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AUF1 Isoform-Specific Regulation of Anti-inflammatory *IL10* Expression in Monocytes

Srijata Sarkar,[1] Kristina S. Sinsimer,[2] Randi L. Foster,[1] Gary Brewer,[1] and Sidney Pestka[1]

IL-10 is an immunomodulatory cytokine that regulates inflammatory responses of mononuclear phagocytes (monocytes and macrophages). Mononuclear cells exposed to microbes or microbial products secrete a host of proinflammatory cytokines followed by delayed onset of anti-inflammatory IL-10. IL-10 suppresses immune responses by inhibiting cytokine production by mononuclear phagocytes. Using THP-1, a human promonocytic leukemia cell line, we show that endotoxin/lipopolysaccharide (LPS) exposure induces *IL10* expression while IFN-γ blocks this LPS-mediated effect. IFN-γ is an important modulator of IL-10 production during infectious diseases. We show that LPS and IFN-γ regulate *IL10* expression in THP-1 cells in part through posttranscriptional mechanisms. Our results demonstrate that 3′-untranslated region (3′-UTR) AU-rich elements (AREs) decrease expression of a chimeric luciferase reporter gene in THP-1 cells. The ARE-binding protein AUF1 binds the *IL10* 3′-UTR. Depletion of AUF1 by RNAi suppresses LPS-mediated induction of *IL10* mRNA and protein without affecting LPS-mediated stabilization of *IL10* mRNA. Upon complementation with either RNAi-refractory p37 or p40 AUF1 plasmids, only p40 restores LPS-mediated induction of *IL10* mRNA and protein to near normal levels. Thus, the p40 AUF1 isoform selectively plays a critical, positive role in *IL10* expression upon LPS exposure.

Introduction

Inflammation, an important host response to injury and infection, causes tissue damage if prolonged. Therefore, it is crucial to preserve a balance between inflammation and tissue homeostasis (Adams and Hamilton 1992; Nathan and Cohn 1995). IL-10 is an immunomodulatory cytokine that plays an important role in suppression of inflammatory responses (Moore and others 2001). Mononuclear phagocytes (macrophages and monocytes), when exposed to microbes or microbial products, produce a host of proinflammatory mediators such as TNF-α, IL-1, and IL-12, followed by delayed onset of the anti-inflammatory cytokine IL-10. IL-10 exerts its anti-inflammatory actions by blocking both production of proinflammatory cytokines by macrophages and their ability to serve as antigen-presenting or costimulatory cells (Bogdan and others 1991; de Waal Malefyt and others 1991; D'Andrea and others 1993).

IFN-γ is an important modulator of IL-10 biosynthesis during infectious diseases (Libraty and others 1997; Chomarat and others 1993; Donnelly and others 1995). IFN-γ, a potent activator of mononuclear phagocytes, enhances their tumoricidal and microbicidal activities (Adams and Hamilton 1984), thus allowing them to play a crucial role in acute and chronic inflammatory responses. IFN-γ increases cytokine production by activated monocytes during inflammation, partly through silencing of *IL10* gene expression. However, the molecular basis of IFN-γ-mediated regulation of IL-10 expression is largely unknown. Because IL-10 plays a pivotal role in regulation of inflammatory processes and pathogenesis of various diseases, it is of critical importance to determine how inflammatory stimuli regulate IL-10 expression.

During the course of an inflammatory response, a balance between transcriptional induction and posttranscriptional gene silencing (mRNA degradation and translation repression) regulates cytokine gene expression. Interactions of 3′-untranslated region (3′-UTR) AU-rich elements (AREs) with ARE-binding proteins and microRNAs control degradation and translation of numerous cytokine mRNAs (Brewer 1991; Schiavi and others 1992; Zhang and others 1993; Ehrenman and others 1994; Chen and Shyu 1995; Buzby and others 1996; DeMaria and Brewer 1996; Kiledjian and

[1]Department of Molecular Genetics, Microbiology, and Immunology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, New Jersey.
[2]Department of Molecular Biology, Princeton University, Princeton, New Jersey.

others 1997; Wilson and Brewer 1999; Bakheet and others 2001; Jing and others 2005; Ouellet and others 2006; Engels and Hutvagner 2006; Zhang and others 2007). AREs contain a variable number of often overlapping AUUUA pentamers, frequently surrounded by U-rich sequences. Many ARE-binding proteins are known. These include AUF1 (Zhang and others 1993; Ehrenman and others 1994; Buzby and others 1996; DeMaria and Brewer 1996; Kiledjian and others 1997), ELAV-like proteins Hel-N1, HuC, HuD, and HuR (Levine and others 1993; Chung and others 1996; Ma and others 1996), Tristetraprolin (TTP) (Carballo and others 1998; Carballo and others 2000), TIA-1/TIAR (Kedersha and others 1999; Mazan-Mamczarz and others 2006), and Hsp70 (Laroia and others 1999). Among these, AUF1 and TTP destabilize ARE-mRNAs while HuR stabilizes mRNA. Current models of ARE-mediated mRNA decay (AMD) suggest that ARE-binding proteins target specific mRNAs to cellular ribonucleolytic activities that remove the 3′-poly (A) tail and 5′-cap, followed by exoribonucleolytic degradation (Decker and Parker 1994; Wilusz and others 2001).

To define molecular mechanisms responsible for *IL10* expression in response to inflammatory stimuli such as lipopolysaccharide (LPS) and IFN-γ, we used the human promonocytic leukemia cell line THP-1. Many transcription factors including STAT3, Sp1, Sp3, IRAK1, C/EBPβ, and C/EBPδ promote LPS-mediated transcriptional induction of *IL10* expression (Benkhart and others 2000; Tone and others 2000; Liu and others 2003; Huang and others 2004). Powell and others (2000) reported that the 3′-UTR directs posttranscriptional regulation of *IL10* in murine macrophages. Our previous study demonstrated that AUF1 binds *IL10* AREs and implicated AUF1 in constitutive expression of *IL10* in the MNT-1 melanoma cell line (Brewer and others 2003). In the present study, we focused on posttranscriptional regulation of human *IL10* in monocytes. We show that LPS and IFN-γ modulate *IL10* mRNA degradation. Moreover, using RNAi to reduce AUF1 abundance followed by complementation with specific AUF1 isoforms, we show that the p40 AUF1 isoform plays a vital role in LPS-mediated induction of *IL10* in monocytes.

Materials and Methods

Reagents

LPS (*Escherichia coli* 055:B5 and 0127:B8; Sigma) was dissolved in complete medium, aliquoted, and stored at –20°C. Other reagents were obtained from the following sources: IFN-γ (PBL Biomedical Laboratories), RPMI-1640 medium (Invitrogen), Pen/Strep/l-glutamine (Invitrogen), fetal bovine serum (Hyclone), and strepavidin-HRP (Jackson Immunoresearch). Rat antihuman IL-10 antibody 9D7 and rat antihuman IL-10 (biotinylated) 12G8 were kindly provided by Dr. Satwant Narula (Schering Plough Corporation).

Cell culture and transfection

THP-1, a human promonocytic leukemia cell line, was maintained in RPMI-1640 medium, 10% fetal bovine serum, and pen/strep/l-glutamine in a 5% CO_2 environment at 37°C. HeLa S3 cells were cultured in DMEM and 10% fetal bovine serum in a 5% CO_2 environment at 37°C. THP-1 cells (2×10^6) were transfected with 1 μg of firefly luciferase-3′-UTR constructs and 40 ng of *Renilla* luciferase plasmid as an internal control using Effectene reagent (Qiagen).

RNA extraction and real-time RT-PCR

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen) and DNA was removed from RNA samples by RNase-free DNase treatment (Qiagen). Total RNA (250–1,000 ng) was reverse transcribed to cDNA with the Taqman reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol. The resulting cDNA was analyzed by PCR with Platinum SYBR® green qPCR SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Specific primer sets used for human *IL10, IL10R1,* and β-*actin* mRNAs were designed using Oligoperfect™ Designer software (Invitrogen). Forward and reverse primers for each are listed in Table 1. Target gene expression levels were normalized to β-*actin* mRNA in all experiments. Levels of cDNA were calculated by the relative quantitation (ΔΔ*Ct*) method (Pfaffl 2001). Data were plotted relative to the untreated control.

