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Early Exposure to IL-4 Stabilizes IL-4 mRNA in CD4+ T Cells via RNA-Binding Protein HuR

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The mechanisms regulating IL-4 mRNA stability in differentiated T cells are not known. We found that early exposure of CD4+ T cells to endogenous IL-4 increased IL-4 mRNA stability. This effect of IL-4 was mediated by the RNA-binding protein HuR. IL-4 mRNA interacted with HuR and the dominant binding site was shown within the coding region of IL-4 mRNA. Exposure of CD4+ T cells to IL-4 had no effects on HuR expression or subcellular localization, but triggered HuR binding to IL-4 mRNA. Thus, IL-4 plays a positive role in maintaining IL-4 mRNA stability in CD4+ T cells via a HuR-mediated mechanism. The Journal of Immunology, 2006, 177: 4426–4435.

Several RNA-binding proteins have been implicated in the regulation of mRNA stability in response to extracellular stimuli (9). Tristetraproline (TTP)4 and AUF1 proteins are implicated in mRNA degradation (15, 16). In contrast, a ubiquitously expressed RNA-binding protein HuR, also known as ELAV-like 1 or HuA, promotes mRNA stability (17, 18). However, to our best knowledge, none of these RNA-binding proteins have previously been shown to interact with murine IL-4 mRNA.

The structure of mRNA determines its fate and half-life in the cell (8, 19). Particularly, the presence of AU-rich elements in the 3′-untranslated region (UTR) facilitates mRNA interaction with RNA-binding proteins. The target RNA motif for HuR binding has been described by two independent groups (20, 21). The first report proposed the sequence of a HuR motif based on the nucleotide frequency, and validated it for a number of mRNAs (20). However, a computational analysis conducted in the laboratory of Dr. M. Gorospe (Laboratory of Cellular and Molecular Biology, National Institute on Aging-Intramural Research Program, National Institutes of Health, Bethesda, MD) revealed no potential binding sites within murine IL-4 mRNA (M. Gorospe, unpublished observation). The other report described the HuR motif as the sequence NNUUNNUUU presented in a single-stranded conformation (21). Although this HuR motif was found in human IL-4 mRNA (21), murine IL-4 mRNA has not been formally analyzed for the presence of this HuR motif.

A previous report showed that expression of HuR is increased following activation of murine splenocytes (22). Activation of human T cells is also accompanied by HuR induction (23). A recent study implicated HuR in stabilization of eotaxin mRNA in human epithelial cells in response to IL-4 treatment (24). Based on these studies, we hypothesized that early exposure of T lymphocytes to IL-4 positively regulates IL-4 mRNA stability via HuR. To test this hypothesis, we determined the effects of IL-4 on IL-4 mRNA stability in T lymphocytes. We found that IL-4 triggers HuR binding to the coding region of IL-4 mRNA and mediates increased stability of the mRNA.

Materials and Methods

Mice and cells

The animal use protocol was approved by the University of Iowa Institutional Animal Care and Use Committee. Female C57BL/6 and DBA/2 mice were purchased from Harlan Sprague-Dawley. TCR-DO11.10/Rag2

\[4\] Abbreviations used in this paper: TTP, tristetraproline; AUF1, actinomycin D; HPRT, hypoxanthine phosphoribosyltransferase; UTR, untranslated region; KO, knockout; shRNA, short hairpin RNA.
knockout (KO) mice on BALB/c background were purchased from Taconic Farms. All mice (females, 6 to 12 wk old) were housed in the specific pathogen-free facility and fed standard mouse chow and water ad libitum. Spleens were aseptically removed from euthanized animals and used as the source of CD4+ T cells.

CD4+ T cells were isolated to >90% purity from C57BL/6 and DBA/2 mice using CD4+ CD62L- T cell isolation kit (Miltenyi Biotec) or mouse CD4+ Negative Isolation kit (Dynal Biotech). The cells were stimulated with plate-bound anti-CD3 (clone 145-2C11; BD Biosciences) plus anti-CD28 (clone 37.51; BD Biosciences) mAbs (2.5 μg/ml each) for 3 days, washed with the fresh medium, and expanded in the presence of 20 μM murine rIL-2 (Roche Applied Science).

CD4+ T cells from TCR-DO11.10/Rag2(KO) mice were obtained by stimulation of splenocytes with 7 μM OVA232-339 peptide (Bachem Biosciences) for 3 days, followed by expansion in the presence of IL-2, and removal of non-CD4+ T cells with Ab-coated magnetic beads.

Endogenous IL-4 was neutralized with 10 μg/ml monoclonal anti-IL-4 Ab (clone 30340.11; R&D Systems). Control cells were cultured in the presence of 10 μg/ml isotype control Ab. In some experiments, CD4+ T cells from C57BL/6 mice were treated with 10 ng/ml IL-4 (R&D Systems).

