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Nuclear to Cytoplasmic Translocation of Heterogeneous Nuclear Ribonucleoprotein U Enhances TLR-Induced Proinflammatory Cytokine Production by Stabilizing mRNAs in Macrophages

Wei Zhao,* Lijuan Wang,* Meng Zhang,* Peng Wang,* Jianni Qi,* Lei Zhang,* and Chengjiang Gao*§

TLR signaling is associated with the transcription of various proinflammatory cytokines, including TNF-α, IL-6, and IL-1β. After transcription, the mRNA of these proinflammatory cytokines needs to be tightly controlled at the posttranscriptional level to achieve an optimal expression. However, the precise mechanism of posttranscriptional regulation is not fully understood. In the current study, we found the expression of heterogeneous nuclear ribonucleoprotein U (hnRNP U), also termed scaffold attachment factor A, was greatly induced by TLR stimulation in macrophages. Knockdown of hnRNP U expression greatly attenuated TLR-induced expression of TNF-α, IL-6, and IL-1β, but not IL-12, whereas hnRNP U overexpression greatly increased TLR-induced expression of TNF-α, IL-6, and IL-1β. Furthermore, hnRNP U knockdown accelerated the turnover and decreased the t1/2 of TNF-α, IL-6, and IL-1β mRNA. RNA immunoprecipitation demonstrated that hnRNP U bound to the mRNA of these proinflammatory cytokines through the RGG motif. Importantly, we showed that TLR stimulation provided a stimulus for hnRNP U nuclear to cytoplasmic translocation. Therefore, we propose that hnRNP U induced by TLR signaling binds to the mRNA of a subset of proinflammatory cytokines and positively regulates the expression of these cytokines by stabilizing mRNA. The Journal of Immunology, 2012, 188: 000–000.

Heterogeneous nuclear ribonucleoprotein U (hnRNP U) is a major constituent of nuclear matrix or scaffold, and is therefore termed scaffold attachment factor A (6). It has been shown that hnRNP U binds directly to DNA sequences termed scaffold attachment regions through the N-terminal acidic region named serum amyloid P (SAP) (7). hnRNP U also contains an arginine- and glycine-rich region termed the RGG box in the C terminus, which is responsible for RNA binding (8). Mice carrying a hypomorphic mutation in the hnRNP U gene exhibit postimplantation lethality (9), suggesting that hnRNP U contributes to a variety of essential biological functions, including transcriptional regulation and RNA metabolism. hnRNP U has been shown to interact with various transcriptional cofactors and modulate their transcriptional activation, such as transcriptional coactivator p300 (10), glucocorticoid receptor (11), Yes-associated protein (12), heterochromatin protein 1α (13), DNA topoisomerase II β (14), and PCAF (15). hnRNP U also can interact with the SCAF-TrCP ubiquitin ligase complex to regulate its E3 ligase activity (16, 17). Recent studies demonstrated hnRNP U is involved in the formation of the inactive X chromosome (18) by facilitating the accumulation of Xist RNA on the (19). However, other functions of hnRNP U, especially in the regulation of immune response, have never been investigated. In this regard, we previously demonstrated that hnRNP U binds to the promoter region of OPN gene through the SAP domain to increase LPS-induced OPN expression in macrophages (20).

As a follow-up study, the function of hnRNP U in TLR signaling was studied in macrophages. We find hnRNP U protein expression is induced by TLR stimulation in macrophages. hnRNP U knockdown and overexpression experiments demonstrate hnRNP U positively regulates TLR-induced expression of proinflammatory cytokines. hnRNP U knockdown accelerated the turnover of TNF-α, IL-6, and IL-1β mRNA. RNA immunoprecipitation (RIP)
demonstrates that hnRNP U binds to the mRNA of these proinflammatory cytokines through the RGG motif. We also provide evidence to show that TLR signaling stimulates hnRNP U nuclear to cytoplasmic translocation.

Materials and Methods

Mice and reagents

C57BL/6j mice were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Medical School of Shandong University (Jinan, Shandong Province, China). Actinomycin D, LPS (Escherichia coli, 055:B5), lipoteichoic acid (LTA), peptidoglycan (PGN), and polysaccharide-polysaccharide-dylic acid [poly(I:C)] were purchased from Sigma-Aldrich (St. Louis, MO), and LPS was repurified, as described (21). The Abs for β-tubulin and Lamin A/C were from BioWorld Technology. The Abs for polyclonal and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). The Abs for hnRNP U and Flag were from Sigma-Aldrich. Their respective HRP-conjugated secondary Abs were purchased from Santa Cruz Biotechnology. DAPI was from Molecular Probes (Invitrogen).