ELISA

Cells (3×10^6) were plated into six-well dishes and treated with appropriate stimuli for different intervals. Culture supernatants were collected at various time points. IL-10 concentration was determined by ELISA. In brief, Immulon4 (Labcor Products, Inc.) plates were coated with rat monoclonal antibody 9D7 (rat antihuman IL-10) at 2.5 μg/mL in PBS overnight at room temperature. Plates were blocked with 3% BSA in PBS for 1 h at room temperature followed by incubation with cell supernatant overnight at room temperature. Plates were washed with 0.1% Tween20 in PBS, incubated with monoclonal antibody 12G8 (rat antihuman IL-10 biotinylated) at 1:20,000 dilution at room temperature for 1 h, incubated with strepavidin-HRP (1 μg/mL in PBS/1% BSA), washed extensively with 0.1% Tween20 in PBS, developed with TMB substrate, and read at 450 nm.

Table 1. Sequence of Real-Time RT-PCR Primers

Gene	*Forward primer*	*Reverse primer*
hsa-*IL10*	5′ CTTTAAGGGTTACCTGGGTTGC 3′	5′ CCTTGATGTCTGGGTCTTGGT 3′
hsa IL10R1	5′ TTCAGCCTCCTAACCTCTGGA 3′	5′ AATACTGCCTGGTGAGGGAGA 3′
hsa β-*actin*	5′ CCATCATGAAGTGTGACGTGGA3′	5′ TTCTGCATCCTGTCGGCAA 3′

mRNA decay assays

Cells were incubated with 5 μg/mL actinomycin D (Calbiochem) to inhibit transcription. At various times, cells were harvested and total RNA was purified. Levels of *IL10* mRNA were quantified at each time point by real-time RT-PCR and were normalized to β-*actin* mRNA levels. First-order decay constants (*k*) and associated mRNA half-lives were calculated from plots of percent *IL10* mRNA remaining *versus* time of actinomycin D treatment by nonlinear regression analyses with Prism 4 software (GraphPad).

Plasmids

Various regions of human *IL10* 3′-UTR indicated in Table 2 (nt 574–980, 933–1,560, 933–1,370, 574–1,563; GenBank accession number M57627) were generated by PCR using primer sets shown in Table 2. PCR fragments were digested with XbaI and cloned into the XbaI site in plasmid pGL3-Promoter (Promega). pEF3neo was constructed from pcDEF3 (Goldman and others 1996) by excising a 347-bp fragment with SacI, and religation of the SacI-linearized plasmid. Plasmid pEF3neo was kindly provided by Dr. Christopher Krause (Robert Wood Johnson Medical School).

Luciferase assay

Lysates were prepared from duplicate samples 24 h after transfection and luciferase activities were determined using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Luminescence was measured using a Turner Design Luminometer (model TD-20/20).

Generation of AUF1 knockdown cells

Plasmids expressing short-hairpin (sh)RNAs targeting *AUF1* mRNAs encoding all four isoforms of AUF1 or expressing a random sequence were constructed as described (Lal and others 2004). To complement expression of single AUF1 isoforms in an *AUF1*-knockdown background, the following strategy was utilized. Six silent point mutations were introduced into the anti-*AUF1* shRNA annealing site of plasmids pBAD/HisB/p37^{AUF1} and pBAD/HisB/p40^{AUF1} to render the encoded mRNAs refractory to RNAi-mediated knockdown (AUF1^{R}). Three sequential rounds of mutations (underlined) were performed utilizing the Stratagene QuikChange Multi Site-Directed Mutagenesis Kit and the following primers (IDT, Coralville, IA, USA) (mutated nucleotide is underlined): Round 1: 5′-GGT GAA GTT GTA GAC TGC ACC CTA AAG TTA GAT CCT-3′; Round 2: 5′-GGT GAA GTT GTA GAT TGT ACC CTA AAG TTA GAT CCT ATC ACA GGG-3′; Round 3: 5′-GGT GAA GTG GTA GAT TGT ACC CTA AAG TTA GAT CCT ATC ACA GGG-3′. Products were sequenced following each round of mutagenesis for confirmation. cDNAs encoding AUF1^{R} isoforms devoid of the His_6-tag were amplified using primers incorporating 5′- KpnI and 3′- EcoRI sites (underlined): forward primer: 5′-GCA CGG TAC CAC TAT GTC GGA GGA GCA GTT C-3′; reverse: 5′-GCA CGA ATT CTT AGT ATG GTT TGT AGC TAT TTT G-3′. PCR products were cloned into pEF3neo digested with the same enzymes to generate pEF3neo/AUF1^{R} plasmids. pEF3neo/AUF1^{R} plasmids were linearized with PvuI and transfected into THP-1 cells using Effectene reagent. Cells were selected with 500 μg/mL Geneticin (GIBCO). Expression of AUF1^{R} was confirmed by Western blot.

shRNA-expressing vectors were linearized with XmnI and transfected into THP-1/AUF1^{R} cells using Effectene reagent. Cells were selected with 500 μg/mL Geneticin and 250 units/mL hygromycin B (Calbiochem). Expression of a single AUF1^{R} isoform was verified by Western blot.

Preparation of cytoplasmic extracts

Cells (2×10^7) were exposed to LPS and/or IFN-γ for 2.5 h or left untreated. Cells were washed with PBS and lysed in 200 μL lysis buffer (40 mM KCl, 10 mM HEPES pH 7.9, 3 mM $MgCl_2$, and 5% glycerol). Following 30-min incubation on ice, the supernatant was clarified by centrifugation for 10 min at 12,000*g*. Protein concentration of the supernatant was determined by Bradford assay (Bio-Rad). Cytoplasmic extracts were aliquoted and stored at –70°C.

Generation of RNA substrate

Plasmid pGEM- *IL10* (574–920) was linearized using HindIII. Labeled RNA was synthesized by incubating 0.5 μg DNA template, 40 mM each of ATP, GTP, and CTP, 4 mM UTP, 10 mM DTT, 40 units RNase inhibitor (Applied Biosystems), 1× transcription buffer (Promega), 50 μCi [α-^{32}P]-UTP (800 Ci/mmol), and 10 units T7 RNA polymerase (Promega) at 37°C for 1 h. DNA template was removed by treating with RQ RNase-free DNase I (Promega) at 37°C for 30 min. Unincorporated nucleotides were removed using G25 spin columns (Amersham).

Electrophoretic mobility shift and supershift assays

In vitro synthesized RNA was diluted to 1×10^5 cpm/μL in 10 mM Tris, pH 8.0, and denatured. RNA (1×10^5 cpm) was incubated with cytoplasmic extract (24–27 μg) in 10 μL

Table 2. Sequences of Primers for Generating *IL10* 3′-UTR Segments

Fragments	*Forward primer*	*Reverse primer*
574–980	5′ACATCTAGAAGGGTGGCGACTCTATAG3′	5′AATTCTAGATAGAGGGAGGTCAGG3′
933–1560	5′AGCTCTAGACTGACCACGCTTTCTAGC3′	5′GCTCTAGAGCTATGAAGACAGACAA3′
933–1370	5′AGCTCTAGACTGACCACGCTTTCTAGC3′	5′AGCTCTAGAAATTCTCTTGCCTCAGCC3′
574–1563	5′ACATCAAGAAGGGTGGCGACTCTATAG3′	5′AAATCTAGATCTGCTATGAAGACAGAC3′

RNA-binding buffer (40 mM KCl, 10 mM HEPES pH 7.9, 3 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 2.5 mM EDTA, 5 µg/mL Pepstatin A, 0.5 mM PMSF, 100 ng yeast tRNA, and 10 units RNasin) for 30 min on ice. The mixture was subsequently treated with RNase T1 (100 units) and RNase A (100 pg) for 15 min on ice followed by addition of heparin (50 µg) and incubation for 10 min on ice. Samples were fractionated in a 5% polyacrylamide gel in 0.25× TBE (22.5 mM Tris-Borate, 0.5 mM EDTA) at 200 V for 3 h at 4°C. Gels were dried and analyzed using a phosphorimager. For antibody supershift assays, cytoplasmic extract was preincubated with 1 µL of antisera for 15 min on ice prior to addition of RNA substrate.