All primary cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS (HyClone Laboratories), 2 mM l-glutamine (Life Technologies), 50 μM 2-ME (Sigma-Aldrich), and 80 μg/ml gentamicin at 37°C in 5% CO2. EL4 (TIB-39) and D10.G4.1 (TIB-224) cell lines were purchased from American Type Culture Collection and cultured following their guidelines.

mRNA stability assay

Primary T lymphocytes were washed at least twice in complete medium to remove exogenous added Abs before expansion with 10 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (EMD Biosciences) or with immobilized anti-CD3/CD28 Abs in the presence of 20 μM IL-2. Following restimulation for the indicated period of time, cells were treated with 10 μg/ml actinomycin D (ActD) or 2 μM α-aminitin (both from EMD Biosciences) to inhibit transcription. Total RNA was isolated using Absolutely RNA Miniprep kit (Stratagene) or RNAqueous-4PCR kit (Ambion). RNA abundance was measured by quantitative RT-PCR as described. The mRNA fold enrichment was calculated by the formula (CtHuR - CtActin) / (CtActin - CtH19). The percentage of remaining IL-4 mRNA relative to HPRT mRNA at 1–3 h after ActD treatment is shown (A, C, and E). The fold change in the abundance of IL-4 mRNA relative to HPRT mRNA before ActD treatment is also shown (B, D, and F). The data are mean ± SEM of four (A, B, D, and F) or three (C, E, and F) independent experiments. Significant change (*, p < 0.05) in IL-4 mRNA abundance relative to control groups is indicated. Ab was purchased from Upstate Biotechnology. Mouse anti-β-actin Ab (clone AC-74) was purchased from Sigma-Aldrich. Immunoblotting analysis and densitometry were performed as previously described (27).

Immunofluorescence staining, confocal microscopy, and flow cytometry

The cells were washed with PBS and fixed with ice-cold methanol. Following blocking with normal goat serum (Chemicon International), the cells were incubated with mouse anti-HuR or isotype control Abs, washed and incubated with goat anti-mouse Alexa Fluor 488 secondary Ab (Invitrogen Life Technologies). Stained cells were immobilized on poly-L-lysine slides and analyzed by confocal microscopy as previously described (27).

HuR expression in CD4+ T cells was also measured by flow cytometry. Freshly isolated or differentiated CD4+ T cells were fixed with methanol and stained with FITC-conjugated anti-HuR Ab. Staining with unconjugated anti-HuR or isotype control Ab followed by PE-conjugated goat anti-mouse Ab was used to measure HuR expression in CD4+ T cells by two-color flow cytometry using a FACSscan (BD Biosciences) at the University of Iowa Flow Cytometry Facility (Iowa City, IA).

FIGURE 1. IL-4 increases IL-4 mRNA stability. Anti-IL-4 Ab decreased IL-4 mRNA stability (A) and abundance (B) in activated CD4+ T cells from DBA/2 mice. Anti-IL-4 Ab also decreased IL-4 mRNA stability (C) and abundance (D) in OVA232-339 peptide-activated CD4+ T cells from TCR-DO11.10/Rag2(KO) transgenic mice. Exogenous IL-4 increased IL-4 mRNA stability (E) and abundance (F) in activated CD4+ T cells from C57BL/6 mice. The percentage of remaining IL-4 mRNA relative to HPRT mRNA at 1–3 h after ActD treatment is shown (A, C, and E). The fold change in the abundance of IL-4 mRNA relative to HPRT mRNA before ActD treatment is also shown (B, D, and F). The data are mean ± SEM of four (A, B, D, and F) or three (C, E, and F) independent experiments. Significant change (*, p < 0.05) in IL-4 mRNA abundance relative to control groups is indicated.
Analysis of HuR binding to in vitro-transcribed RNA probes

A murine IL-4 cDNA clone was purchased from Open Biosystems. PCR amplification of plasmid DNA was used to obtain fragments corresponding to the full-length IL-4 cDNA, coding region plus 3' UTR, coding region alone or 3' UTR alone. The mutation in the coding region (U324C) was made by overlap extension.

GAPDH cDNA (positions 319-1047; GenBank accession no. NM_008084) was obtained by amplification with specific primers from the cDNA pool of EL4 cells. Topoisomerase-activated adapters were used to incorporate bacteriophage T7 RNA polymerase promoters into the PCR products to make templates for in vitro transcription as previously described (28). All templates were sequenced with T7 promoters for quality control at the University of Iowa DNA Facility.

Biotin pull-down assays were performed using 2 μg of biotinylated riboprobes, 30 μg of cytosolic protein extracts, and 20 μl of streptavidin-coated magnetic beads M-280 (Dynal Biotech) following a previously published protocol (26). Following extensive washing, complexes were subjected to immunoblotting to detect bound HuR protein.

RNA EMSA

RNA oligonucleotides were synthesized, labeled with biotin at 5' position and HPLC-purified at Integrated DNA Technologies. These oligonucleotides were as follows: IL-4-CDS (5'-GCUUCGCAUAUUUAUUUAA-3', corresponding to the HuR binding site within the coding region of IL-4 mRNA); IL-4mut (5'-GCUUCGCAUAUUUAUUUAA-3', corresponding to the mutant U324C HuR binding site within the coding region of IL-4 mRNA); and IL-4 3'UTR (5'-AUGGUUUUAUUUAAUUU-3', corresponding to the HuR binding site within the 3'UTR). Cytosolic protein extracts (10 μg) were incubated with 500 nM of the probes in binding buffer (40 mM KCl, 10 mM HEPES (pH 7.9), 3 mM MgCl2, 5% glycerol, 0.1% Nonidet P-40) for 10 minutes at room temperature.

