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Coordinate Regulation of GATA-3 and Th2 Cytokine Gene Expression by the RNA-Binding Protein HuR

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The posttranscriptional mechanisms whereby RNA-binding proteins (RBPs) regulate T cell differentiation remain unclear. RBPs can coordinateantly regulate the expression of functionally related genes via binding to shared regulatory sequences, such as the adenyate-uridylate–rich elements (AREs) present in the 3′ untranslated region (UTR) of mRNA. The RBP HuR posttranscriptionally regulates IL-4, IL-13, and other Th2 cell-restricted transcripts. We hypothesized that the ARE-bearing GATA-3 gene, a critical regulator of Th2 polarization, is under HuR control as part of its coordinate posttranscriptional regulation of the Th2 program. We report that in parallel with stimulus-induced increase in GATA-3 mRNA and protein levels, GATA-3 mRNA half-life is increased after restimulation in the human T cell line Jurkat, in human memory and Th2 cells, and in murine Th2-skewed cells. We demonstrate by immunoprecipitation of ribonucleoprotein complexes that HuR associates with the GATA-3 endogenous transcript in human T cells and found, using biotin pulldown assay, that HuR specifically interacts with its 3′UTR. Using both loss-of-function and gain-of-function approaches in vitro and in animal models, we show that HuR is a critical mediator of stimulus-induced increase in GATA-3 mRNA and protein expression and that it positively influences GATA-3 mRNA turnover, in parallel with selective promotion of Th2 cytokine overexpression. These results suggest that HuR-driven posttranscriptional control plays a significant role in T cell development and effector function in both murine and human systems. A better understanding of HuR-mediated control of Th2 polarization may have utility in altering allergic airway inflammation in human asthmatic patients. The Journal of Immunology, 2011, 187: 000–000.

The process of T cell activation involves complex phenotypic and functional changes that are dynamically modulated throughout the different stages of an immune response. Coordinate action of multiple gene regulatory pathways ensures that the different molecular species involved in the T cell response are expressed with the proper timing, magnitude, and duration. To this end, chromatin remodeling and transcriptional activation are highly integrated with posttranscriptional mechanisms, which specifically regulate the rate of mRNA transport, turnover, and translation. It has been established that the activation of human T cells determines global, coordinate changes of mRNA turnover rates that affect more than half of the induced gene pool (1–3), and research in the past decade has identified several cis-elements and trans-factors that mediate these processes in T cells (4).

mRNA molecules exist within the cell as components of ribonucleoprotein (RNP) complexes in association with a host of RNA-binding proteins (RBPs) that dynamically interact with the mRNAs and modulate their correct splicing, nucleocytoplasmic shuttling, turnover, and translation rates (5). Several conserved regions have been identified and recently defined as USER (untranslated sequence elements for regulations), as they are preferentially located in the mRNA untranslated regions (UTRs), particularly in the 3′UTR (4, 6, 7). Among these, the adenyate-uridylate–rich elements (AREs) are the most conserved and well-characterized regulatory elements mediating changes in mRNA turnover and translation in immune genes (8). ARE-mediated gene regulation occurs for many T cell cytokine genes such as IL-2, IL-3, CSF2, IL-4, and IL-13, although alternative USER do play a role in T cell gene regulation (4). Multiple ARE-binding proteins, acting either in a cooperative or exclusive fashion, regulate mRNA stability and translation and adapt the amplitude and duration of gene expression according to the cellular environment (5).

Genome-wide studies examining the transcript pools selectively associated with distinct RBPs have established that functionally related mRNAs that share a USER, such as the ARE, can be coordinately regulated by one or more cognate RBPs (6, 9). The ubiquitous RBP HuR binds to a heterogeneous group of AREs and is functionally characterized as a positive regulator of mRNA stability and/or translation, acting through multiple, often independent mechanisms yet to be fully characterized (10). HuR has been characterized as a critical positive regulator of genes transcribed in effector T cell polarized subsets and in the generation of
T cells in the thymus (11–13). T cell activation induces HuR nucleocytoplasmic shuttling (12, 14–17), an event reflecting its functional activation, and positively regulates the mRNA turnover of several T cell-derived genes (15, 18–24). The complex role of HuR in T cell biology is also revealed by recent conditional knockout mouse models. When the HuR gene was ablated in T cells using an lck cru recombinase system, the mouse phenotype was characterized by numerous abnormalities of T cell ontogeny (13). Another model of tamoxifen-inducible HuR conditional knockout displayed atrophy of multiple organ systems due to apoptosis of progenitor cells in thymus, bone marrow, and intestine (25).