Western blotting

Proteins were fractionated by 7.5% SDS-PAGE, transferred to a PVDF (polyvinylidene difluoride; Bio-Rad) membrane, and incubated for 1 h in blocking solution (3% nonfat dry milk, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.05% Tween20) at room temperature. Blots were incubated with AUF1 anti-serum (1:15,000) or anti-α-tubulin (1:5,000) for 1 h at room temperature, extensively washed in TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.05% Tween20), incubated with HRP-conjugated secondary antibody for 1 hr at room temperature, and visualized with enhanced chemiluminescence reagent Plus (PerkinElmer Life Sciences, Inc.).

Statistical analysis

Data are shown as mean ± standard deviation. Pair-wise Student's *t*-test was performed and all data are considered statistically significant with a one-sided $p < 0.05$.

Results

Regulation of IL10 expression by LPS and IFN-γ in THP-1 cells

THP-1 cells, like primary monocytes, secrete a variety of inflammatory cytokines in response to appropriate stimuli. To examine the effect of LPS and IFN-γ on *IL10* expression, THP-1 cells were exposed to LPS, and/or IFN-γ, or left untreated. Cells and culture supernatant were collected at regular intervals for assay of *IL10* mRNA and protein abundance, respectively. Abundance of *IL10* mRNA was measured by real-time RT-PCR with primer pairs specific for human *IL10* mRNA (Fig. 1A; Table 1). Exposure of THP-1 cells to LPS increased *IL10* mRNA levels between 4 and 24 h compared to untreated cells. Exposure to LPS and IFN-γ inhibited LPS-mediated induction of *IL10* mRNA by 75–80% by 6–8 h. By contrast, at 24 h, *IL10* mRNA abundance was the same with or without IFN-γ. Treatment of THP-1 cells with IFN-γ alone had little effect on abundance of *IL10* mRNA throughout the time course. We conclude that IFN-γ blocks LPS-mediated induction of *IL10* mRNA at earlier time points (4–11 h).

IL-10 accumulation in the culture medium was determined by ELISA (Fig. 1B). IL-10 from cells exposed to LPS continued to increase up to 24 h. Similar to *IL10* mRNA kinetics, exposure to a combination of LPS and IFN-γ significantly inhibited IL-10 accumulation. It is noteworthy, that IFN-γ inhibited LPS-mediated IL-10 expression to a lesser extent at 24 h as compared to the earlier time points (Fig. 1B) while no effect of IFN-γ was seen on the level of *IL10* mRNA at 24 h (Fig. 1A). There was very little IL-10 secretion in either untreated cells or cells exposed to IFN-γ alone (Fig. 1B). Together, these data indicate that LPS exposure enhances

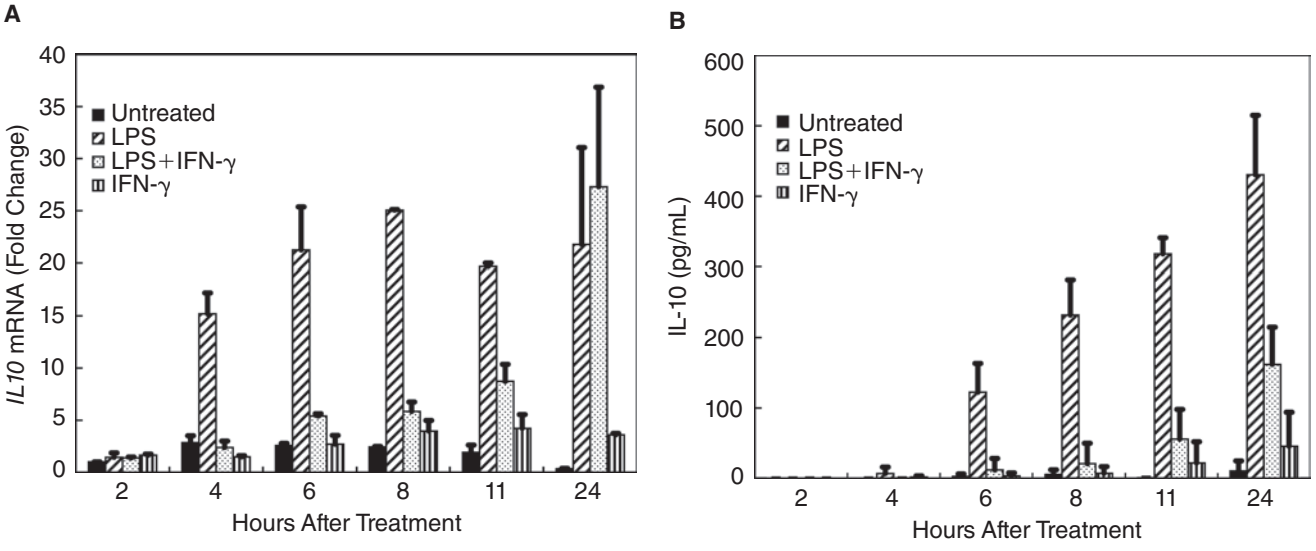

FIG. 1. Kinetics of *IL10* gene expression. THP-1 cells were exposed to LPS and/or IFN-γ or left untreated. Cells and culture supernatants were collected at different time points. Total RNA was extracted and the level of *IL10* mRNA was measured by real-time RT-PCR and expressed as fold change over mRNA level in untreated cells. (**A**) IL-10 in culture supernatants was measured by ELISA. (**B**) Values are the means of two independent experiments with standard deviations shown as error bars.

Table 3. Decay Kinetics of *IL10* mRNA in THP-1 Cells

mRNA	*Treatment*	$k \pm SE$[a]	*Half-life (h)*
IL10	Untreated	0.32 ± 0.04	2.1
	LPS	0.15 ± 0.07	4.6
	LPS + IFN-γ	0.33 ± 0.04	2.0
	IFN-γ	0.41 ± 0.10	1.6

[a]The first-order decay constants (*k*) are shown SE.

abundance of *IL10* mRNA and protein with similar kinetics and that IFN-γ reduces LPS-mediated induction of IL-10. As well, they are consistent with IFN-γ being a suppressor of IL-10 synthesis, thereby permitting a proinflammatory response.

LPS and IFN-γ modulate IL10 mRNA degradation in opposite fashion

LPS and IFN-γ might achieve their differential effects on *IL10* expression partly through regulation of mRNA decay, as 3′-UTR sequences control many cytokine mRNAs in response to external stimuli. As reported earlier, the *IL10* 3′-UTR contains one or more AREs (Brewer and others 2003). To examine the effects on mRNA degradation, THP-1 cells were exposed to LPS and/or IFN-γ for 2.5 h or left untreated. Transcription was then inhibited using actinomycin D to assess mRNA decay kinetics. First-order decay constants (*k*) and half-lives of *IL10* mRNA for each treatment of THP-1 cells are shown in Table 3.

Exposure of THP-1 cells to LPS for 2.5 h increased mRNA half-life approximately 2.2-fold compared to untreated cells (Fig. 2 and Table 3; $p = 0.02$; $n = 3$). This stabilization effect likely contributes to induction of *IL10* mRNA abundance upon exposure of cells to LPS (*cf.* Fig. 1A). By contrast, the differences in half-lives of *IL10* mRNA in cells exposed to both LPS and IFN-γ, or IFN-γ alone, were not statistically significant compared to untreated cells (Fig. 2 and Table 3; $p = 0.48$ and $p = 0.34$, respectively; $n = 3$ for both). Thus, while IFN-γ alone has no effect upon *IL10* mRNA half-life, it blocks the mRNA stabilizing effect of LPS exposure. This observation is again consistent with the ability of IFN-γ to promote a proinflammatory response. It is noteworthy, however, that after 6-hr exposure to LPS, stabilization of *IL10* mRNA was no longer evident (data not shown). Hence, mRNA stabilization contributes to IL-10 synthesis during the acute phase of LPS exposure. This is not too surprising given that IL-10 modulates biosynthesis of a variety of cytokines, including itself, upon exposure to LPS (Brown and others 1996). Perhaps, reversal of LPS-induced stabilization of *IL10* mRNA after 6 h could be because of IL-10-mediated autoregulation via negative feedback through the IL-10 receptor. However, the exact mechanism is not known.