Cell culture

Female C57BL/6j mice (5–6 wk old) were used for the preparation of primary mouse macrophages. Thioglycolate-elicited mouse peritoneal macrophages were prepared as described (21). Mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (Manassas, VA). HEK293-TLR4 cell line was obtained from InvivoGen (San Diego, CA). The cells were cultured at 37°C under 5% CO2 in DMEM supplemented with 10% FCS (Invitrogen-Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin. LPS, LTA, PGN, and poly(I:C) were used at a final concentration of 100 ng/ml, 5 μg/ml, 10 μg/ml, and 20 μg/ml, respectively.

Plasmid constructs

pCMV6-Flag-hnRNP U expression plasmid was purchased from OriGene. hnRNP U domain deletion plasmids pCMV6-Flag-N689 and pCMV6-Flag-C564 were constructed using PCR-generated fragments encoding the aa residues 1–689 and 564–800 of mouse hnRNP U (800 aa), respectively. The PCR products were inserted in frame between the EcoRI/EcoRV sites of the pCMV6-Flag expression vector (OriGene). For the luciferase reporter plasmids containing full-length TNF-α-3′-untranslated region (UTR), IL-6 3′-UTR, and IL-12p40 3′-UTR, the 3′-UTRs were amplified from RAW264.7 cells by PCR and inserted into the pGL3 basic plasmid (Promega). The primers used were 5′-GCTCTAGAGGAGGTTGCTCTGTCGAC (forward) and 5′-ACCCTTCCAGGCTTCCCTAC (antisense) for IL-6; and 5′-AGGCCGTCAGCTTTGAGGCTTGAAGACAAGCCGGA-3′ (forward) and 5′-ACCTTCCAGGCTTCCCTAC (antisense) for IL-12p40. To construct luciferase reporter plasmids without adenylate-uridylate-rich elements (AREs) TNF-α 3′-UTR ΔARE and IL-6 3′-UTR ΔARE, the ARE region was deleted by PCR-mediated mediatiation of the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) with the following primers: 5′-TTGGCTATTTGAATGTTATTTGGAAG (forward) and 5′-AAGTGCTAAATTAATTTAAAGGCCG (reverse) for TNF-α; 5′-TGAAGTTGCTCTAAGGTGTTGTTCTTCTC (forward) and 5′-AGTCTCCCAACATCATTAGTCTGAC (reverse) for IL-6. All constructs were confirmed by DNA sequencing. The NF-κB luciferase reporter was purchased from Stratagene.

Transfection

For transient transfection, small interfering RNA (siRNA) specific for hnRNP U (sc-38299) and negative control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. For 1 × 10^6 cells, 0.4 μmol hnRNP U siRNA was mixed with 15 μl Geneporter 2 Transfection Reagent (QIAGEN, San Diego, CA) and transfected into the cells. After 6 h, the supernatant was removed and fresh medium was added. The cells were cultured for another 36 h before further experiments. For stable transfection, short hairpin RNA (shRNA) plasmid specific for hnRNP U (sc-38299-5H) and control shRNA plasmid (sc-108060) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The plasmids were transfected into RAW264.7 macrophages using JetPEI-Macrophage reagent (Polyplus), according to the standard protocol, and selected with 800 μg/ml G418 (Invitrogen) for 2–3 wk, and then the cells were pooled, expanded, and used for the following experiments. The cell viability was not changed between stably transfected RAW264.7 cells and parental RAW264.7 cells.

Detection of cytokine production

A total of 2 × 10^5 macrophages was seeded into 24-well plates and incubated overnight. Cells were stimulated with LPS, LTA, or poly(I:C) for indicated time periods. The concentrations of TNF-α, IL-6, IL-1β, and IL-12p70 in culture supernatants were measured by ELISA kits (R&D Systems, Minneapolis, MN).