The role of HuR in Th2-restricted gene expression is supported by its established role in promoting IL-4 and IL-13 mRNA stability (14, 26, 27). We also showed that another selective marker of Th2 cells, the chemotactic PGD2 receptor CRTH2, is also regulated at the level of mRNA turnover (28). Notably, CRTH2 transcript (i.e., the product of the GPR44 gene), also harbors HuR binding sites (29). On this basis, we could infer that HuR may represent the posttranscriptional counterpart for nuclear proteins directing lineage-restricted transcription of these genes. One such protein is GATA-3, a factor that controls the coordinate expression of Th2 genes at both the epigenetic and transcriptional levels (30, 31). GATA-3 is itself a Th2-selective marker, and its lineage-restricted induction is thought to be mediated by both intrinsic and extrinsic, instructive pathways (32, 33). Although IL-4 is primarily involved in GATA-3 transcriptional activation via the JAK3–STAT6 pathway (32, 34), little is known about the contribution of posttranscriptional pathways to GATA-3 expression. We and others have shown previously that, in airway epithelial cells, IL-4 can contribute to STAT6-dependent transcriptional signals and STAT6-independent posttranscriptional signals, the latter through the activation of HuR, in the regulation of CCL11 (24, 35, 36). It can be hypothesized then that GATA-3, being an ARE-bearing gene activated by IL-4, might be regulated in a similar fashion. The regulation of a critical transcriptional effector of the Th2 gene program such as GATA-3 would provide an additional level at which HuR participates in the regulation of the coordinate expression of Th2-restricted genes, and of lineage-restricted genes more in general. In light of these considerations, we investigated whether the GATA-3 gene is part of a transcript pool under coordinate posttranscriptional control exerted by HuR and evaluated the functional outcome of HuR regulation. Our results implicate HuR as a novel determinant of GATA-3 mRNA and protein levels in human and mouse T cells that is involved in the control of GATA-3 mRNA stability as one of potentially multiple means of HuR-driven posttranscriptional regulation in T cells.

Materials and Methods

Cell culture

Blood was obtained from healthy subjects under a protocol approved by a Johns Hopkins University School of Medicine institutional review board and from buffy coats (Biological Specialty Corporation, Colmar, PA). Th2-skewed cells were generated in vitro as described (27, 37), and intracellular staining for IL-13, IL-4, and IFN-γ to quantify cell polarization was performed as established (27, 37). Human memory T cells were isolated using Memory CD4+ T cell Isolation Kit (Miltenyi Biotec) and following the manufacturer’s protocol. The human Jurkat T cell line was cultured in RPMI 1640, 10% FBS, 2 mM L-glutamine (Invitrogen), and 100 μg/ml gentamicin (Quality Biological, Gaithersburg, MD). The H2 cells, a clone of the human cell line H1299, a non-small cell lung carcinoma stably transfected with the erbB-2 (HER2) plasmid (5’,-AAGUGAAGGAGUUAACGACU-3’) were cultured in DMEM (Invitrogen), 10% FBS, and penicillin (100 U/ml)/streptomycin (100 μg/ml) (27). NIH/3T3 cells were maintained in DMEM (Invitrogen) and 10% BCS.

Marine T cell polarization in vitro

Naïve splenocytes were isolated from 8-wk-old female FVB HuR transgenic and female wild-type FVB mice. CD4+ T cells were isolated using CD4 (L3T4) MicroBeads (Miltenyi Biotec) following the manufacturer’s protocol. Cells were cultured with anti-CD3 anti-CD28 (5 μg/ml each) for 5 d in 10% FCS-DMEM media under Th1 polarizing (20 ng/ml IL-12 and 20 μg/ml anti-IL-4 Ab), Th2 polarizing (20 ng/ml IL-4 and 20 μg/ml anti-IFN-γ Ab), or nonpolarizing (no cytokines and blocking Abs) conditions.