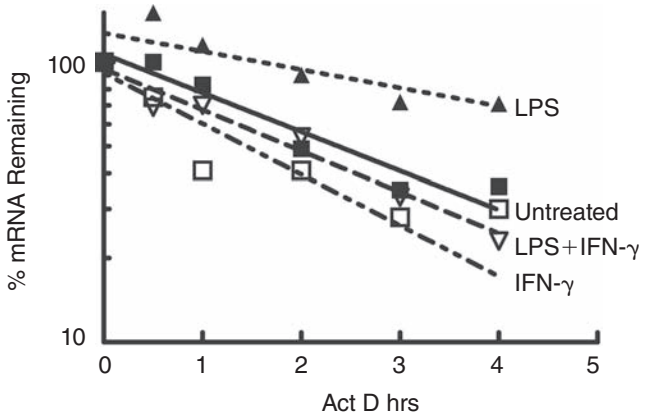

FIG. 2. Decay kinetics of *IL10* mRNA. THP-1 cells were exposed to LPS and/or IFN-γ, or left untreated for 2.5 h followed by addition of actinomycin D. Cells were collected at 0, 0.5, 1, 2, 3, and 4 h after addition of the drug and total RNA was analyzed by real-time RT-PCR as described in Materials and Methods. *IL10* mRNA levels were assessed at each time point, normalized to β-*actin* mRNA, and plotted relative to *IL10* mRNA abundance at time zero. A representative of three independent experiments is shown.

IL10 3′-UTR is a potent posttranscriptional regulator

To assess the contributions of the *IL10* 3′-UTR to observed posttranscriptional regulation (*cf.* Fig. 2), we designed a series of chimeric constructs linking an SV40 promoter-luciferase open reading frame with different segments of the *IL10* 3′-UTR (Fig. 3A). THP-1 cells were cotransfected with a *Renilla* luciferase internal control plasmid and the *IL10* 3′-UTR constructs or the pGL3-Promoter control. Ratios of firefly and *Renilla* luciferase were determined. Luciferase activity for cells transfected with the plasmid containing the entire *IL10* 3′-UTR (nt 574–1,563) was less than 10% of the control level (Fig. 3B; $p = 0.001$, $n = 2$). Lysates of cells transfected with the construct containing the first five AUUUA motifs (nt 574–980) showed about 25% activity compared to cells transfected with the control plasmid (Fig. 3B; $p = 0.001$, $n = 2$). Luciferase activity for cells transfected with the construct containing AUUUA motifs 6–8 (nt 933–1,560) was about 50% of the control level (Fig. 3B; $p = 0.005$, $n = 2$). Finally, the construct containing nt 933–1,370, which contains AUUUA motifs 6 and 7, yielded luciferase activity indistinguishable from the pGL3-Promoter control (Fig. 3B; $p = 0.137$; $n = 2$). Comparable results were obtained upon transfections of HeLa S3 cells (data not shown). We conclude that the region spanning nt 574–980, which contains AUUUA motifs 1–5 and U-rich flanking sequence, and the region spanning nt 1,370–1,560, which contains an AUUUA pentamer motif, provide effective (≥50%) posttranscriptional silencing of reporter gene expression. The full-length 3′-UTR, however, provides the highest level of silencing capability (>90%) in at least the two cell types tested.

ARE-binding protein AUF1 binds IL10 3′-UTR sequences

Our previous study (Brewer and others 2003) showed that the *IL10* 3′-UTR segment containing the first five AUUUA pentamers forms ribonucleoprotein complexes when incubated with cytoplasmic extracts of normal neonatal melanocytes. One of the components of these complexes is AUF1. To

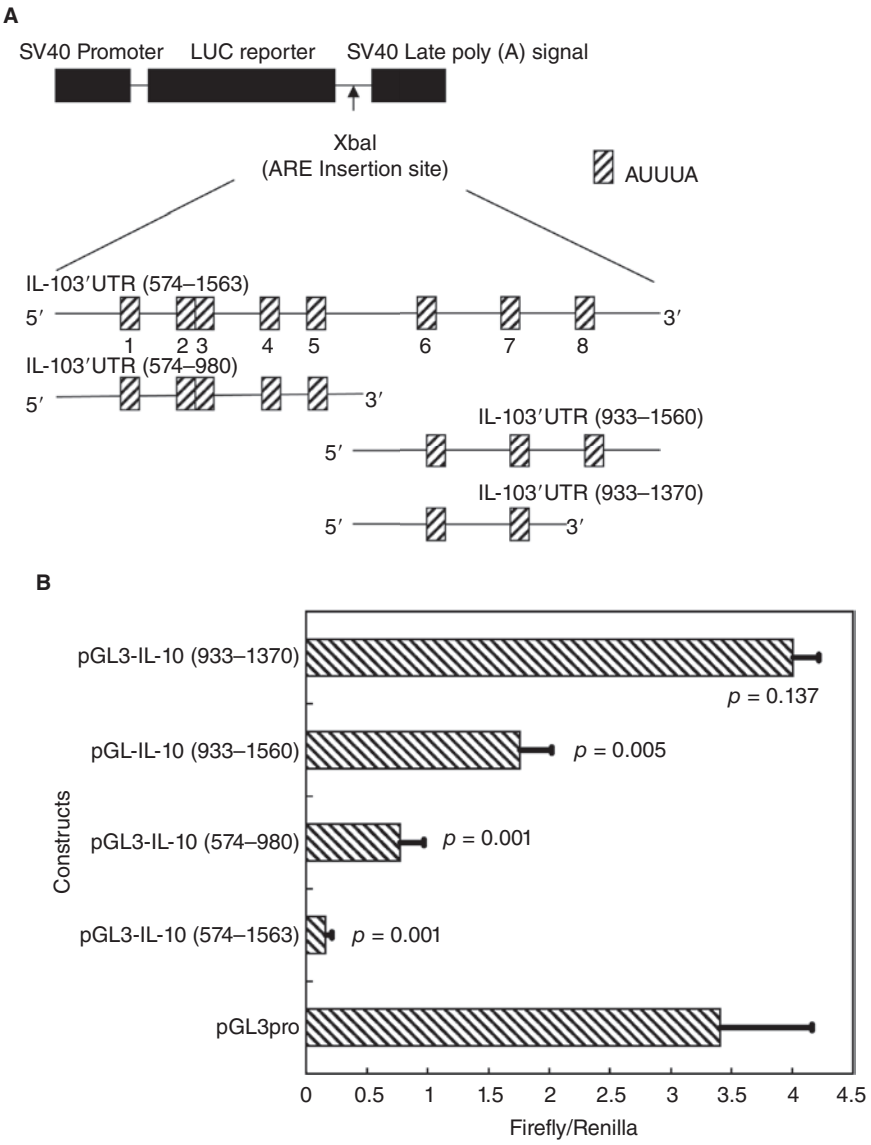

FIG. 3. Role of *IL10* 3′-UTR in post-transcriptional regulation. (**A**) Different regions (indicated by nucleotide number relative to transcription start site, GenBank accession number M57627) of the 3′-UTR were inserted downstream of the luciferase open reading frame in plasmid pGL3-Promoter. AUUUA pentamers are shown as hatched boxes. (**B**) Chimeric luciferase-*IL10*-3′-UTR constructs were transfected into THP-1 cells and luciferase activity as the ratio of firefly and *Renilla* luciferase was measured in cell lysates 24 h after transfection. Data represent mean standard deviations from two independent experiments. *P*-values with respect to the control are shown.

determine whether the *IL10* 3′-UTR associates with specific mRNA-binding proteins, including AUF1, in THP-1 cells, we examined cytoplasmic extracts from cells exposed to LPS and/or IFN-γ for 2.5 h, or left untreated. Equal amounts of cytoplasmic proteins were incubated with *in vitro* transcribed, radiolabeled RNA containing the first five AUUUA motifs and flanking U-rich sequence spanning nucleotides 574–920. Following RNase digestion, RNA-protein complexes were resolved in a nondenaturing polyacrylamide gel and detected by phosphorimager analysis. Three ribonucleoprotein complexes assembled from all protein extracts; their intensities and mobilities were comparable between all samples (Fig. 4A). Ribonucleoprotein complex formation with radiolabeled RNA was competed by increasing amounts of identical, unlabeled RNA (Fig. 4B, lanes 3 and 4). Likewise, unlabeled RNA spanning nucleotides 930–1,240 harboring two AUUUA motifs inhibited ribonucleoprotein complex formation, though to a lesser extent (Fig. 4B, lanes 5 and 6). By contrast, poly(C) did not affect ribonucleoprotein complex formation (Fig. 4B, lanes 7–9). Together, these results indicate specific RNA-protein interactions. However, exposure of cells to LPS and/or IFN-γ did not affect these interactions (Fig. 4A).

