisms by which AUF1 controls NF- κ B signaling.

LPS interaction with CD14 promotes dimerization of Toll-like receptor 4 (TLR4) and the subsequent recruitment of adaptor molecule MyD88. MyD88 then recruits the serine/threonine kinases interleukin-1 receptor-associated kinase 4 (IRAK4) and IRAK1 to initiate the activation of a cascade of kinases. IRAK4 phosphorylates IRAK1, which mediates the recruitment of tumor necrosis factor receptor-associated factor

FIG. 10. Effects of AUF1 on *TAK1* gene expression. (A) Total RNA was extracted from cells expressing shCTRL, shAUF1, or shAUF1 plus shRNA-refractory p40^{AUF1} (p40^R) following LPS exposure for 0, 2, or 4 h. RNAs were analyzed by qRT-PCR with *TAK1*-specific primers. RNA levels are relative to the levels in untreated cells. The mRNA levels of untreated cells were set to 1. Values are the means of two independent experiments, with standard deviations shown as error bars. (B) shCTRL- and shAUF1-expressing cells were cultured with cycloheximide for the indicated times. Cell lysates were prepared and analyzed by Western blotting with anti-TAK1 and anti-tubulin antibodies. Signals were quantified to plot data as percent protein remaining versus time. Nonlinear regressions yield first-order decay constants and protein half-lives. (C) *TAK1* mRNA associates with AUF1 in cells. Cytoplasmic lysates from THP-1 cells were used for mRNP immunoprecipitation assays with preimmune and AUF1 antisera. Precipitated RNA was quantitated by qRT-PCR with *TAK1*-

6 (TRAF6) to the receptor complex. The IRAK1-TRAF6 complex then dissociates from the receptor to interact with and activate TAK1. TRAF6 is a ubiquitin ligase that may catalyze autopolyubiquitination by Lys63-linked ubiquitin chains. TAK1-interacting proteins TAB1 and TAB2 then bind these chains, initiating the formation and activation of the TAK1 complex by phosphorylation of TAK1. TAK1 in turn phosphorylates the IKK β subunit of IKK, which phosphorylates I κ B, leading to its destruction within the NF- κ B/I κ B complex by proteasomes. NF- κ B subsequently translocates to the nucleus, where it activates its target genes, including *IL10*. Indeed, cells from cells from *Tak1*^{-/-} mice present impaired activation of the IKK complex when exposed to proinflammatory cytokines compared to the activation of the IKK complex in cells from wild-type mice. Furthermore, ectopic expression of TAK1 restores the activation of the IKK complex in *Tak1*^{-/-} cells (35). TAK1 functions as a branching signal transducer downstream of MyD88, IRAK1, and TRAF6 in the NF- κ B and MAPK pathways.

Our key initial finding was that knockdown of AUF1 reduces the levels of phosphorylated I κ B α in response to LPS exposure compared to the levels in cells expressing a control shRNA (Fig. 4A and 7A). This would serve to maintain NF- κ B in the cytoplasm and dampen the activation of its target genes (e.g., *IL10*). We thus worked our way backward through the signaling pathway to determine the upstream protein(s) whose expression is affected by AUF1 knockdown and could therefore account for the reduced I κ B α phosphorylation. Indeed, AUF1 knockdown significantly reduced the levels of the kinase TAK1 (Fig. 7B) without significantly affecting its associated proteins, TAB1 and TAB2, or its upstream activator, TRAF6. As noted above, the TAK1 kinase normally phosphorylates the IKK α / β subunit of IKK, but AUF1 knockdown reduced phospho-IKK α / β levels and, thus, reduced both I κ B α phosphorylation and NF- κ B binding. Complementation with a TAK1 expression vector in cells with AUF1 knockdown restored NF- κ B activation in these cells. This indicates that the regulation of *TAK1* gene expression is the focal point of AUF1 control of NF- κ B signaling.

We note that, in extracts of cells expressing either shAUF1 alone or shAUF1 plus TAK1 cDNA, DNA binding by NF- κ B is less transient than that in control cells (Fig. 6A and 9B). In shAUF1-expressing cells, low-level but sustained binding may be due to the failure of I κ B to remove NF- κ B from the nucleus. Upon LPS binding to TLR4, I κ B, encoded by a canonical NF- κ B-responsive gene, is phosphorylated and degraded, allowing NF- κ B to translocate to the nucleus. I κ B is resynthesized later in a NF- κ B-dependent manner and transports NF- κ B back to the cytoplasm. It is notable that the level of I κ B is reduced in untreated shAUF1-expressing cells (Fig. 4A, I κ B α). Due to impaired NF- κ B signaling in shAUF1-express-

and *GAPDH*-specific primers to determine mRNA levels as the ratio between immune/preimmune precipitations, i.e., fold enrichment. As expected for *GAPDH*, there was no difference in *C_T* values between immune and preimmune precipitations, hence its ratio is 1. Values are the means of three independent experiments, with standard deviations shown as error bars.