HuR silencing

Jurkat T cells were stably transfected using a lentivirus expressing an HuR short hairpin RNA (shRNA) as described (39). Alternatively, Jurkat T cells were transiently transfected using a previously described HuR small interfering RNA (siRNA) (siRNA) (G-025935, Santa Cruz Biotechnology, Santa Cruz, CA), anti-GATA-3 (Sigma–Aldrich), anti–GATA-3 (Santa Cruz Biotechnology), and anti–β-tubulin (1 μg/ml) (Sigma–Aldrich). HuR and GATA-3 densitometric levels were determined using Quantity One software (Bio-Rad) after normalization to GAPDH levels and calculated using the comparative cycle threshold (Ct) method (24, 42) using GAPDH mRNA expression for normalization.

Western blot analysis

Western blot analysis was performed as described (27, 39) using anti-HuR clone 3A2 (1 μg/ml) (43) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GATA-3 (Santa Cruz Biotechnology), and anti–β-tubulin (1 μg/ml) (Sigma–Aldrich). HuR and GATA-3 densitometric levels were determined using Quantity One software (Bio-Rad) after normalization to β-tubulin.

Immunoprecipitation of endogenous messenger RNA complexes

We used a modification of an established protocol (24, 27, 44). Jurkat cell lysates were obtained using polysome lysis buffer. For immunoprecipitation (IP), Protein A-Sepharose beads (Sigma) were swollen 1:1 (v/v) in NT2 buffer. A 100-μl aliquot of the preswollen protein A bead slurry was used for IP reaction and incubated 4 h at room temperature with excess immunoprecipitating Ab, using the 3A2 anti-HuR Ab or an IgGl isotype control Ab (B&L Life Sciences). The Ab-coated beads were washed with ice-cold NT2 buffer and resuspended in 900 μl NT2 buffer supplemented with 100 U/ml RNaseOUT, 0.2% vanadyl-ribonucleoside complex, 1 mM DTT, and 20 mM EDTA. The IP reactions were tumbled at room temperature for 2 h, and washed beads were resuspended in 100 μl NT2 buffer supplemented with 0.1% SDS and 30 μg proteinase K and incubated for 30 min at 55˚C. The cytoplasmic RNA was extracted using phenol–chloroform–isoamyl alcohol and precipitated in ethanol.

In vitro biotin pulldown assay

Biotinylated transcripts were generated by RT-PCR of Jurkat RNA for human transcripts or cDNA from pUX-Asc GATA-3 CDS BC062915 (Open
BioSystems) for murine transcripts using forward primers that contained a T7 transcription initiation site (5′-GTGAAATGTAATACGACTACA-TAGGG-3′) as described (27). The primers used to generate cDNA are listed in Supplemental Table I. PCR products were purified from agarose gels as described (45) and used as templates for the synthesis of biotinylated RNAs using T7 RNA polymerase and biotin-conjugated CTP for human RNAs or UTP for murine RNAs. Following an established protocol (29), cytoplasmic fractions of unstimulated Jurkat cells (40 μg) or NIH/3T3 cells (40 μg) were incubated for 1 h at room temperature with 1 μg biotinylated transcripts, then RNP complexes were isolated with streptavidin-conjugated Dynabeads (Invitrogen). The presence of HuR in the pulldown pellet was verified by Western blot analysis (27). For generation of mutant biotinylated transcripts, AU-rich elements in the GATA-3 UTR from the pYX-Asc GATA-3 CDS plasmid were mutated from 5′-AAATTGTTGTTTGTATG-3′ to 5′-TTATATTATGTTTAGTTG-3′ at 2270–2286 and from 5′-TGGAATAATCTAATAAA-3′ to 5′-ATAATDAGTGA-TAACTA-3′ at 2762–2778 by site-directed mutagenesis (Mutagenex, Hillsborough, NJ).

Generation of the HuR transgenic mouse

A hemagglutinin (HA)-tagged HuR fragment containing Sall sites on both the 5′ and 3′ end was cloned in the Sall site of the mCD4.e/p-Sall(−) plasmid (46), which replaced the CD2 gene with HA–HuR. Plasmids were digested with NotI to remove bacterial genes. Fragments were then microinjected into FVB pronuclei and transplanted into an FVB founder. Founder mice and their progeny were screened for the presence of the transgene using primers specific for HA–HuR. No gross abnormalities in organ architecture were found as determined by the University of Missouri Veterinary School Research Animal Diagnostic Laboratory.