To assess whether assembled RNA-protein complexes contained AUF1, all binding reactions were incubated with either anti-AUF1 antibody that recognizes all four isoforms of AUF1 or preimmune serum. Anti-AUF1 antibody produced super-shifted complexes identically, regardless of the LPS/IFN-γ exposure regimen (Fig. 5, lanes 5–8); however, there was no corresponding decrease in the faster migrating bands, as has been observed previously in super-shift assays with the *GMCSF* ARE (Buzby and others 1996). This effect could be in part because of the relatively small percentage of total RNA-protein complexes super-shifted by the antibody against AUF1. These RNA-protein complexes are undoubtedly composed of other ARE-binding proteins as well (see Discussion section). In any event, no super-shifted bands appeared with preimmune serum (Fig. 5, lanes 9–12) or with

no serum additions (Fig. 5, lanes 1–4). Therefore, AUF1 is a component of at least a portion of RNA-protein complexes that assemble *in vitro* with RNA harboring the first five AUUUA motifs.

AUF1 knockdown silences IL10 expression in THP-1 cells

To gain further insight into the role of AUF1 in *IL10* expression, we generated THP-1 cells with reduced AUF1 abundance by RNA interference. AUF1 consists of four isoforms (37-, 40-, 42- and 45-kD) generated by alternative pre-mRNA splicing (Zhang and others 1993; Ehrenman and others1994; DeMaria and Brewer 1996). THP-1 cells were transfected with plasmids encoding shRNAs with either scrambled sequence (shCRTL) or sequence that targets all four AUF1 isoforms (shAUF1). Drug selection generated pools of stably transfected cells. Western blot analysis revealed that AUF1 abundance was reduced >90% in cells transfected with *AUF1*-specific shRNA compared to control shRNA and parental THP-1 cells (K.S. and G.B, manuscript in preparation; see Fig. 6A). We first examined whether AUF1 knockdown affected *IL10* mRNA half-life in nonstimulated cells or cells exposed to LPS. AUF1 knockdown had no effect upon mRNA half-life; likewise, knockdown did not affect LPS-mediated stabilization of *IL10* mRNA (data not shown).

On the basis of these results, we examined whether AUF1 knockdown affected *IL10* mRNA or protein abundance under any conditions. shAUF1- and shCTRL-expressing cells and parental THP-1 cells were exposed to LPS and/or IFN-γ for indicated time or left untreated (Figs. 6B and C). Cells and culture supernatants were collected for measuring *IL10* mRNA and secreted protein abundance, respectively. In shAUF1-expressing cells, abundance of *IL10* mRNA increased by only 3-fold upon 6 h LPS exposure compared to a 10- to 14-fold increase in parental THP-1 (data not shown) and shCTRL-expressing cells (Fig.6B). As expected, IFN-γ blunted LPS-mediated induction of *IL10* mRNA in both parental THP-1 and shCRTL-expressing cells. However, IFN-γ did not significantly reduce *IL10* mRNA levels further in shAUF1-expressing cells compared to LPS exposure alone; thus, AUF1 likely does not play a role in the effects of IFN-γ on *IL10* expression. Likewise, AUF1 knockdown reduced accumulation of secreted IL-10 normally seen upon 24-h LPS exposure (Fig. 6C). Together, these results suggest that AUF1 is required for LPS-mediated induction of *IL10* mRNA and protein abundance. To determine if silencing of *IL10* gene expression upon AUF1 knockdown might be simply mediated by effects upon expression of the IL-10 receptor, IL-10R1, we examined abundance of *IL10R1* mRNA upon AUF1 knockdown. LPS induces *IL10R1* gene expression in THP-1 cells, and a combination of LPS and IFN-γ induces expression further (S.S. and S.P., unpublished observations).

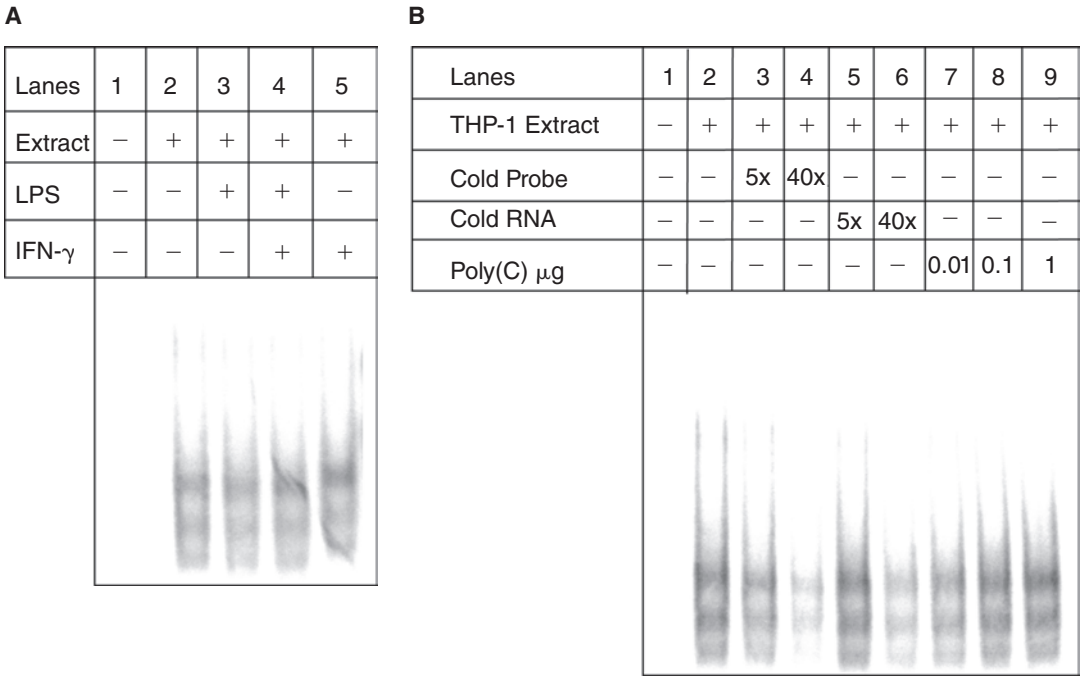

FIG. 4. Interaction of proteins with the *IL10* 3′-UTR. (**A**) Radiolabeled RNA (1×10^5 cpm) containing nucleotides 574–920 of *IL10* mRNA was generated by *in vitro* transcription and incubated with cytoplasmic extracts prepared from THP-1 cells with indicated treatments. Complexes were separated in a 5% native polyacrylamide gel and analyzed with a phosphorimager. (**B**) A competition assay was performed with 5-fold and 40-fold molar excess of unlabeled probe (lanes 3 and 4), *in vitro* transcribed RNA corresponding to nucleotides 930–1240 (lanes 5 and 6), or varying amounts of poly(C) (lanes 7–9). Extracts from untreated THP-1 cells were used.