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1 mM DTT) for 30 min at room temperature. Following non-denaturing gel electrophoresis and transfer to nylon membranes, biotinylated RNA probes were visualized using Chemiluminescent Nucleic Acid Detection Module (Invitrogen Life Technologies). A 50-fold excess of non-biotinylated RNA oligonucleotide IL-4-CDS was used for competition experiments. Cytosolic protein extracts were preincubated with 4 μg of monoclonal anti-HuR Ab or isotype control Ab before addition of the RNA in supershift RNA EMSAs.

Cell transfections and RNA interference

Mouse IL-4 cDNA (spanning positions 50–603 of full-length IL-4 cDNA; GenBank accession no. NM_021283) was subcloned into pcDNA3.1V5HisTOPO expression vector (Invitrogen Life Technologies). To generate a reporter vector, the 3′UTR of IL-4 cDNA (positions 511–605) was ligated downstream of the open reading frame of the firefly luciferase gene driven by the CMV promoter. Control construct consisted of the CMV-driven luciferase gene with the SV40 polyadenylation signal. Both constructs were subcloned into pCR-TOPO4 vector (Invitrogen Life Technologies) and validated by sequencing. RNA interference studies were performed using short hairpin RNA (shRNA) constructs from Open Biosystems. NIH-3T3 cells (5 × 10^5 cells/well in 24-well plates) were transfected with 0.75 μg of control or anti-HuR shRNA expressing plasmid and 0.25 μg of IL-4 expressing vector or luciferase reporter vectors. Transfection efficiency (~30%) was estimated using a GFP expression vector pcDNA3.1, which also served as a negative control. Cell lysates were used to estimate HuR ablation by Western blot and luciferase activity from the reporter vector. Cell culture supernatants were used to evaluate IL-4 production.

Statistical analyses

The data were expressed as the mean ± SEM. The half-life of cytokine mRNAs was calculated for each experiment and the differences between the groups in repeated experiments were analyzed by two-tailed paired Student’s t test. Unpaired Student’s t test assuming equal variances was used to analyze the difference between the groups within the same experiments. All calculations were performed with GraphPad Prism software version 4.0 (GraphPad Software).

Results

Exposure of CD4+ T cells to IL-4 increases IL-4 mRNA stability

We have previously shown that activated CD4+ T cells from DBA/2 mice secrete more IL-4 than CD4+ T cells from C57BL/6 mice within the first 24 h of activation (14). This finding suggests that CD4+ T cells from DBA/2 mice are exposed to higher amounts of IL-4. Therefore, to determine the role of endogenous IL-4 in the regulation of IL-4 mRNA stability, we stimulated and expanded CD4+ T cells from DBA/2 mice in the presence of neutralizing anti-IL-4 or isotype control Ab. Following restimulation with PMA/ionomycin for 4 h and transcriptional inhibition with ActD, we used quantitative real-time RT-PCR to measure the amount of remaining IL-4 mRNA relative to HPRT mRNA.

We observed that anti-IL-4 Ab accelerated IL-4 mRNA decay (Fig. 1A). Because ActD started to show cytotoxic effects after 3 h, we had to determine IL-4 mRNA half-life in the group treated with isotype control Ab by extrapolation and estimated it at 3.8 ± 0.7 h (mean ± SEM from four independent experiments). IL-4 mRNA half-life was significantly decreased to 2.1 ± 0.3 h (p < 0.05; n = 4) in the group treated with anti-IL-4 Ab (Fig. 1A). Furthermore, treatment of DBA/2-derived CD4+ T cells with anti-IL-4 Ab decreased the abundance of IL-4 mRNA (Fig. 1B).

To determine whether endogenous IL-4 regulates IL-4 mRNA stability under more physiological conditions of Ag-specific T cell activation, we planned to stimulate CD4+ T cells from TCR-DO11.10/Rag2(KO) transgenic mice with the specific Ag (ova323-339 Peptide) in the presence of neutralizing anti-IL-4 Ab. Because the TCR-DO11.10/Rag2(KO) transgenic mice are on BALB/c background, we initially tested the effects of anti-IL-4 Ab treatment on IL-4 mRNA stability in polyclonally activated CD4+ T cells from wild-type BALB/c mice. We found that anti-IL-4 Ab decreased IL-4 mRNA half-life from 3.1 to 2.5 h, suggesting that endogenous IL-4 has similar effects on IL-4 mRNA stability in DBA/2 and BALB/c strains.

Using Ag-stimulated CD4+ T cells from the TCR-DO11.10/Rag2(KO) transgenic mice, we found that anti-IL-4 Ab significantly decreased IL-4 protein secretion following restimulation with anti-CD3/CD28 Ab (from 30683 ± 3698 pg/ml in isotype control-treated group to 316 ± 14 pg/ml in anti-IL-4-treated group; p < 0.01; n = 3), which is consistent with a previous study using this model (29). We estimated IL-4 mRNA half-life at 3.6 ± 0.8 h in the group treated with isotype control Ab (Fig. 1C). Treatment with anti-IL-4 Ab significantly decreased IL-4 mRNA half-life to 1.8 ± 0.4 h (p < 0.05; n = 3). Anti-IL-4 Ab also significantly decreased the abundance of...
IL-4 mRNA (Fig. 1D). Thus, decreased IL-4 mRNA stability following treatment with anti-IL-4 Ab suggested that endogenous IL-4 promotes its own mRNA stability in Ag-specific CD4\(^+\) T cells.