Intracellular staining and flow cytometry

In vitro-polarized T cells were restimulated with 50 ng/ml PMA, 500 ng/ml ionomycin, and 1 μg/ml brefeldin A for 6 h. Cells were fixed in 2% paraformaldehyde and permeabilized with 0.2% saponin, then stained with anti-IL-4 FITC and anti-IFN allopurinol (BD Bioscience). For GATA-3 intracellular staining, cells were fixed and permeabilized with Foxp3 Fix/Perm buffer set (BD Biosciences) and then stained with anti-GATA-3 (eBioscience). Cells were analyzed on the CflowAn flow cytometer (Becton Coulter) using FlowJo software. For detection of HA and HuR by intracellular staining, cells were fixed and permeabilized with Fix/Perm buffer set (BD Biosciences), stained with either 2 μg anti-HA (Sigma-Aldrich) or 2 μg anti-HuR (3A2), and then incubated with PE-conjugated anti-mouse IgG to detect either HA or HuR. Cells were analyzed on a FACScan flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences).

Cytokine assays

Supernatants (diluted 1:200) were used for cytokine detection using mIL-4, IL-13, or IFN-γ ELISA Ready-SET-Go kit (eBioscience).

Statistical analysis

The p values were calculated using the two-tailed Student t test.

Results

Activation-induced stabilization of GATA-3 mRNA

To test whether T cell activation would regulate GATA-3 expression through posttranscriptional pathways, we first examined the expression and decay rate of GATA-3 mRNA turnover in human, in vitro Th2-skewed cells. Cells were left unchallenged or were restimulated for 3 h with PMA (50 ng/ml) and the Ca2+ ionophore ionomycin (250 ng/ml), alone or in combination. steady-state levels of GATA-3 mRNA were increased by cell treatment, as well as GATA-3 protein levels, as detected by Western blot (Fig. 1A). To measure mRNA turnover, stimulated cells were either collected at the end of the stimulation (as time 0) or further treated with the transcriptional inhibitor actinomycin D (Act D; 3 μg/ml) for 1, 3, or 5 h. Total RNA was isolated for analysis of GATA-3 mRNA expression by real-time RT-PCR. Results were normalized to housekeeping mRNA levels and expressed as percentage of maximum (i.e., mRNA at time 0). GATA-3 mRNA was detectable at baseline (Ct = 23.5) and displayed a fast turnover, as only 33% of the initial pool was detectable within 1 h from transcriptional termination (t1/2: 1.5 h; Fig. 1B). Treatment with PMA plus ionomycin induced a stimulus-dependent stabilization of the GATA-3 mRNA, with 53% of the mRNA still detectable at the same 1-h time point (t1/2: 2 h). The stabilizing effect was transient and peaked soon after activation, as the decay curves showed at later time points smaller differences between unstimulated and activated cells. The flattening of the decay curve at the later experimental time points could also be due to cell type- and time-dependent toxicity related to the Act D, as after longer incubation with this drug there is an increasing percentage of nonviable T cells that do not process RNA efficiently. However, stimulus-induced stabilization of GATA-3 mRNA occurred in parallel with that of another known HuR mRNA target, IL-13 (27).