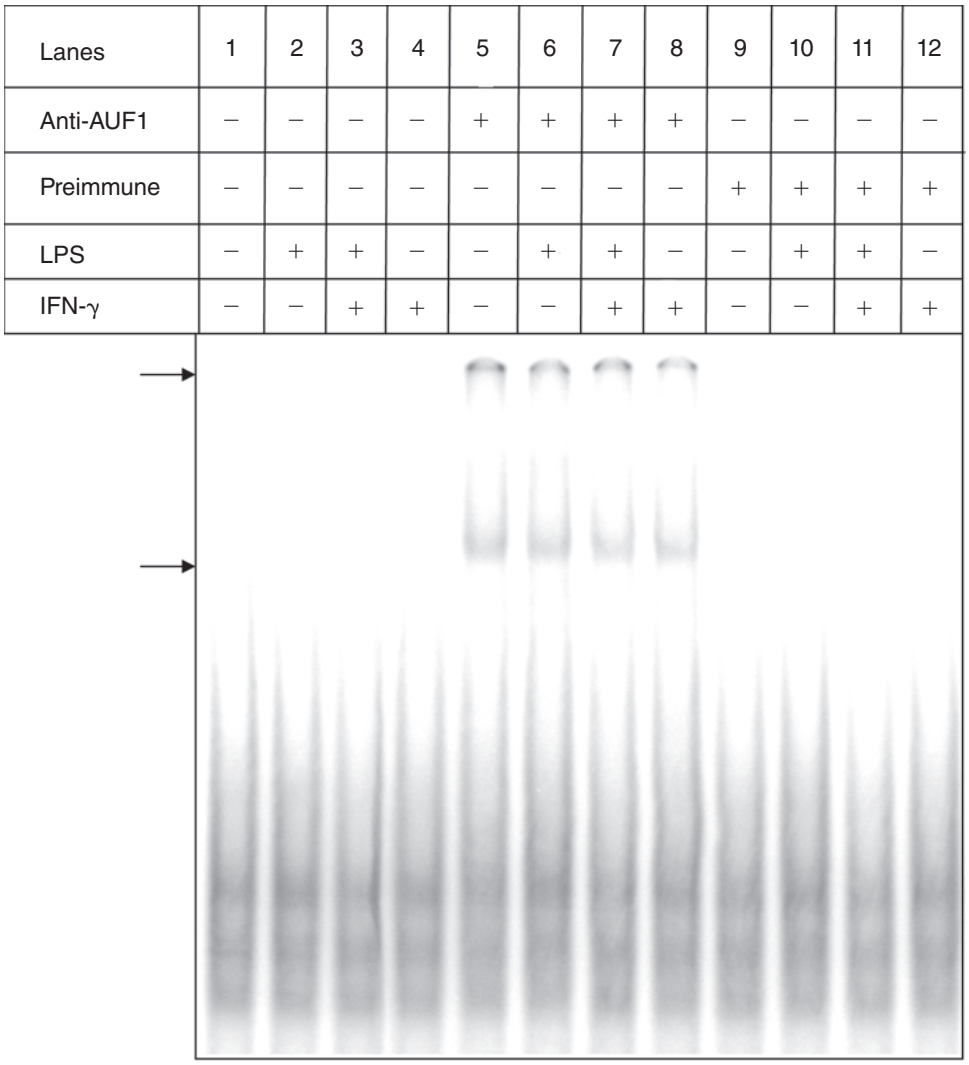

FIG. 5. AUF1 in cytoplasmic extracts from THP-1 cells binds the *IL10* 3′-UTR. Labeled RNA probe containing the five proximal AUUUA motifs was incubated with extracts from THP-1 cells treated as indicated. Antibodies (1:10 dilution) added to the binding reactions are also indicated. Super-shifted complexes are indicated by arrows.

In contrast to *IL10* mRNA, abundance of *IL10R1* mRNA increased almost 2-fold upon exposure of shCTRL-, and shAUF1-expressing cells to LPS (Fig. 6D). A combination of LPS and IFN-γ had no statistically significant effect upon *IL10R1* mRNA abundance compared to LPS alone in cells expressing either control shRNA or shAUF1 (Fig. 6D). Thus, AUF1 knockdown blocks LPS-mediated induction of *IL10* mRNA abundance while it has no effect upon *IL10R1* mRNA levels. Therefore, the effects of AUF1 knockdown on *IL10* gene expression are likely not because of reduced *IL10R1* expression levels.

p40 AUF1 isoform rescues IL-10 induction upon LPS exposure

To determine whether expression of specific AUF1 isoforms could restore LPS-mediated induction of IL-10 in an AUF1 knockdown background, we expressed shRNA-resistant p37 or p40 AUF1 cDNAs ($p37^R$ and $p40^R$, respectively) in cells expressing shAUF1. We chose these isoforms since they are selectively cytoplasmic compared to p42 and p45 in THP-1 cells (Wilson and others 2003). $p37^R$ and $p40^R$ proteins were readily apparent compared to shAUF1-expressing cells (Fig. 7A, compare lanes 3 and 4 to lane 1). The higher expression of p40 compared to p37 reflects the relative differences normally seen in THP-1 cells (Wilson and others 2003). Cells expressing shCTRL, shAUF1, shAUF1/$p37^R$, or shAUF1/$p40^R$ were exposed to LPS and/or IFN-γ for indicated time or left untreated. Ectopic expression of $p40^R$, but not $p37^R$, in shAUF1-expressing cells restored LPS-mediated induction of both *IL10* mRNA abundance (Fig. 7B) and secreted protein (Fig. 7C). These results demonstrate that the p40 AUF1 isoform selectively plays a critical role in *IL10* gene expression in response to LPS exposure.

Discussion

Monocytes/macrophages form the first line of defense against invading pathogens or bacterial endotoxin (LPS) by secreting proinflammatory mediators such as TNFα, IL-1, and IL-12. These cells either destroy the pathogen directly or recruit other immune cells to do so (Adams and Hamilton