RNA quantitation and immunoblot analysis

Total RNA was extracted with TRIzol reagent, according to the manufacturer’s instructions (Invitrogen). A LightCycler (ABI PRISM 7000) and a SYBR RT-PCR kit (Takara) were used for quantitative real-time RT-PCR analysis. Specific primers used for RT-PCR assays were 5′-GCCAC-CAACATTCTCTTGCTG-3′ (sense) and 5′-TACCTTCAAGTGAGATTGGAAT-3′ (antisense) for TNF-α; 5′-ACCTTTCAACACCCAGAAGTTA-3′ (sense) and 5′-AAATGACCCGTAG-3′ (antisense) for IL-1β; 5′-AAACACCCCGCTTTCACGTA-3′ (sense) and 5′-TTATGGAAGTGGAC-3′ (antisense) for IL-6; and 5′-TGTGTTACCAG-3′ (sense) and 5′-GCGGAACGCAGA-3′ (antisense) for TNF-α. Data are normalized to β-actin expression in each sample. For immunoblot analysis, cells were lysed with M-Per Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with a protease inhibitor mixture. Nuclear proteins were extracted by NE-PER Protein Extraction Reagent (Pierce), according to the manufacturer’s instructions. Protein concentrations in the extracts were measured with a bicinchoninic acid assay (Pierce) and were made equal with extraction reagent. Equal amounts of extracts were separated by SDS-PAGE, and then were transferred onto nitrocellulose membranes for immunoblot analysis, as described previously (21).

Stability of mRNA in macrophages

RAW264.7 macrophages were stimulated with 100 ng/ml LPS for 2 h. Actinomycin D (5 μg/ml) was then added to the culture medium to stop transcription, and total RNAs were prepared at the indicated time periods. The turnover of mRNA is determined by quantitative RT-PCR, as described above.

RIP assay

RAW264.7 cells or RAW264.7 cells stably transfected with plasmids coding for hnRNP U, N689, and C564 were stimulated with LPS for 2 h and then fixed and immunoprecipitated using the RIP assay kit (Upstate Biotechnology), according to the manufacturer’s instructions. The purified cell lysates were immunoprecipitated using 2 μg anti-hnRNP U or anti-Flag and anti-small nuclear ribonucleoprotein (snRNPs) 70 Ab. After 1-h incubation, protein A agarose beads were added. After 3 h of incubation at 4°C, the immunoprecipitates were centrifuged and washed six times with RIP wash buffer. Then RNA was isolated from the immunoprecipitates. The RNA obtained was reverse transcribed, and PCRs were performed using TNF-α, IL-6, IL-1β, and IL-12p40-specific primers (sequences given above). U1-specific primers were provided by the RIP assay kit.

Immunofluorescence staining

The 293-TLR4 cells transiently transfected with plasmids encoding Flag-hnRNP U were cultured for 24 h and then stimulated with LPS for 1 h. Flag-tagged hnRNP U was detected directly following fixation and washing. Anti-Flag Ab was used at 1:1000 dilutions in the blocking solution. The cells were then incubated with Alexa Fluor 568-conjugated secondary Ab (Molecular Probes, Invitrogen) diluted 1:1000 in blocking solution. Nuclei were stained with DAPI. Cells were examined with an Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan). For primary peritoneal macrophages, anti-hnRNP U Ab was used.

Assay of luciferase reporter gene expression

RAW264.7 macrophages were cotransfected with the mixture of indicated luciferase reporter plasmid, pRL-TK-Renilla-luciferase plasmid, and indicated amount of hnRNP U construct using Jet-PEI transfection reagent (Polyplus). Total amounts of plasmid DNA were evaluated via empty control vector. After 24 h, the cells were left untreated or treated with TLR agonists. Luciferase activities were measured with Dual-Luciferase Re-
porter Assay System (Promega), according to the manufacturer’s instructions. Data are normalized for transfection efficiency by dividing Firefly luciferase activity with that of Renilla luciferase.

**RNA EMSA**

Cytoplasmic extracts were prepared from HEK293 cells transfected with Flag-tagged hnRNP-U expression plasmid with M-PER Protein Extraction Reagent (Pierce). The ARE probes were labeled with biotin using the biotin 3'-end RNA labeling kit (Pierce), according to the manufacturer’s instructions. The sequences of the ARE probes used were 5'-UAU-UAUUUAUUAUUUAUUAUUUAUUUAU-3' from the TNF-α 3' UTR. The sequences of non-ARE probes were 5'-CCAUUCUCUAC-CCAGCCCCACUGCACC-3' from the TNF-α 3' UTR. RNA EMSA assay was performed with LightShift Chemiluminescent RNA EMSA Kit (Pierce), according to the manufacturer’s instructions. In competitive binding assays, unlabeled probes were added at 500-fold. Supershift assays were performed by preincubating cell extract with Flag Ab.

**Statistical analysis**

All data are presented as means ± SD of three experiments. Analysis was performed using a Student t test. The p values <0.05 were considered significant.