Cis-elements and trans-factors involved in GATA-3 posttranscriptional regulation

We used established models for the study of the cis-element (the ARE) and the trans-factor (HuR) that we hypothesized to be involved in the posttranscriptional regulation of GATA-3. As in the case of IL-13 and IL-4 (14, 27), the 3′ UTR of GATA-3 mRNA harbors “class 1” AREs (47), with four scattered AUUUA pentamers embedded in an A- and U-rich milieu. In addition, it displays two adjacent AUUUA pentamers and two additional described HuR consensus sites (29) (Fig. 1C). We subcloned the full-length GATA-3 3′ UTR in a Tet-Off promoter-driven reporter system expressing the rabbit β-globin gene (pTet-BBB) (38, 48). The construct expressing the GATA-3 UTR-bearing transcript, and its parent vector as control, were transiently transfected in parallel in H2 cells, which are stably transfected with the rTet factor (38), together with a GFP expression vector for normalization. The turnover of the β-globin mRNA, assessed by Northern blot after transcriptional shut-off with doxycycline (Fig. 1D), was markedly accelerated by the insertion of the GATA-3 3′ UTR, displaying a half-life of 1.5 h compared with 9.6 h in cells transfected with the control vector. The HuR-mediated stabilization of GATA-3 mRNA appears to rely on cell activation, whereas in unstimulated conditions the GATA-3 mRNA is fairly labile (Fig. 1B). Therefore, the behavior observed with the chimeric mRNA reporter supports the hypothesis that GATA-3 can be regulated at the level of mRNA turnover through its 3′ UTR and is consistent with the rapid decay observed for the endogenous GATA-3 mRNA in unstimulated Th2 cells.

As the GATA-3 mRNA displays dynamic changes in decay after cell activation, including a transient stabilization, we investigated whether HuR associated with endogenous GATA-3 transcript. We performed IP of messenger ribonucleoprotein (mRNP) complexes isolated from Jurkat T cells either unstimulated or treated with PMA and ionomycin for 3 h using a monoclonal anti-HuR (3A2) or an isotype-matched Ab. Western blot analysis confirmed that the IP with anti-HuR was specific (Fig. 2A). As the GATA-3 mRNA displays dynamic changes in decay after cell activation, including a transient stabilization, we investigated whether HuR associated with endogenous GATA-3 transcript. We performed IP of messenger ribonucleoprotein (mRNP) complexes isolated from Jurkat T cells either unstimulated or treated with PMA and ionomycin for 3 h using a monoclonal anti-HuR (3A2) or an isotype-matched Ab. Western blot analysis confirmed that the IP with anti-HuR was specific (Fig. 2A). As the GATA-3 mRNA displays dynamic changes in decay after cell activation, including a transient stabilization, we investigated whether HuR associated with endogenous GATA-3 transcript. We performed IP of messenger ribonucleoprotein (mRNP) complexes isolated from Jurkat T cells either unstimulated or treated with PMA and ionomycin for 3 h using a monoclonal anti-HuR (3A2) or an isotype-matched Ab. Western blot analysis confirmed that the IP with anti-HuR was specific (Fig. 2A).
HuR REGULATES GATA-3 EXPRESSION AT POSTTRANSCRIPTIONAL LEVEL

We further validated the interaction of HuR with the 3′UTR of GATA-3 mRNA using a biotin pulldown approach (Fig. 2B). In vitro-transcribed, biotinylated transcripts spanning the coding region or the full-length 3′UTR of GATA-3 mRNA half-life, calculated as the time required for the transcript to decrease to 50% of its initial abundance. The full 3′UTR of the GATA-3 mRNA (common to transcript variants 1 (NM_001002295) and 2 (NM_002051)). AU-rich elements are set in boldface; underlined are other putative HuR recognition sites as described (29). This fragment was subcloned in the unique BglII site of the pTet-BBB reporter construct. Exons and introns in the rabbit β-globin gene are shown in white and black. TetOp, tetracycline operator sequences; TATA, minimal CMV promoter. D, Representative Northern blot (top) and densitometric analysis (bottom) (mean ± SEM of n = 3) of β-globin mRNA expression in H2 cells transfected with the indicated plasmids. Cytoplasmic RNA was harvested at baseline (time 0) or at the indicated times after transcriptional shutoff induced by doxycycline (Dox). In parentheses: β-globin mRNA half-life, calculated as the time required for the transcript to decrease to 50% of its initial abundance. *p < 0.05 (β-globin mRNA in pTet-BBB–GATA-3–transfected cells compared with pTet-BBB transfectants).