Next, we determined whether exogenous IL-4 may increase IL-4 mRNA stability in CD4\(^+\) T cells from C57BL/6 mice, which produce very low amounts of endogenous IL-4. Control CD4\(^+\) T cells from C57BL/6 mice expressed IL-4 mRNA with a relatively shorter half-life (1.1 ± 0.2 h; n = 3). Exogenous IL-4 stabilized IL-4 mRNA (Fig. 1E) and increased IL-4 mRNA half-life to 2.4 ± 0.2 h (n = 3). Furthermore, exogenous IL-4 increased IL-4 mRNA abundance in CD4\(^+\) T cells from C57BL/6 mice (Fig. 1F). Thus, low IL-4 mRNA stability and abundance in C57BL/6 derived CD4\(^+\) T cells may be partially corrected by adding exogenous IL-4.

**IL-4 mRNA stability and HuR expression in lymphocyte cell lines**

To elucidate the mechanisms that maintain IL-4 mRNA stability in Th2 lymphocytes, we used established cell lines EL4 and D10.G4.1, which are known to secrete IL-4 upon stimulation (30–32). These cells have been used in a number of studies to evaluate mRNA stability for other cytokines and allowed us to avoid potential problems due to heterogeneity of primary cells (30–33).

As expected, PMA/ionomycin stimulation of EL4 cells resulted in considerable induction of IL-4 mRNA (79 ± 21-fold increase over nonstimulated cells; n = 3) that reached steady levels by 20 h poststimulation (data not shown). When we treated EL4 cells with PMA/ionomycin for 16 h, induced IL-4 mRNA remained relatively stable after 3 h of ActD treatment (Fig. 2A). IL-2 mRNA was induced by PMA/ionomycin (2821 ± 705-fold increase over nonstimulated cells; n = 3), but appeared to be less stable than IL-4 mRNA (Fig. 2A). TNF-\(\alpha\) mRNA was also induced by PMA/ionomycin (7 ± 1-fold increase over nonstimulated cells; n = 3), but decayed relatively faster (Fig. 2A). Similar results were obtained when the cytokine mRNA amounts were normalized to GAPDH mRNA (data not shown). Furthermore, when we treated activated EL4 cells with \(\alpha\)-amanitin, which inhibits RNA polymerase II with minimal cytotoxic effects, IL-4 mRNA remained quite stable for up to 8 h (data not shown). Thus, using two different inhibitors of transcription, we determined that IL-4 mRNA is relatively stable in EL4 cells.

In addition, we evaluated IL-4 mRNA stability in a cloned Th2 cell line D10.G4.1, which is a better model for differentiated CD4\(^+\) T lymphocytes (34). Following PMA/ionomycin and ActD treatment, IL-4 mRNA was also more stable than IL-2 mRNA in D10.G4.1 cells (Fig. 2B). Thus, we showed that IL-4 mRNA is relatively stable in two unrelated lymphocyte cell lines.

Expression of the RNA-binding protein HuR, particularly in the cytosol, has been shown to increase mRNA stability (22, 35, 36). To evaluate expression and localization of HuR in EL4 and D10.G4.1 cells, we used immunofluorescence staining followed by confocal microscopy. Both cell lines showed robust nuclear staining with a HuR-specific mAb, but not with the isotype control Ab (Fig. 2C). Lower intensity, but still detectable, HuR signal was evident in the cytosol in the majority of the cells from both lines (Fig. 2C, arrows). Moreover, immunoblotting of cytosolic and nuclear cell extracts confirmed that HuR was predominantly localized in the nucleus, but could also be detected in the cytosol (Fig. 2D). Stimulation of EL4 or D10.G4.1 cells with PMA/ionomycin had no apparent effect on subcellular localization of HuR (data not shown). Thus, using immunofluorescence and immunoblotting, we showed that HuR is constitutively present in the cytosol of EL4 and D10.G4.1 cells.

To determine whether HuR binds to IL-4 mRNA in EL4 cells, we immunoprecipitated ribonucleoprotein complexes from EL4 cell lysates using a HuR-specific polyclonal Ab and measured the relative amounts of IL-4 mRNA in the immunoprecipitated complexes. Low amounts of IL-4 and GAPDH mRNA could be detected using isotype-specific antibodies (Fig. 3), but not using preimmune serum (data not shown). Furthermore, we verified by Northern analysis that HuR did not increase IL-4 mRNA abundance in EL4 cells (data not shown). Immunoblotting of the IP and input lysates confirmed that the IP contained HuR (Fig. 3). Thus, using immunoprecipitation and immunoblotting, we showed that HuR selectively binds to endogenous IL-4 mRNA in EL4 cells, but not in D10.G4.1 cells.