**FIGURE 1.** TLR-induced hnRNP U expression in macrophages. (A) RAW264.7 cells were stimulated with 100 ng/ml LPS for the indicated time periods. (B) RAW264.7 cells were stimulated with 5 μg/ml LTA or 20 μg/ml poly(I:C) for 12 h. Mice peritoneal macrophages were stimulated with 100 ng/ml LPS for the indicated time periods. (C) Mouse peritoneal macrophages were stimulated with 10 μg/ml PGN or 20 μg/ml poly(I:C) for 12 h. hnRNP U expression in the cells was detected by immunoblotting. Similar results were obtained in three independent experiments.

**FIGURE 2.** Knockdown of hnRNP U attenuates TLR-induced cytokine expression. (A) Mouse peritoneal macrophages were transfected with control siRNA (Ctrl) or hnRNP U siRNA (siRNA). After 36 h, hnRNP U expression in the cells was detected by immunoblotting. (B) A total of 2 × 10⁶ mouse peritoneal macrophages was transfected with control siRNA (Ctrl) or hnRNP U siRNA (siRNA) and then stimulated with 100 ng/ml LPS, 20 μg/ml poly(I:C), or 5 μg/ml LTA for 12 h. TNF-α, IL-6, IL-1β, and IL-12p70 in the supernatants were measured by ELISA. (C) RAW264.7 cells stably transfected with control shRNA (Ctrl) or hnRNP U shRNA (shRNA). hnRNP U expression in the cells was detected by immunoblotting. (D) A total of 3 × 10⁶ RAW264.7 cells stably transfected with control shRNA (Ctrl) or hnRNP U shRNA (shRNA) was stimulated with 100 ng/ml LPS, 20 μg/ml poly(I:C), or 5 μg/ml LTA for 12 h. TNF-α, IL-6, IL-1β, and IL-12p70 in the supernatants were measured by ELISA. (E) A total of 3 × 10⁶ RAW264.7 cells stably transfected with control shRNA (Ctrl) or hnRNP U shRNA (shRNA) was stimulated with 100 ng/ml LPS for the indicated time periods. Expression level of TNF-α, IL-6, IL-1β, and IL-12p40 mRNA was examined by quantitative PCR. For quantitative PCR, the results were presented as fold expression to that of β-actin. Data are shown as mean ± SD (n = 3) of one representative experiment (*p < 0.01).
Results

**TLR-induced hnRNP U expression**

To investigate the functions of hnRNP U in TLR signaling, hnRNP U protein expression was examined in macrophages stimulated with LPS (TLR4 ligand) for various times by immunoblotting. As shown in Fig. 1A, upon stimulation with LPS, a significant increase in expression of hnRNP U protein was detected in RAW264.7 macrophages. Induced expression of hnRNP U protein in RAW264.7 macrophages was detected after stimulation with LPS for 4 h and reached the peak level after stimulation with LPS for 12–18 h (Fig. 1A). To further confirm LPS-induced hnRNP U protein expression in macrophages, thioglycolate-elicited mouse primary peritoneal macrophages were used. In a similar fashion, expression of hnRNP U protein was upregulated 4 and 8 h after LPS treatment (Fig. 1C).

To confirm whether other TLR agonists can also induce hnRNP U protein expression in macrophages, LTA or PGN (TLR2 ligand) and poly(I:C) (TLR3 ligand) were used to stimulate macrophages. Similarly, stimulation with both LTA or PGN and poly(I:C) greatly enhanced hnRNP U protein expression 4 h after stimulation in both RAW 264.7 macrophages and peritoneal macrophages (Fig. 1B, 1D). Taken together, these data indicate that TLR signaling is associated with significantly increased hnRNP U protein expression in macrophages.

**Knockdown of hnRNP U attenuates TLR-induced cytokine expression**

TLR-induced hnRNP U protein expression indicates hnRNP U may play an important role in TLR signaling. To investigate the functions of hnRNP U in TLR signaling under physiological condition, hnRNP U-specific siRNA was transfected transiently into peritoneal primary macrophages. The production of TLR-induced cytokines, including TNF-α, IL-6, IL-1β, and IL-12, was measured by ELISA. hnRNP U protein was decreased ~60% by transfection of hnRNP U-specific siRNA, as measured by immunoblotting (Fig. 2A). After hnRNP U siRNA transfection, LPS-induced TNF-α, IL-6, and IL-1β secretion was decreased significantly, compared with control siRNA-transfected macrophages (Fig. 2B, p < 0.01). Similarly, LTA- and poly(I:C)-induced TNF-α, IL-6, and IL-1β expression was also greatly decreased, compared with control siRNA-transfected macrophages (Fig. 2B, p < 0.01). However, LPS-, LTA-, and poly(I:C)-induced IL-12 p70 was not significantly affected by hnRNP U siRNA transfection (Fig. 2B).