HuR siRNA knockdown results in reduced GATA-3 expression in human Th2-polarized T and memory cells

To probe further the relevance of HuR in human T cell responses, we examined Gata-3 expression in human peripheral T cells in which HuR was silenced using HuR-specific siRNA. Silencing of HuR was implemented in both CD45RO+ memory T cells and in Th2-skewed CD4+ T cells, where levels of HuR were decreased by 67 and 82%, respectively (Fig. 3A, 3B). Levels of Gata-3 protein were reduced as well by 57 and 71%, respectively (Fig. 3A, 3B). In these cells, steady-state mRNA levels of GATA-3, IL-4, and IL-13, but not IFN-γ, were significantly reduced as well compared with those of cells transfected with the scrambled siRNA control (Fig. 3C, 3D). HuR silencing also consistently reduced GATA-3 transcript stability in both cell types (Fig. 3E, 3F), though to a lesser extent than that documented in Jurkat cells (Fig. 2, Supplemental Fig. 1).
Activated transgenic T cells express increased GATA-3 mRNA and protein levels

To investigate further the functional outcome of HuR association with GATA-3 mRNA, we generated a transgenic mouse model of HuR overexpression, in which HA-tagged HuR was expressed in CD4+ T cell-restricted fashion. CD4+ T cells purified from transgenic mice expressed anti-HA Ab or with an anti-HuR Ab, together with the endogenous HA–HuR (Fig. 4). Transgenic animal GATA-3 mRNA levels and stability were consistently increased in Th2 HA–HuR transgenic animals compared with CD4+ T cells from WT controls (Fig. 4C). In contrast, when naïve CD4+ T cells from HA–HuR mice and WT control mice were cultured under Th2-polarizing conditions, we observed a significant increase in GATA-3 protein expression in cells from HA–HuR mice compared with cells from WT littermates (Fig. 4D). Th2-polarized cells from HA–HuR transgenic animals displayed greater frequencies of GATA-3+ cells (80 versus 53% in WT), as well as significantly higher GATA-3 mRNA and protein levels present in wild-type (WT) littermates (Fig. 4). Overall, these data indicate that a relatively modest increase in HuR levels brought by overexpression (20% higher than WT; Fig. 4) led to a significant increase in HuR–derived cells compared with those of cells from WT (n = 3), *p < 0.05 (for half-life) for both genes.

Activated transgenic T cells express increased GATA-3 mRNA in Jurkat cell cytoplasmic extracts immunoprecipitated with anti-HuR or isotype-matched Ab. Upper panel, Western blot analysis showing specific HuR detection in the IP samples. Lower panel, Real-time PCR plot showing GATA-3 mRNA enrichment in the anti-HuR IP samples compared with isotype-matched IgG Ab (representative of n = 3). B, HuR protein expression by Western blot after biotin pulldown assay (representative of n = 3) with biotinylated transcripts spanning the GATA-3 3′UTR and coding region (CR) and the 3′UTR of GAPDH, used as negative control. C, Western blot (representative of n = 2) of HuR, GATA-3, and β-tubulin (as loading control) expression in Jurkat cell clones (G11 and D2) stably infected with HuR shRNA lentivirus knockdown and in control clone G4 stably infected with empty vector. Bar graphs represent the mean densitometric analysis normalized to β-tubulin. D, Mean ± SEM (n = 3) of HuR and GATA-3 mRNA steady-state levels as measured by real-time PCR in Jurkat cell clones G4 (control) and D2 (HuR shRNA). E and F, GATA-3 (E) and IL-13 (F) mRNA decay measured by real-time PCR in G4 and D2 cells after treatment with Act D (n = 3), *p < 0.05 (for half-life for both genes).

FIGURE 2. Functional association of HuR with GATA-3 mRNA. A, IP of mRNP complexes assay for detection of GATA-3 mRNA in Jurkat cell cytoplasmic extracts immunoprecipitated with anti-HuR or isotype-matched Ab. B, Western blot analysis showing specific HuR detection in the IP samples. Lower panel, Real-time PCR plot showing GATA-3 mRNA enrichment in the anti-HuR IP samples compared with isotype-matched IgG Ab (representative of n = 3). C, HuR protein expression by Western blot after biotin pulldown assay (representative of n = 3) with biotinylated transcripts spanning the GATA-3 3′UTR and coding region (CR) and the 3′UTR of GAPDH, used as negative control. D, Mean ± SEM (n = 3) of HuR and GATA-3 mRNA steady-state levels as measured by real-time PCR in Jurkat cell clones G4 (control) and D2 (HuR shRNA). E and F, GATA-3 (E) and IL-13 (F) mRNA decay measured by real-time PCR in G4 and D2 cells after treatment with Act D (n = 3), *p < 0.05 (for half-life for both genes).
HuR regulates GATA-3 expression at posttranscriptional level

3'UTRs as indicated in Materials and Methods. This strategy abrogated HuR binding to murine GATA-3 3'UTR (Supplemental Fig. 2B). Taken together, these data suggest that HuR is capable of regulating GATA-3 gene expression in mouse CD4+ T cells and that this biological action is likely to be at least partially mediated by ARE-mediated changes in mRNA stability.