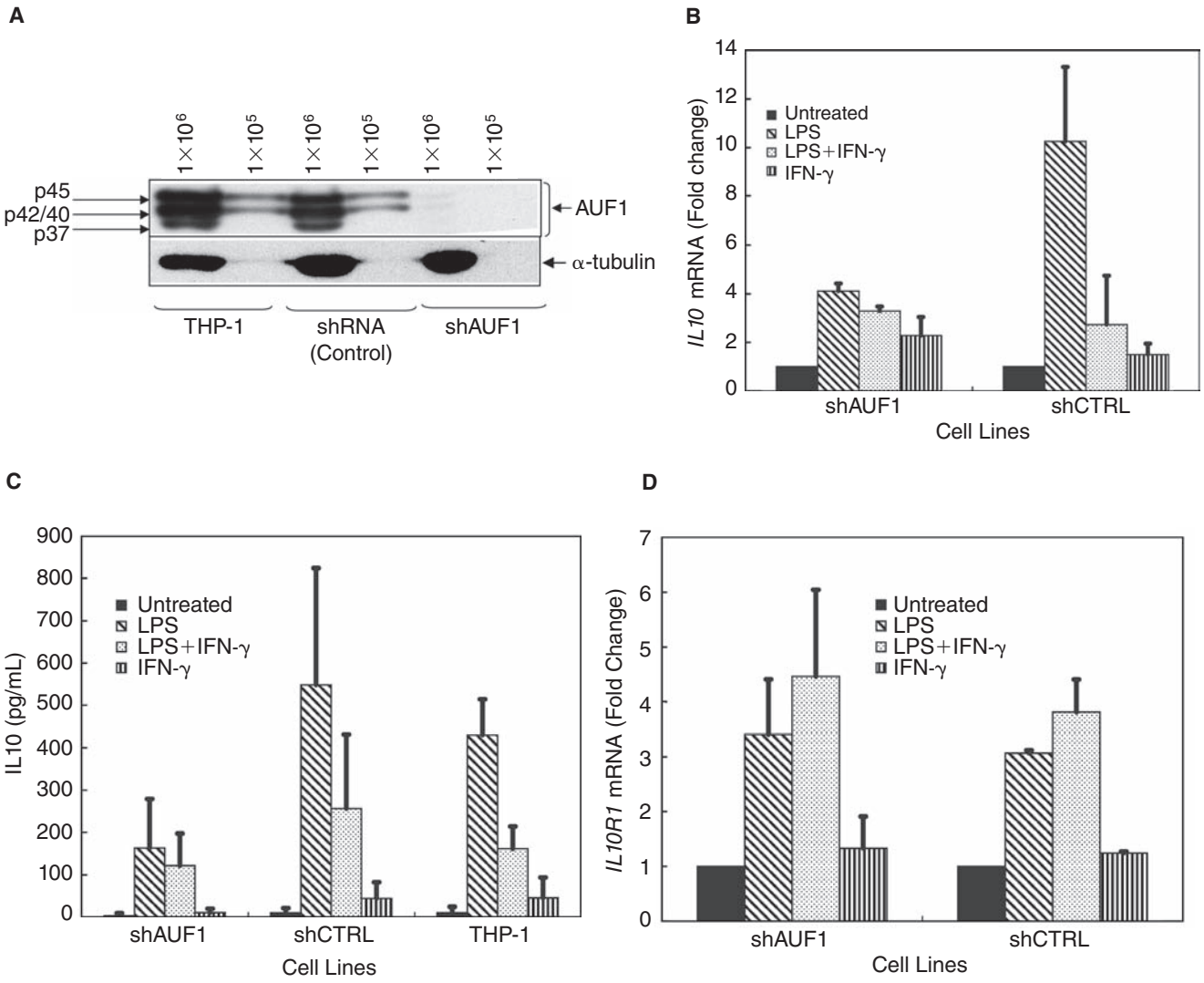

FIG. 6. Levels of IL-10 expression following AUF1 knockdown. (**A**) Extracts were prepared from 1×10^5 and 1×10^6 parental THP-1 cells and cells transfected with indicated shRNA vectors. Extracts were analyzed by Western blot using anti-AUF1 antibody (upper panel). The same blot was probed with anti-α-tubulin as a loading control (lower panel). Individual AUF1 isoforms are indicated on the left of the panel by arrows. (**B**) Parental THP-1, shCTRL-, and shAUF1-expressing THP-1 cells (2×10^6) were exposed to LPS and/or IFN-γ for 6 h or left untreated. Total RNA was extracted from cells treated with LPS and/or IFN-γ and levels of *IL10* (**B**) and *IL10R1* mRNAs (**D**) were measured by real-time RT-PCR. (**C**) Culture supernatants from THP-1, shCTRL-, and shAUF1-expressing cells were collected 24 h after exposure to LPS and/or IFN-γ or no treatment. IL-10 was measured by ELISA. Results are from 2 to 4 independent experiments.

1992; Nathan and Cohn 1995). Following production of proinflammatory cytokines, the anti-inflammatory cytokine IL-10 is produced. Since IL-10 plays a pivotal role in the regulation of immune responses, it is critical to understand how inflammatory stimuli, such as LPS and IFN-γ, modulate *IL10* expression in monocytes/macrophages. Most studies examining regulation of *IL10* expression have focused on murine cells (Tone and others 2000; Powell and others 2000; Nemeth and others 2005). For example, in murine macrophages, LPS exposure leads to a 2,000-fold accumulation of *IL10* mRNA while secreted IL-10 increases 10-fold (Nemeth and others 2005). We have not observed such a vast difference in RNA and protein levels in human THP-1 cells. This suggests that murine and human cells might regulate *IL10* in a different manner. In addition, many studies have focused on transcriptional regulation of *IL10* (Benkhart and others 2000; Tone and others 2000; Huang and others 2004; Liu and others 2003; Cao and others 2005) to the exclusion of its posttranscriptional pathways.

In this study, we explored posttranscriptional regulation of human *IL10* expression in response to LPS and IFN-γ in monocytes. THP-1 cells, like primary monocytes, secrete very low constitutive levels of IL-10 that increase rapidly in response to LPS exposure. IFN-γ, a potent activator of monocytes and macrophages, inhibits LPS-mediated induction of IL-10. This serves to augment production of proinflammatory

cytokines largely by inhibiting IL-10 biosynthesis. IFN-γ exerts its regulatory effect in an exocrine fashion since it is not produced by monocytes and macrophages. Among various cytokines tested, only IFN-γ modulates IL-10 production in LPS-activated monocytes (Chomarat and others 1993). The inhibitory effect of IFN-γ on *IL10* expression in monocytes is achieved in part by inhibition of the ability of LPS to enhance stability of *IL10* mRNA; however, IFN-γ does not alter stability of *IL10* mRNA in the absence of LPS (Fig. 2). Although IFN-γ-mediated regulation of IL-10 production in macrophages/monocytes is significant in the pathogenesis of infectious diseases (Chomarat and others 1993; Donnelly and others 1995; Libraty and others 1997), control of IL-10 production in myeloid cells is poorly understood. A recent study showed that IFN-γ modulates transcription of *IL10* in dendritic cells in a STAT-1-dependent manner (Flores and others 2007). Our data indicate that IFN-γ inhibits LPS-mediated induction of IL-10 partially through a posttranscriptional mechanism.

This study provides further insight into our previous finding that AUF1 plays an important role in the regulation of *IL10* gene expression (Brewer and others 2003). The AUF1 family of mRNA destabilizing factors is a target of ubiquitin-proteasome pathways that contribute to rapid