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**FIGURE 4.** Analysis of secondary structure of IL-4 mRNA and HuR binding to in vitro-transcribed RNA probes. A. A representative secondary structure of IL-4 mRNA with free energy: −162.37 kcal/mol predicted by RNA-fold analysis is shown. The HuR binding sites (highlighted by arrows and shaded boxes) are presented in single-stranded conformation in the coding regions (CDS) (1) and double-stranded (2) conformation in the 3'UTR. B. Biotin pull-down assay demonstrated binding of HuR to the coding and 3'UTR regions. In vitro-transcribed biotinylated IL-4 mRNA probes spanned coding regions and 3'UTR, coding regions only, or 3'UTR only. GAPDH riboprobe was used to determine background nonspecific binding. Following incubation with lysates from EL4 cells, the probes were pulled down using streptavidin-coated magnetic beads and bound HuR was detected by immunoblotting. C. PCR mutagenesis was used to introduce indicated single nucleotide mutations. Biotin pull-down assay was used to analyze HuR binding to wild-type and mutant in vitro-transcribed biotinylated probes. The membrane was stripped and reprobed with anti-AUF1 Ab, which recognizes all four isoforms of AUF1 (p37, p40, p42, p45). The data are representative of two experiments.
control Ab, apparently due to nonspecific binding to protein A-agarose (Fig. 2E). The amount of GAPDH mRNA, which does not specifically interact with HuR, changed little when HuR-specific Ab was used (Fig. 2E). In contrast, p21 mRNA, which has been previously shown to be stabilized by HuR (37), was enriched by 15-fold in the HuR immune complexes (data not shown). IL-4 mRNA was enriched even higher in the HuR immune complexes (Fig. 2E, inset), indicating a specific association of HuR with IL-4 mRNA in vivo.

HuR binds to the coding and 3′ UTRs of IL-4 mRNA

Two previous studies identified the target mRNA motifs recognized by HuR (20, 21). However, the interaction of HuR with murine IL-4 mRNA has not been formally investigated. Because the computational analysis conducted in the laboratory of Dr. M. Gorospe revealed no potential HuR binding sites within murine IL-4 mRNA (M. Gorospe, unpublished data), we used an alternative consensus sequence NNUUNNUUU, which was proposed by Meisner et al. (21). We identified two putative HuR binding sites containing the consensus motif within murine IL-4 mRNA (Fig. 3A). The first binding site was found in the coding region (positions 318–326). The second binding site, which actually consisted of two overlapping sequences (positions 549–557 and 550–558) was found in the 3′ UTR.

To determine whether HuR binds to the identified sequences, we used synthetic biotinylated RNA oligonucleotides and RNA-EMSA. RNA probe corresponding to the HuR binding site within the coding region showed nonspecific bands in the absence of the protein extract (Fig. 3B, asterisk). Incubation of this probe with the cytosolic protein extract from EL4 cells formed three distinct RNA-protein complexes with retarded electrophoretic mobility (Fig. 3B, arrows marked 1, 2, and 3). These complexes were diminished by preincubation with a 50-fold excess of unlabeled competitor RNA oligonucleotide of identical sequence. RNA probe corresponding to the HuR binding site in the 3′UTR of IL-4 mRNA also showed some nonspecific bands in the absence of the protein extract (Fig. 3C, arrow). Preincubation of the cytosolic protein extract with a monoclonal anti-HuR Ab (clone 3A2), but not with an isotype control Ab, produced a supershifted complex with both riboprobes (Fig. 3, D and E). Importantly, a single nucleotide mutation in the HuR binding site within the coding region diminished this supershifted complex (Fig. 3D). As a composite, these data suggest that HuR binds to the identified sequences in IL-4 mRNA.

It has been previously reported that mRNA recognition by HuR is dependent on the presentation of the consensus sequence in the single-stranded conformation (21). To predict the conformation of the HuR-binding motifs in murine IL-4 mRNA, we analyzed IL-4 mRNA-fold using mfold Web server (http://www.bioinfo.rpi.edu/applications/mfold) developed by Zuker (38). This analysis predicted 15 possible secondary structures for full-length IL-4 mRNA with the range of free energy from −162.37 to −150.13 kcal/mol. In two of those structures, the HuR-binding motif in the coding region was predicted to exist in the single-stranded conformation (Fig. 4A). In contrast, the HuR-binding motif in the 3′UTR was predicted to be present in the double-stranded conformation in all possible structures (Fig. 4A). To validate the identified HuR-binding motifs in IL-4 mRNA in the context of secondary RNA structure, we used in vitro-transcribed biotinylated RNA probes and biotin pull-down assay. We detected binding of HuR to the IL-4 probe spanning the coding region and 3′UTR (Fig. 4B). The IL-4 probe containing the coding region only appeared to be more efficient in HuR binding than the probe containing the coding region with the 3′UTR or 3′UTR alone. The presence of 3′UTR in IL-4 riboprobes had no effect on binding of HuR (data not shown). GAPDH probe apparently bound some HuR, albeit the signal intensity was low. HuR could not be detected in the samples containing no riboprobe, ruling out the possibility of nonspecific contamination in the biotin pull-down assay.