To further confirm the function of hnRNP U on TLR-induced cytokine production, hnRNP U-interfering RNA-expressing vector (hnRNP U shRNA) was constructed and transfected into RAW264.7 cells to select hnRNP U stably knockdown cells. hnRNP U protein expression in hnRNP U shRNA stably transfected RAW264.7 cells was decreased ~80%, as compared with control shRNA-transfected cells (Fig. 2C). Consistent with the results of hnRNP U siRNA transient transfection, the production of LPS-, LTA-, and poly(I:C)-induced TNF-α, IL-6, and IL-1β in hnRNP U shRNA stably transfected RAW264.7 cells was significantly decreased, as compared with control shRNA-transfected cells (Fig. 2D, p < 0.01). Similarly, IL-12 p70 production could not be affected by hnRNP U shRNA transfection. The steady state mRNA for LPS-induced TNF-α, IL-6, and IL-1β, but not IL-12 p70, was also greatly decreased in hnRNP U stably knockdown cells (Fig. 2E, p < 0.01). Taken together, these data indicate that hnRNP U positively regulates a subset of TLR-induced proinflammatory cytokine expression.
hnRNP U overexpression and proinflammatory cytokine expression

Knockdown of hnRNP U expression by siRNA indicates that hnRNP U positively regulates a subset of TLR-induced proinflammatory cytokine expression. To further confirm the results, stable RAW264.7 cell line with hnRNP U overexpression was constructed by transfection of hnRNP U expression plasmid. hnRNP U overexpression was confirmed by Western blotting (Fig. 3B). Cells were stimulated with LPS, poly(I:C), or LTA for 12 h. TNF-α, IL-1β, IL-6, and IL-12p70 in the supernatants were measured by ELISA. As shown in Fig. 3C–E, LPS-, poly(I:C)-, and LTA-induced TNF-α, IL-6, and IL-1β production was greatly increased in hnRNP U overexpression stable cells, compared with control vector-transfected stable cells (p < 0.01). Similarly, LPS-, poly(I:C)-, and LTA-induced IL-12 p70 production was not enhanced by hnRNP U overexpression (Fig. 3F). These data are consistent with the data obtained from siRNA knockdown experiments, confirming hnRNP U positively regulates a subset of TLR-induced proinflammatory cytokine expression.

hnRNP U is a 120-kDa protein with two functional domains: SAP and RNA-binding RGG box. It has been shown that hnRNP U binds directly to DNA sequences through the SAP domain, whereas the RGG box is responsible for RNA binding (7, 8). To investigate the domain requirement for the positive function of hnRNP U on the production of TLR-induced proinflammatory cytokines, two hnRNP U truncations were constructed, as follows: N689 (containing SAP) and C564 (containing RGG box) (Fig. 3A). These two expression plasmids were transfected into RAW264.7 cells,
and stable cell lines with high expression of hnRNP U N689 and C564 were selected. The expression of N689 and C564 was confirmed by Western blotting (Fig. 3B). Cells were stimulated with LPS, poly(I:C), or LTA for 12 h. TNF-α, IL-6, IL-1β, and IL-12p70 in the supernatants were measured by ELISA. Surprisingly, none of these two mutants have the ability to enhance LPS-, poly(I:C)-, and LTA-induced TNF-α, IL-6, and IL-1β production (Fig. 3C–E), indicating both motifs may be needed for the function of hnRNP U to enhance the production of TLR-induced proinflammatory cytokines.

**hnRNP U stabilizes cytokine mRNAs**

TLR-induced TNF-α, IL-6, and IL-1β transcription requires the activation of NF-κB transcription factor. To evaluate the effect of hnRNP U on LPS-induced NF-κB activation, NF-κB luciferase reporter plasmid and hnRNP U expression plasmid were cotransfected into RAW264.7 cells. Twenty-four hours after transfection, the cells were stimulated with LPS and the luciferase activity was measured. As shown in Fig. 4A, transfection of hnRNP U could not impair the NF-κB activation, indicating hnRNP U may enhance cytokine expression through a posttranscriptional mechanism, not at the transcriptional level.