Activated splenocytes or polarized CD4+ Th2 cells from transgenic mice produce higher levels of Th2 cytokines

RBPs in general coordinate posttranscriptionally the expression of functionally related genes (9, 49) and in particular HuR has been shown to influence the GATA-3–regulated genes IL-4 and IL-13 (14, 26, 27). We asked whether higher expression levels of GATA-3 and HuR could affect levels of Th2 cytokines in both unpolarized and polarized T cells. Splenocytes from HA–HuR transgenic mice showed a significant increase in activation–induced IL-4 and IL-13, but not in IFN-γ expression, at both the mRNA and protein levels (Fig. 5A, 5B) compared with splenocytes from WT control mice. Levels of IL-5 were not reproducibly affected (data not shown). We then polarized naive CD4+ T cells under either Th1 or Th2 conditions (Fig. 6). Although under Th1 conditions there were no appreciable differences among IFN-γ– or IL-4–producing cells between transgenic and WT mice, Th2 polarization led to a small but consistent increase in the frequencies of IL-4–secreting cells, ranging in six experiments from 4 to 16% (mean ± SEM: 7.4 ± 2.3%, p = 0.013, representative plot shown in Fig. 6A), whereas the frequencies of IFN-γ–secreting cells in Th1 cells were unchanged (Fig. 6B).

Furthermore, in agreement with the data generated in unpolarized cells (Fig. 5), HA–HuR CD4+ transgenic Th2 cells secreted significantly higher amounts of IL-4 and IL-13 than the WT-derived Th2 cells, whereas IFN-γ secretion from Th1-polarized cells did not change in the two groups (Fig. 6C). Taken together, these results indicate that in parallel with the increase in GATA-3 levels, HuR overexpression can lead to significant increases in Th2 cytokine production, as previously shown in vitro (14, 27).

Discussion

The molecular mechanisms involved in Th2 differentiation and maintenance have been only partially understood to date. Although much has been learned about the transcriptional programs that determine naive CD4+ T cell fate, very little is known about the role of posttranscriptional gene regulation in controlling T cell lineage commitment. The transcription factor GATA-3 is considered to be one of the most important genes involved in the process.
mice compared with WT mice. Mean sufficient to drive the differentiation of naive CD4+ T cell into the of Th2 polarization, as it has been described to be necessary and increased IL-4 and IL-13 secretion in Th2-polarizing cultures of CD4+ T cells overexpressing HuR. Increased frequencies of IL-4–expressing cells and in-

**FIGURE 6.** Increased frequencies of IL-4–expressing cells and increased IL-4 and IL-13 secretion in Th2-polarizing cultures of CD4+ T cells overexpressing HuR. A and B, Scatterplot of cytokine-associated fluorescence in Th2-polarized (A) and Th1-polarized (B) cells showing an increased frequency of IL-4+ cells but similar frequency of IFN-γ+ cells in FVB transgenic cells (HuR Tg) compared with WT cells. Plots representative of n = 6. C, Increased secretion of IL-4 and IL-13 in Th2-polarized CD4+ T cells but not of IFN-γ in Th1-polarized cells from FVB transgenic mice compared with WT mice. Mean ± SEM of n = 5. *p < 0.05.

do of Th2 polarization, as it has been described to be necessary and sufficient to drive the differentiation of naive CD4+ T cell into the Th2 lineage (50).

Previously, we and others have described the cloning of HuR and demonstrated that its regulation is cell cycle dependent (16, 18, 51). Activation of T cells results in 10- to 14-fold increases in total cellular HuR levels (16), as well as its translacion from the nucleus into the cytoplasm, which correlates with its functional activation (12, 14–17). HuR has been subsequently shown to play an important role in regulation of many genes involved in Th2-driven inflammatory diseases, such as asthma (11, 14–16, 27).