Furthermore, we explored whether mutations in the putative HuR-binding sites could affect HuR binding. The mutation U324C in the coding region altered the sequence of the putative HuR binding site, but did not change its single-stranded conformation (data not shown). Using a biotin pull-down assay, we found that this mutation decreased HuR binding to the coding region of IL-4 mRNA (Fig. 4C, top). Conversely, U556C mutation in the 3′UTR transformed the predicted RNA structure to a single-stranded conformation (data not shown) and increased HuR binding. Importantly, neither mutation had any effect on binding of AUF1, which has been previously shown to antagonize HuR and target mRNAs to degradation (16, 39, 40) (Fig. 4C, bottom). Thus, our results indicate that sequence or secondary structure alterations within the putative HuR binding sites of IL-4 mRNA specifically modulate HuR binding.

Production of IL-4 correlates with HuR expression levels

To determine whether HuR regulates IL-4 production, we attempted to modulate the levels of HuR by using RNA interference. Unfortunately, none of the methods (chemically synthesized short-interfering RNA, transient transfection with plasmids expressing shRNA constructs, and stable transfection with shRNA plasmids followed by positive selection) resulted in a noticeable decrease of HuR levels in T lymphocytes. However, using shRNA constructs targeting HuR, we were able to ablate HuR expression in NIH-3T3 fibroblasts (Fig. 5A). We found that HuR-specific shRNA constructs decreases production of IL-4 from cotransfected expression vector encoding IL-4 cDNA (Fig. 5B). These data suggest that
HuR positively regulates IL-4 production. This possibility is further supported by the finding that HuR-specific shRNA construct decreased luciferase activity of the reporter vector consisting of 3′ UTR of IL-4 cDNA downstream of the firefly luciferase gene (Fig. 5C). The luciferase activity of the control vector consisting of SV40 polyadenylation signal downstream of the luciferase gene was not affected by the shRNA constructs (data not shown).

**Exposure of CD4 T cells to IL-4 increases HuR-binding activity**

To determine whether exposure to IL-4 modulates HuR we determined the effects of anti-IL-4 neutralizing Ab on HuR-binding activity to the full-length IL-4 riboprobe. We found that treatment of CD4+ T cells from TCR-DO11.10/Rag2(KO) mice with anti-IL-4 Ab decreased HuR-binding activity in cytosolic protein extracts shown by densitometry chart (Fig. 6A, top). There was no difference in AUFI binding to IL-4 riboprobe (Fig. 6A, bottom), suggesting that endogenous IL-4 had no effect on AUFI-binding activity. TTP could not be detected in the biotin pull-down assays (data not shown). To supplement the data from the biotin pull-down assays, we used RNA-EMSA analysis with the synthetic RNA probe for the coding region of IL-4 mRNA because it produced distinct RNA-protein complexes. RNA-EMSA analysis showed lower intensity of complex 1 and complex 3 in T cells treated with anti-IL-4 Ab (Fig. 6B), which is consistent with lower HuR-binding activity. Importantly, the amounts of HuR, AUFI, and TTP in the nuclear and cytosolic protein extracts were not affected by the anti-IL-4 Ab (Fig. 6C).

Next, we analyzed association of HuR with endogenous IL-4 mRNA in activated CD4+ T cells from TCR-DO11.10/Rag2(KO) mice. Analysis of the lysates from control cells revealed a 10-fold enrichment of IL-4 mRNA in the HuR immune complexes (Fig. 6D). Treatment of CD4+ T cells with anti-IL-4 Ab decreased the enrichment of IL-4 mRNA in the HuR immune complexes even though the amount of immunoprecipitated HuR protein was apparently higher. These data are consistent with the in vitro-binding activity and suggest that exposure of CD4+ T cells to IL-4 positively regulates HuR binding to IL-4 mRNA. The apparent difference in the amount of immunoprecipitated HuR despite equal levels of HuR expression may be explained by differential availability of the epitopes recognized by the anti-HuR Ab, as many RNA-binding proteins are buried inside of the ribonucleoprotein complexes (25). Thus, it is possible that anti-IL-4 treatment renders HuR more accessible to the anti-HuR Ab by reducing its association with the mRNAs.

In subsequent experiments, we compared HuR-binding activity in CD4+ T cells from DBA/2 (Th2-like phenotype) and C57BL/6 mice (Th1-like phenotype). Cytosolic protein extracts from DBA/2-derived CD4+ T cells showed considerable HuR binding to the IL-4 riboprobe (Fig. 7A, top). In contrast, cytosolic protein extracts from C57BL/6-derived CD4+ T cells showed very low HuR-binding activity to the IL-4 riboprobe, which was just slightly above the nonspecific background binding to the GAPDH riboprobe (Fig. 7A, top). AUFI binding to IL-4 mRNA appeared to be slightly higher in T cells from C57BL/6 mice than from DBA/2 mice in some experiments (Fig. 7A, bottom), albeit the signal was very low, required very long exposure times, and could not be reliably quantified. RNA-EMSA showed lower intensity of complex 1 and complex 2 in T cells derived from C57BL/6 mice compared with DBA/2 mice (Fig. 7B). However, the amounts of HuR in the nuclear and cytosolic protein extracts were similar in both groups (Fig. 7C).