The regulation of mRNA stability is one of the most important posttranscriptional mechanisms to control cytokine expression (4). Cytokine mRNA stability was measured by quantitative RT-PCR after adding actinomycin D, a specific RNA polymerase II inhibitor to arrest transcription. Two hours following LPS stimulation of hnRNP U stably knockdown RAW264.7 macrophages and control cells, actinomycin D was added. Expression of mRNA was normalized to that of the housekeeping gene, 28S RNA, and that of cytokines at time zero. In the macrophages transfected with control siRNA, the t1/2 of TNF-α, IL-6, and IL-1β mRNA was ~36, ~240, and ~30 min, respectively (Fig. 4B). However, in hnRNP U stably knockdown macrophages, the stability of the transcripts of all three cytokines was remarkably decreased following LPS stimulation (Fig. 4B). hnRNP U knockdown decreased the t1/2 of TNF-α, IL-6, and IL-1β mRNA to ~24, ~36, and ~22 min, respectively (Fig. 4B). In contrast, the stability of LPS-induced IL-12p40 mRNA was not remarkably decreased in hnRNP U siRNA-transfected macrophages, compared with that in control macrophages (Fig. 4B). These findings demonstrate that knockdown of hnRNP U expression is associated with a greater degradation rate of TLR-induced proinflammatory cytokine mRNAs. Therefore, hnRNP U promotes the stabilization of TLR-induced proinflammatory cytokine mRNAs.

**hnRNP U binding to cytokine mRNA through RGG motif**

hnRNP U is a RNA-binding protein; the ability of hnRNP U binding to TNF-α, IL-6, IL-1β, and IL-12 mRNA was measured by RIP assays. RAW264.7 macrophage was stimulated with LPS for 2 h, and RIP assays were performed with hnRNP U Ab. Then, RT-PCR was performed to identify hnRNP U binding to cytokine mRNAs. The presence of PCR products indicates the hnRNP U binding to RNA. As shown in Fig. 5A, RT-PCR detected the binding of hnRNP U to TNF-α, IL-6, and IL-1β mRNA, but not to IL-12p40 mRNA. As a control, snRNP70 bound to U1 mRNA, but not to any of the cytokine mRNAs. There is no PCR product with normal IgG in RIP assays (data not shown). These data indicate that hnRNP U can bind to TNF-α, IL-6, and IL-1β mRNA, but not to IL-12p40 mRNA.

To verify hnRNP U binding to TNF-α, IL-6, and IL-1β mRNA through RGG box, hnRNP U wild-type (WT) and truncations (N689 and C564) were transfected into RAW264.7 cells, and stable cell lines with high expression of WT and mutant hnRNP U were selected. After stimulation with LPS for 2 h, RIP assays were performed with FLAG Ab. Again, RT-PCR detected the binding of FLAG-tagged hnRNP U binding to TNF-α, IL-6, and IL-1β mRNA, but not IL-12p40 mRNA (Fig. 5B). In contrast, FLAG-tagged hnRNP U N689 (lacking RGG motif) lost the ability to bind to TNF-α, IL-6, and IL-1β mRNA (Fig. 5B). However, FLAG-tagged hnRNP U C564 (containing the RGG motif) could bind to TNF-α, IL-6, and IL-1β mRNA (Fig. 5B). None of the FLAG-tagged hnRNP U could bind to U1 mRNA, which can bind to snRNP70 efficiently. Collectively, these data indicate that
hnRNP U can bind to TNF-α, IL-6, and IL-1β mRNA through the RGG motif.

**hnRNP U binding to the ARE sequence in the 3′-UTR**

Several RNA-binding proteins have been shown to regulate the stability of cytokine mRNA through binding to the ARE found in the 3′-UTR of cytokine mRNA (22). Typical AREs have been identified in the 3′-UTR of TNF-α, IL-6, and IL-1β, but not in IL-12p40 (23). Therefore, hnRNP U may directly bind to TNF-α, IL-6, and IL-1β mRNA through the AREs. To confirm hnRNP U increases TLR-induced production of proinflammatory cytokines through binding to the 3′-UTR, the 3′-UTRs of TNF-α, IL-6, and IL-12p40 were PCR amplified and inserted into the 3′-UTR of a luciferase reporter pGL3. The resulting luciferase constructs were cotransfected into HEK293 T cells together with hnRNP U WT and truncations; luciferase activity was measured. Cotransfection hnRNP U WT plasmids resulted in ∼2-fold increase in luciferase activity in the TNF-α and IL-6 3′-UTR-inserted pGL3 reporter, respectively (Fig. 6A, p < 0.01). However, hnRNP U WT has no effect on the IL-12p40 3′-UTR-inserted pGL3 reporter, indicating hnRNP U enhances the expression of TNF-α and IL-6, but not IL-12p40 through binding to their 3′-UTR (Fig. 6A). Consistently, hnRNP U mutant N689 (lacking RGG motif) could not increase the luciferase activity of the TNF-α and IL-6 3′-UTR-inserted pGL3 reporter (Fig. 6A). Surprisingly, hnRNP U mutant C564 (containing the RGG motif), which could bind to the mRNA of TNF-α, IL-6, and IL-1β as measured by RIP assays, could not increase the luciferase activity of the TNF-α and IL-6 3′-UTR-inserted pGL3 reporter, indicating hnRNP U may need other domains except RGG box to facilitate the stabilization of TNF-α, IL-6, and IL-1β mRNA (Fig. 6A).