FIG. 11. AUF1 abundance affects polyribosome distribution of *TAK1* mRNA. Cytoplasmic lysates of THP-1 cells expressing shCTRL or shAUF1 were subjected to polyribosome profile analyses. (A, B) Representative polyribosomal profile from cells expressing shCTRL (A) and shAUF1 (B) cells. The 254-nm traces obtained during collection of fractions are shown with positions of free RNP, 40S and 60S ribosomal subunits, 80S ribosome, and polyribosomes indicated at the top. (C, D) Relative distributions of *TAK1* (C) and *GAPDH* mRNAs (D) in polyribosome gradients after AUF1 knockdown. Data shown are means $\pm$ standard deviations from two independent experiments. (E, F) Representative polyribosomal profiles of EDTA-treated lysates from THP-1 cells expressing shCTRL (E) and shAUF1 (F). (G, H) Relative distributions of *TAK1* (G) and *GAPDH* mRNAs (H) in polyribosome gradients with EDTA-treated cell lysates. Data shown are means $\pm$ standard deviations from two independent experiments.

ing cells, I κ B is not resynthesized following degradation. As a result, NF- κ B remains DNA bound longer upon AUF1 knockdown. Complementation of TAK1 expression upon AUF1 knockdown restores NF- κ B DNA binding that is also less transient than that of shCTRL-expressing cells. The transfected *TAK1* transgene is likely not regulated by AUF1, probably due to our intentional exclusion of the 3'-UTR. Together, these results suggest the importance of an AUF1-*TAK1* mRNA interaction for the regulation of LPS-mediated NF- κ B signaling. Thus, this regulation cannot take place, presumably due to reduced abundance of AUF1 in shAUF1-expressing cells and the lack of a *TAK1* 3'-UTR in the *TAK1* expression vector in cells expressing the shAUF1 plus *TAK1* open reading frame.

How does AUF1 control the levels of TAK1? While knockdown of AUF1 reduces TAK1 abundance, it does not affect the levels of *TAK1* mRNA (Fig. 10A and data not shown) or TAK1 protein stability (Fig. 10B). However, mRNP immunoprecipitation with AUF1 antibody revealed that *TAK1* mRNA is an AUF1-associated target (Fig. 10C). The simplest interpretation of these results is that AUF1 promotes the translation of *TAK1* mRNA. Indeed, AUF1 affects the translation of *TAK1* mRNA by promoting the initiation step(s) (Fig. 11). This is not without precedence, since AUF1 promotes the translation of *MYC* mRNA by binding the AU-rich element (ARE) within its 3'-UTR (18). However, future work will be required to fully elucidate the mechanisms by which AUF1 controls the translation of *TAK1* mRNA.

Nonetheless, transfection of an shRNA-refractory p40^{AUF1} cDNA but not of p37^{AUF1} selectively restores the LPS-mediated induction of *IL10* (33). Significantly, p40^{AUF1} elevates TAK1 protein levels and, consequently, the levels of phosphorylated TAK1 (Fig. 8B). As expected, p40^{AUF1} also restores the LPS-induced activation of IKK α / β (Fig. 7A) and *IL10* expression (Fig. 8D). Also, p40^{AUF1} elevates p38 MAPK signaling (i.e., phospho-MAPKAPK-2) (Fig. 8A). However, identification of AUF1 targets within the p38 MAPK pathway and how AUF1 controls these signaling events will require future work. Finally, p40^{AUF1} does not alter the cytoplasmic levels of *TAK1* mRNA; also, AUF1 knockdown does not alter the stability of TAK1 protein. These observations are consistent with the aforementioned translational-activation mechanism.

Overproduction of proinflammatory cytokines due to mRNA stabilization occurs in AUF1 knockout mice (22). Thus, AUF1 plays a critical role in limiting proinflammatory cytokine production upon LPS challenge. Our research has led to a novel mechanism of AUF1-mediated regulation of inflammatory responses by modulation of the NF- κ B and p38 MAPK pathways through regulation of the upstream mediator TAK1. Proinflammatory stimuli like cytokines and microbial products activate TAK1 upstream of IKK and MAPKs (JNK and p38 MAPK). The inducible transcription factors NF- κ B, which is activated by IKK, and AP-1, which is activated by JNK and p38 MAPK, are crucial mediators of multiple aspects of inflammatory responses. Thus, p40^{AUF1}, via its interaction with *TAK1* mRNA, regulates inflammatory responses. Further studies will address issues such as how LPS affects this interaction. In conclusion, our data indicate that functional interactions between RNA-binding protein AUF1 and TAK1, a critical component of inflammatory signaling pathways, has profound

effects upon cytokine production during inflammatory responses.

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