To confirm that IL-4 had no effect on the expression of HuR in CD4+ T cells from DBA/2 or C57BL/6 mice, we used two-color flow cytometry analysis following intracellular staining for HuR. We found that resting CD4+ T cells from C57BL/6 and DBA/2 mice showed low intensity of HuR staining (Fig. 7D). Following activation of CD4+ T cells, the HuR expression similarly increased in CD4+ T cells from both strains (Fig. 7D). Exposure of CD4+ T cells from C57BL/6 mice to exogenous IL-4 had no effects on HuR expression (data not shown).

**FIGURE 6.** Exposure of CD4+ T cells from TCR-DO11.10/Rag2(KO) mice to IL-4 increases HuR-binding activity. A, Cytosolic protein extracts obtained from CD4+ T cells that were activated and expanded in the presence of isotype control or anti-IL-4 Abs were analyzed for HuR and AUFI-binding activity with biotin pull-down assay. The densitometry analysis of the biotin pull-down assays shows significantly lower HuR binding to IL-4 mRNA in anti-IL-4-treated cells. *p < 0.05. The mean ± SEM of three independent experiments is shown. B, RNA-EMSA was performed using IL-4 RNA probe corresponding to the HuR binding site within the coding region and the indicated cytosolic protein extract. C, HuR expression in cytosolic and nuclear protein extracts was analyzed by immunoblotting. Immunoblotting scans are representative of three independent experiments. D, Ribonucleoprotein complexes were immunoprecipitated using lysates from TCR-DO11.10/Rag2(KO) CD4+ T cells treated with isotype control or anti-IL-4 Ab. Immunoprecipitation of HuR was confirmed by Western blot analyses of 1/10 of the reaction. Association of HuR with endogenous IL-4 mRNA was analyzed by RT-PCR as described in Materials and Methods and in Fig. 2E. Data are representative of two experiments.
Finally, we evaluated the effects of exogenous IL-4 on HuR-binding activity CD4\(^+\) T cells from C57BL/6 mice. Treatment with IL-4 resulted in increased HuR-binding activity (Fig. 8A). RNA-EMSA confirmed higher HuR-binding activity in C57BL/6 T cells treated with exogenous IL-4 (Fig. 8B). However, IL-4 had no effects on the amounts of HuR in the nuclear and cytosolic protein extracts (Fig. 8C). Thus, our data showed that exposure of CD4\(^+\) T cells to IL-4 has no apparent effects on HuR expression or localization, but increases HuR-binding activity.

**Discussion**

In the present report, we propose a novel mechanism of positive feedback regulation of *Il4* gene expression: IL-4 promotes IL-4 mRNA stability via up-regulation of the binding activity of mRNA stabilizing protein HuR. Although the role of IL-4 in the transcriptional regulation of the *Il4* gene is well established, the autocrine effects of IL-4 on its own mRNA stability have not been previously described. Although not arguing against the role of IL-4 in the regulation of *Il4* gene transcription, we suggest that endogenous IL-4 also contributes to enhanced IL-4 production via increased IL-4 mRNA stability.

In support of our hypothesis, we showed that neutralization of endogenous IL-4 during activation and expansion of CD4\(^+\) T cells decreases IL-4 mRNA stability and abundance. A previous study using CD4\(^+\) T cells from TCR-DO11.10/Rag2(KO) mice showed that anti-IL-4 Ab decreases IL-4 secretion upon restimulation (29). Using the same model, we showed that the decrease in IL-4 mRNA abundance following treatment with anti-IL-4 Ab is accompanied by decreased IL-4 mRNA stability.

Furthermore, we previously showed that increased IL-4 mRNA stability determines a Th2 bias of DBA/2 mice relative to C57BL/6 mice (14). In this study, we have shown that anti-IL-4 Ab decreases IL-4 mRNA stability and abundance in CD4\(^+\) T cells from DBA/2 mice. In addition, exogenous IL-4 increases IL-4 mRNA stability and abundance in CD4\(^+\) T cells from C57BL/6 mice. Thus, we provide evidence that the difference in IL-4 mRNA stability between the two strains could be explained, in part, by differential exposure to early IL-4.

It appears that IL-4 exerts differential effects on mRNAs associated with Th1 or Th2 responses. In fact, IL-4, in synergy with TNF-\(\alpha\), increases stability of eotaxin mRNA in human epithelial cells (24). In contrast, several other studies previously showed that IL-4 decreases mRNA stability for IL-12p40 (41), MIP-1\(\alpha\) (42), and TNF-\(\alpha\) (43). The destabilizing effects of IL-4 on TNF-\(\alpha\) mRNA have been explained by a STAT6-dependent induction of TTP (43), whereas the stabilization of eotaxin mRNA is achieved.
via involvement of HuR (24). We also found that IL-4 increases IL-4 mRNA stability via a HuR-mediated mechanism.

The exposure to IL-4 is most likely required for HuR-mediated IL-4 mRNA stabilization only during initial stimulation of naïve CD4+ T cells. Addition of anti-IL-4 Ab to the cultures of established T cell lines EL4 and D10.G4.1 had no effect on IL-4 mRNA abundance or stability (data not shown). This finding is consistent with a previous study that showed no effect of IL-4 on production of IL-4 in differentiated Th2 cells (44). We speculate that early exposure to IL-4 imprints high HuR-binding activity and IL-4 mRNA stability in developing Th2 cells. It was recently shown that differentiated Th2 cells maintain IL-4 mRNA expression following primary immune responses and elimination of infection (1). Further studies are required to determine whether HuR expression and increased RNA-binding activity in differentiated Th2 cells facilitates rapid IL-4 production upon recall responses.