To further confirm ARE is essential for hnRNP U to increase TLR-induced production of proinflammatory cytokines, the AREs from the 3′-UTR of TNF-α and IL-6 were deleted in the pGL3-TNF-α 3′-UTR and pGL3-IL-6 3′-UTR reporters. In accordance with the data in Fig. 6A, transfection of hnRNP U WT plasmids greatly increased the luciferase activity of the reporters with WT 3′-UTR of TNF-α and IL-6 (p < 0.01). Deletion of ARE from 3′-UTR from TNF-α and IL-6 ablated hnRNP U-induced increase of luciferase activity (Fig. 6B).

To directly confirm hnRNP U binding to ARE, RNA EMSA was performed using biotin-labeled ARE from TNF-α 3′-UTR with cytoplasmic extracts from HEK293 cells transfected with hnRNP U expression plasmid. As shown in Fig. 6C, no RNA and protein complex was detected without cytoplasmic extracts (lane 1). Addition of cytoplasmic extracts resulted in the formation of RNA and protein complex (lane 2). This RNA and protein binding was specific because it was eliminated by the addition of excess unlabeled TNF-α ARE (lane 3), but not by the addition of non-ARE sequence from TNF-α 3′-UTR (lane 4). To determine whether this ARE-binding activity was attributable to hnRNP U, supershift experiments were conducted (Fig. 6C). Incubation with an anti-Flag Ab completely supershifted the RNA and protein complex (lane 6). All together, these data indicate hnRNP U binds to the 3′-UTR through the ARE sequence to regulate the stability of TNF-α and IL-6.

**TLR-induced hnRNP U nuclear to cytoplasmic translocation**

hnRNP U is a nuclear protein, and mRNA degradation occurs in the cytoplasm. To evaluate whether TLR stimulation provides a stimulus for hnRNP U nuclear to cytoplasmic translocation, immunofluorescence microscopy was performed in LPS-stimulated peritoneal macrophages with hnRNP U Ab. DAPI staining was performed for nuclear definition. In unstimulated macrophages, hnRNP U remained exclusively nuclear (Fig. 7A). However, in LPS-stimulated macrophages, hnRNP U was distributed throughout the cytoplasm and nucleus (Fig. 7A). To further confirm the translocation of hnRNP U, FLAG-hnRNP U fusion plasmid was constructed and transfected into HEK293T/TLR4 cells, which stably express TLR4. Twenty-four hours after transfection, hnRNP U was detected by immunoblotting. The purity of the obtained cytoplasmic and nuclear fractions was confirmed with anti-tubulin β (marker for the cytoplasmic fraction) and anti-poly(ADP-ribose) polymerase and Lamin A/B (marker for the nuclear fraction) Abs.
fection, immunofluorescence microscopy was performed with FLAG Ab. Similarly, FLAG-hnRNP U remained exclusively nuclear without LPS stimulation. With LPS stimulation, FLAG-hnRNP U was distributed throughout the cytoplasm and nucleus (Fig. 7B). These data suggest that LPS stimulation provides a stimulus for hnRNP U nuclear to cytoplasmic translocation.

To biochemically confirm LPS-stimulated hnRNP U nuclear to cytoplasmic translocation, nuclear and cytoplasmic fractions were carefully separated and Western blot analysis was performed for hnRNP U. As expected, hnRNP U was present predominantly in nucleus in unstimulated macrophages. However, cytoplasmic hnRNP U protein level was gradually increased in LPS-stimulated macrophages. At the same time, nuclear hnRNP U protein level was gradually decreased (Fig. 7C). In murine embryonic fibroblast, LPS stimulation can also induce hnRNP U nuclear to cytoplasmic translocation (Fig. 7D). These findings are consistent with the immunofluorescent analysis and confirm that LPS stimulation can promote nuclear export of hnRNP U from nucleus into cytoplasm.