Genes affecting Th2-polarized function, such as GATA-3, IL-4, and IL-13, harbor in their 3'UTR AREs and other sequences regulating mRNA turnover and translation, which are highly enriched in immune genes. RBPs like HuR, which bind the ARE in the 3'UTR regions of these genes, can powerfully affect the rates of mRNA transport in the cytoplasm, transcript stability, and/or translation. Because HuR is known to regulate posttranscriptionally the expression of IL-4 and IL-13 by increasing the stability of their mRNA (14, 27), we hypothesized that it may also regulate GATA-3 expression as well, via interacting with specific AREs present in its 3'UTR, as part of a global, coordinate action on the Th2 phenotype. Our results demonstrate that the stability of GATA-3 mRNA is increased, along with its steady-state and with protein levels, in stimulated human and murine Th2-skewed cells, and that the prolonged mRNA turnover is modified accordingly in conditions of relative HuR overexpression and silencing, both in vitro and ex vivo (Figs. 1, 2, 3, 4, Supplemental Fig. 1). In conjunction with the data showing association of HuR with both endogenous and synthetic GATA-3 mRNA shown in Fig. 2, Supplemental Fig. 1, we provide strong evidence indicating that HuR directly regulates GATA-3 by association with its 3'UTR and stabilization of its transcript. Along the same lines, HuR silencing in Jurkat cells reduced GATA-3 mRNA steady-state levels and stability, as well as protein levels, similarly to what was observed for GATA-3 mRNA and protein after HuR silencing in the human mammary epithelial cell line MCF-7 (52). We have also recently demonstrated by HuR RIP-Chip that GATA-3 mRNA is a HuR target in MCF-7 estrogen-positive breast cancer (39). Of larger potential relevance to human disease, HuR silencing obtained in human CD45RO+ memory T cells as well as in human Th2-polarized cells significantly decreased GATA-3 mRNA and protein levels.

As a corollary to the data generated in human cells, even relatively modest levels of HuR overexpression generated in our transgenic mouse model resulted in increased GATA-3 mRNA steady state levels and stability along with increased GATA-3 protein expression. Importantly, increased levels of GATA-3 protein, as well as that of Th2 cytokine secretion, were paralleled in the transgenic model by a lack of effect on IFN-γ secretion in either Th1- or Th2-polarized cells. Notably, GATA-3 is the most highly expressed transcription factor in normal mammary epithelium and is required for its maintenance (53–55). Exogenous changes in the levels of HuR in our experimental models confirmed that this factor regulates GATA-3 mRNA stability. In general, the decay profiles obtained with transcriptional inhibitors like Act D are better suited to document the occurrence of relative changes in decay induced by cell stimulation or by altered levels of regulatory factors—HuR in this study—rather than tracing the exact kinetics of decay, as there are limitations due to the toxicity of Act D (56). Overall, our mRNA decay data show in different experimental settings (Figs. 1, 2, 3, 4, Supplemental Fig. 1) a significant, consistent stimulus-dependent change in GATA-3 mRNA stability that is susceptible to HuR regulation. However, we cannot exclude that HuR may regulate the overall levels of GATA-3 by other concurrent mechanisms. In fact, besides its positive effect on mRNA stabilization, HuR has been found to modulate, for other T cell targets, either cytoplasmic accumulation or translation (2, 14, 15, 57, 58).

Although our results reveal a direct, functional interaction of HuR with GATA-3 mRNA, it remains to be established to what extent HuR participates in the regulation of Th2 polarization and function via controlling GATA-3 expression, as opposed to its direct role on IL-4 and IL-13 expression. Taken together, the results of our study highlight the central importance that HuR may play in CD4+ T cell differentiation and function through the coordinated regulation of its targets and point to posttranscriptional regulation as a critical yet still largely uncharacterized component of T cell gene regulation.

In future studies, it will be important to perform more specific, targeted deletions of HuR in specific T cell subsets. Such approaches will help to elucidate more fully the effect of posttranscriptional gene regulation in phenotype initiation and maintenance during T cell differentiation. These efforts can potentially shed light on the mechanisms of Th2-driven inflammatory diseases such as asthma.
Disclosures
The authors have no financial conflicts of interest.

References