There are multiple steps in the regulation of HuR-mediated mRNA stabilization. First, induction of HuR is required for mRNA stabilization in proliferating cells (22, 26). Expression of HuR is increased during T cell activation (22, 23). We also found that activated CD4+ T cells express higher amounts of HuR than resting CD4+ T cells. However, IL-4 does not have an effect on the expression of HuR. The second step in the regulation of HuR involves translocation of HuR between the nucleus and cytoplasm (45, 46). Again, we found that IL-4 has no effects on HuR localization. Finally, there is evidence that HuR binding to target mRNAs may be regulated by ERK independently from its localization (37). The effects of ERK are likely to be indirect because HuR has no phosphorylation sites (47). Although ERK is involved in T cell activation, IL-4 does not signal via ERK in T lymphocytes and we found no effects of IL-4 on ERK phosphorylation in CD4+ T cells (data not shown). Atasoy et al. (24) established that HuR-mediated stabilization of eotaxin mRNA by synergistic action of IL-4, and TNF-α is associated with HuR translocation from the nucleus to cytoplasm. Because unstimulated epithelial cells express HuR almost exclusively in the nucleus (24), it remained unclear whether TNF-α or IL-4 was responsible for HuR translocation. The fact that proliferating T cells already express detectable amounts of HuR in the cytosol allowed us to determine that IL-4 has no effects on HuR localization, but increases HuR-binding activity.

Although we were not able to decrease HuR expression in T cells, we showed that ablation of HuR decreased ectopic IL-4 production in NIH-3T3 fibroblasts using RNA interference and co-transfection with IL-4 expression vector. The presence of IL-4 plasmid DNA, which could not be removed even by vigorous DNase treatment, interfered with RT-PCR assays. This result prevented us from determining the direct effects of HuR on IL-4 mRNA stability. However, HuR ablation decreased activity of the reporter vector consisting of the 3’UTR of IL-4 cDNA downstream of luciferase gene. As a composite, these data favor a positive role for HuR in the regulation of IL-4 biosynthesis.

We determined that murine IL-4 mRNA contains two putative HuR binding sites. Both sites conform to the consensus sequence NNUUUNNUUU, but were missed by the computational analysis using the alternative model for HuR-binding motif. However, this fact is still in agreement with the notion that some HuR target mRNAs identified on microarrays did not contain computer-predicted motifs (20). Identification of HuR binding site within the coding region was rather unexpected because the frequency of the HuR-binding motifs is lowest in the coding regions (20) and there are very few examples of mRNAs that have regulatory cis-elements within the coding region. In vitro-binding assays indicate that HuR binds both to the coding region and 3’UTR of IL-4 mRNA. The fact that HuR ablation decreased activity of the reporter vector expressing the 3’UTR of IL-4 mRNA downstream of luciferase suggests that HuR binding to the 3’UTR of IL-4 mRNA regulates IL-4 production. The role of the HuR binding site within the coding region is currently under investigation.

The presence of AU-rich motifs in the 3’UTR of many cytokine and chemokine mRNAs has been linked to rapid mRNA degradation (19). Murine IL-4 mRNA contains a 58-nt long AU-rich sequence within its 3’UTR (48). However, a previous study showed that the 3’UTR of IL-4 mRNA did not target a reporter transcript for degradation, suggesting that IL-4 mRNA is not likely to interact with TTP (48). This suggestion is also supported by the fact that we could not detect TTP in biotin pull-down assays. Several studies established the antagonistic effects of HuR and AUF1 on mRNA stability. Using biotin pull-down assays, we demonstrated binding of AUF1 to IL-4 mRNA. It is possible that HuR binding protects IL-4 mRNA from the destabilizing effects of AUF1, as it has been shown for some other mRNAs (40).

In conclusion, the findings reported in our study add an additional level to the complex regulation of If4 gene expression in Th2 lymphocytes. IL-4 plays a positive feedback role in If4 gene expression not only at the level of transcription, but also by stabilizing its own mRNA via increased binding activity of HuR.

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FIGURE 8. Exogenous IL-4 increases HuR-binding activity in activated CD4+ T cells from C57BL/6 mice. A, Cytosolic protein extracts obtained from C57BL/6 CD4+ T cells that were activated and expanded in the presence of exogenous IL-4 were analyzed for HuR and AUF1-binding activity with biotin pull-down assay. The densitometry analysis of the biotin pull-down assays shows increased HuR binding to IL-4 mRNA in cells treated with exogenous IL-4. B, RNA-EMSA was performed using synthetic RNA oligonucleotide corresponding to the HuR binding site within the coding region and cytosolic protein extracts from control and IL-4-treated C57BL/6 CD4+ T cells. C, Expression and localization of HuR in the cytosolic and nuclear extracts from DBA/2 or C57BL/6 CD4+ T cells were analyzed by immunoblotting. The data are representative of two independent experiments.
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Disclosures

The authors have no financial conflict of interest.

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