Discussion
TLR signaling can induce the transcription of varieties of genes. Some of the gene products have been demonstrated to modify the TLR-induced immune response at different levels (24). In this study, we found hnRNP U protein expression and nuclear to cytoplasmic translocation were greatly increased by TLR stimulation; therefore, hnRNP U can be classified as a TLR-inducible gene. The function of TLR-induced hnRNP U was further investigated by knockdown and overexpression experiments. These data indicate hnRNP U is a positive regulator for TLR-induced expression of proinflammatory cytokines. We continue to show that hnRNP U can bind to proinflammatory cytokine mRNAs through the RGG box. Knockdown of hnRNP U expression accelerated the degradation of proinflammatory cytokine mRNAs. Therefore, we propose that TLR-induced hnRNP U protein expression and cytoplasmic translocation provide a forward positive regulatory mechanism to enhance the production of proinflammatory cytokines by binding and stabilizing the mRNAs.

Heterogeneous nuclear ribonucleoproteins (hnRNP) were originally described as chromatin-associated RNA-binding proteins that form complexes with RNA polymerase II transcripts. The hnRNP family is comprised of ∼20 proteins that are able to modulate RNA processing (25). But, the precise mechanism for hnRNP U to modulate RNA processing remains elusive. In this study, we identified hnRNP U as a mRNA-stabilizing modifier for TLR-induced proinflammatory cytokine mRNAs. Another member of the hnRNP family that is affecting proinflammatory mRNA stability and studied extensively is hnRNP D, also called AUF1 (26). But, AUF1 is principally associated with destabilization of proinflammatory cytokine mRNAs by binding to the so-called cis-acting AREs present in the 3′-UTR of certain mRNAs (27, 28). These studies indicate that different hnRNP proteins have different functions to regulate the production of proinflammatory cytokines by either increasing or decreasing the stabilization of mRNA. However, the functional relationship between hnRNP U and hnRNP D in regulating the mRNA stability needs further investigation.

hnRNP U is a 120-kDa protein with two functional domains: SAP and RNA-binding RGG box. It has been shown that hnRNP U binds directly to DNA sequences through the SAP domain, whereas the RGG box is responsible for RNA binding (7, 8). We found hnRNP U binding to the proinflammatory cytokines mRNA is through the C-terminal RGG box because the hnRNP U truncation plasmid N689 (lacking RGG box) lost the binding ability, whereas C564 (containing RGG box) could bind to the mRNA of proinflammatory cytokines. Although C564 can bind to the mRNA of proinflammatory cytokines, this expression vector lost the ability to enhance TLR-induced proinflammatory cytokine expression. C564 also lost the ability to increase the luciferase activity in the reporter plasmids with the insertion of the 3′-UTRs of TNF-α and IL-6. These data indicate that except for the RGG box for RNA binding, hnRNP U still needs other motifs for its full function. Structural analysis identified another domain called B30.2/SPRY in the middle part of hnRNP U. The B30.2/SPRY domain has been indicated in protein-protein interaction (29). So, hnRNP U stabilization of mRNAs may need other interacting proteins through B30.2/SPRY. Indeed, a recent study found hnRNP U interacts with the Wilms’ tumor suppressor gene, WT1, through the middle domain of hnRNP U (30).

hnRNP U is a nuclear protein, whereas mRNA degradation is a cytoplasmic event. We found TLR stimulation provides a stimulus for hnRNP U nuclear to cytoplasmic translocation with both immunofluorescence staining and Western blotting with nuclear and cytoplasmic fractions in macrophages and HEK293/TLR4 cells. However, how TLR stimulation can cause the cytoplasmic translocation is not clear. Recent reports have confirmed scaffold attachment factor A/hnRNP U is phosphorylated at S59 position by DNA-dependent protein kinase in response to DNA double-strand break inducers (31, 32). TLR signaling can induce the activation of varieties of kinases, which may in turn phosphorylate hnRNP U and induce the translocation. However, the precise mechanism for hnRNP U translocation needs further investigation.

In conclusion, our results demonstrate that hnRNP U expression and nuclear to cytoplasm translocation were induced by TLR signaling. By binding to the mRNAs of proinflammatory cytokine and preventing them from degradation, hnRNP U positively regulates TLR-induced proinflammatory cytokine production in macrophages. Therefore, hnRNP U is an important immune response modifier and plays an important role in TLR signaling.

Disclosures
The authors have no financial conflicts of interest.

References