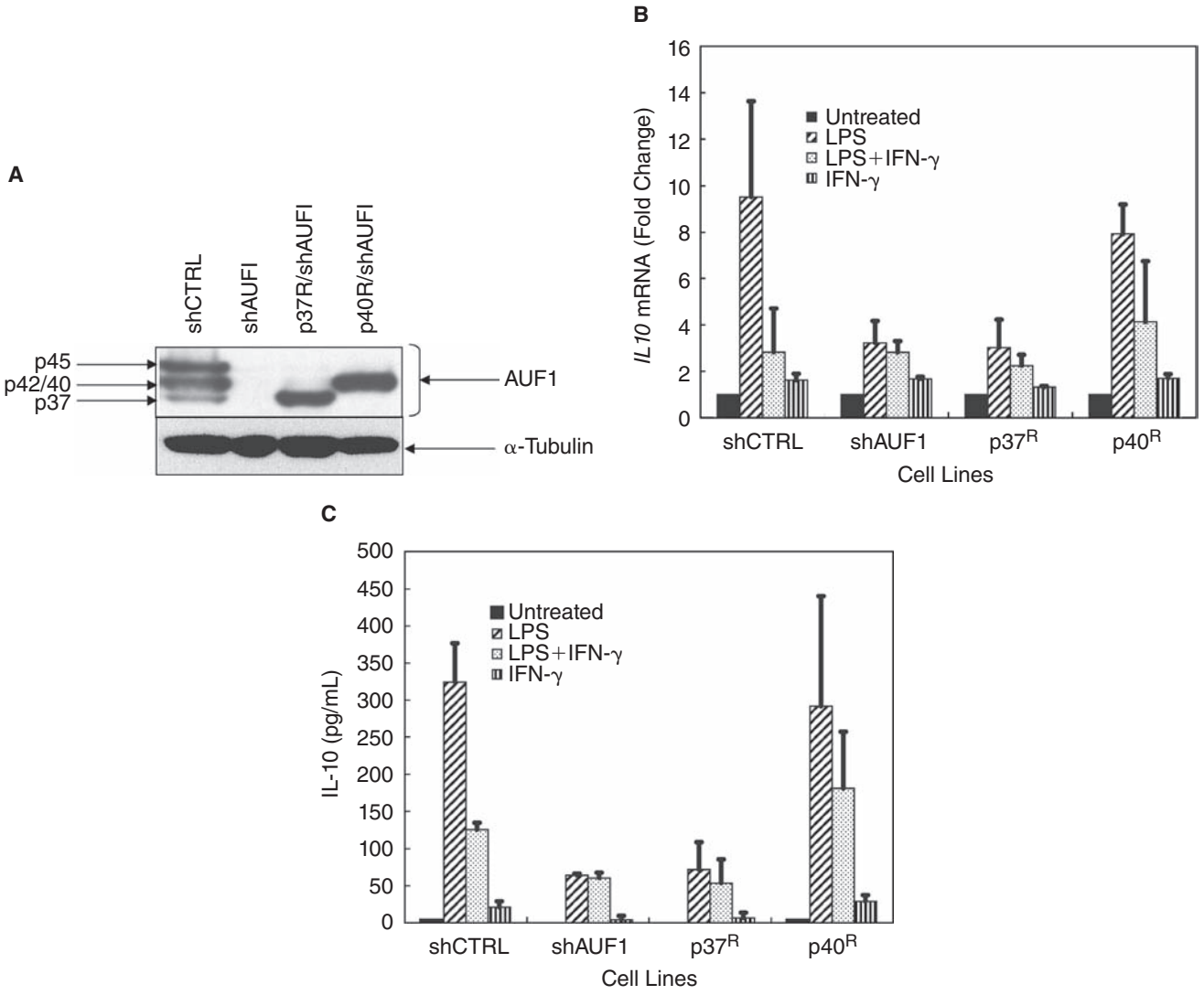

FIG. 7. Complementation with p40 AUF1 restores IL-10 expression. THP-1 cells expressing shCTRL, shAUF1, p37^R/shAUF1, or p40^R/shAUF1 were exposed to LPS and/or IFN-γ or left untreated. (**A**) Western blot analysis of cells transfected with indicated shRNA vectors with anti-AUF1 antibody (upper panel) and anti-α-tubulin antibody (lower panel). Bands representing AUF1 isoforms and α-tubulin protein are indicated by arrows on the right of the panel; individual AUF1 isoforms are indicated on the left of the panel by arrows. (**B**) Cells were exposed to LPS and/or IFN-γ for 6 h or left untreated. *IL10* mRNA was measured by real-time RT-PCR. (**C**) THP-1 cells expressing shCTRL, shAUF1, p37^R/shAUF1, or p40^R/shAUF1 were exposed to LPS and/or IFN-γ or left untreated. Culture supernatants were collected after 24 h. IL-10 was measured by ELISA. Results from two independent experiments are shown.

silencing of ARE-containing mRNAs (Loroia and others 1999). Cytoplasmic AUF1 in THP-1 lysate bound the *IL10* 3′-UTR, but there was no apparent difference in the intensity or distribution of RNA-protein complexes in extracts from untreated, LPS and/or IFN-γ treated cells (Fig. 5). This, however, does not exclude the possibility of posttranslational modifications of AUF1 that affect its effector functions or of modulation of additional proteins or cofactors after LPS and/or IFN-γ exposure. For example, Stoecklin and others (2008) reported that in murine, bone marrow-derived macrophages, TTP modulates expression of basal and LPS-induced levels of *IL10* mRNA by regulating mRNA degradation. Thus, it is highly likely that AUF1 and TTP together act to control *IL10* expression.

To examine the significance of AUF1 in the regulation of *IL10* expression, we took a genetic approach by shRNA-mediated AUF1 knockdown in THP-1 cells. On the basis of previous studies (Pende and others 1996; Sirenko and others 1997; Buzby and others 1999; Sarkar and others 2003; Paschoud and others 2006), we expected a more stable and higher level of *IL10* mRNA upon AUF1 knockdown. On the contrary, AUF1 knockdown had no discernable effects on either mRNA half-life or on LPS-mediated stabilization of *IL10* mRNA compared to cells expressing a scrambled shRNA (data not shown). However, abundances of *IL10* mRNA and protein were reduced in AUF1 knockdown cells relative to control cells (Figs. 6B and C). Thus, the mechanisms by which AUF1 controls *IL10* expression are not yet clear. Nonetheless, these experiments clearly indicate a role for AUF1 in *IL10* expression in response to LPS exposure.

The AUF1 protein family consists of four isoforms (p37, p40, p42, and p45) generated by alternative pre-mRNA splicing (Wagner and others 1998). In THP-1 cells, p42 and p45 are predominantly nuclear while p37 and p40 are predominantly cytoplasmic (Wilson and others 2003). To address the question whether p37 and/or p40 plays a role in LPS-mediated IL-10 induction in THP-1 cells, expression of p37 or p40 was restored in shAUF1-expressing cells by transfecting cDNAs encoding mRNAs refractory to the shAUF1 ($p37^R$ and $p40^R$, respectively). Expression of $p40^R$ partially restored LPS-mediated induction of IL-10 while $p37^R$ expression had only a minimal effect (Fig. 7). Wilson and others (2003) reported that activation of THP-1 cells with phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate led to loss of phosphorylation of p40 at Ser^{83} and Ser^{87} concomitant with stabilization of ARE-mRNAs encoding TNF-α and IL-1β. By contrast, p37, which lacks these serines because of alternative pre-mRNA splicing, cannot be phosphorylated at comparable positions. Examining potential roles for p40 phosphorylation in *IL10* gene expression could clarify this.

The mechanisms of *IL10* regulation by AUF1 are clearly complex. Since there is no significant difference in the half-life of constitutive or induced *IL10* mRNA following AUF1 knockdown, other factors may be involved in LPS-mediated stabilization of *IL10* mRNA. Furthermore, AUF1 might control *IL10* mRNA levels through alternate mechanisms. One mechanism might include promoting nucleocytoplasmic transport of *IL10* mRNA (a direct mechanism); that AUF1 can bind the *IL10* 3′-UTR suggests some kind of direct mechanism. On the other hand, the fact that AUF1 knockdown does not alter *IL10* mRNA half-life (data not shown) suggests that AUF1 modulates an LPS-responsive inhibitor of *IL10* transcription (an indirect mechanism). For example, bacterial products and various cytokines induce SOCS3 (suppressor of cytokine signaling) synthesis, which in turn inhibits activation of transcription factor STAT3 and in turn, gene transcription (Yasukawa and others 2000). Thus, AUF1 might affect expression of a SOCS3-like factor. In any event, future experiments will be required to fully elucidate the mechanisms of AUF1 action in *IL10* gene expression.

Finally, studies of $AUF1^{-/-}$ mice underscore its significance for properly regulated inflammatory responses (Lu and others 2006). These mice present symptoms of severe endotoxemia when exposed to very low dose endotoxins because of the failure to attenuate proinflammatory cytokine secretion (Lu and others 2006). Normally, following LPS-induced secretion of proinflammatory cytokines by monocytes and macrophages, the immune system attenuates this proinflammatory response in part via AUF1 binding to and silencing of ARE-mRNAs encoding the TNF-α and IL-1β proinflammatory mediators (Lu and others 2006). Since AUF1 plays a critical role in regulation of anti-inflammatory cytokine IL-10 as described in this work, we speculate that LPS exposure fails to adequately induce IL-10 production by monocytes/macrophages in $AUF1^{-/-}$ mice. Consequently, low IL-10 levels fail to suppress proinflammatory cytokine production that contributes to endotoxemia. Future studies will examine this hypothesis and focus on signaling pathways involved in AUF1-mediated *IL10* regulation.

In conclusion, we believe our experiments have unexpectedly revealed isoform-specific roles for AUF1 in immune regulation. While the p37 isoform plays a central role in silencing cytokine gene expression (Sarkar and others 2003), our work demonstrates that the p40 isoform may serve to promote an anti-inflammatory response via its induction of IL-10 synthesis. As p40 contains an additional 19 amino acids in RNA-recognition motif-1 compared to p37, perhaps these two proteins possess specificities for distinct subsets of cytokine mRNAs. In any event, future studies will address the molecular basis for differential cytokine regulation by p37 and p40 AUF1.

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Address reprint requests or correspondence to:
Dr. Sidney Pestka
Professor and Chairman
Department of Molecular Genetics,
Microbiology, and Immunology
UMDNJ-Robert Wood Johnson Medical School
675 Hoes Lane West, R727
Piscataway, NJ 08854

Tel: (732) 235-4567
Fax: (732) 235-5223
E-mail: pestka@umdnj.edu

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