MolecularMedicine

Conditional Knockout of the RNA-Binding Protein HuR in CD4+ T Cells Reveals a Gene Dosage Effect on Cytokine Production

Matthew M Gubin,[1] Patsharaporn Techasintana,[1*] Joseph D Magee,[1] Garrett M Dahm,[1] Robert Calaluce,[1] Jennifer L Martindale,[2] Maryln S Whitney,[1] Craig L Franklin,[1] Cindy Besch-Williford,[1] John W Hollingsworth,[3] Kotb Abdelmohsen,[2] Myriam Gorospe,[2] and Ulus Atasoy[1]*

[1]University of Missouri, Columbia, Missouri, United States of America; [2]National Institute on Aging, National Institutes of Health, Baltimore, Maryland, United States of America; and [3]Department of Medicine, Duke University, Durham, North Carolina, United States of America

The posttranscriptional mechanisms by which RNA binding proteins (RBPs) regulate T-cell differentiation and cytokine production *in vivo* remain unclear. The RBP HuR binds to labile mRNAs, usually leading to increases in mRNA stability and/or translation. Previous work demonstrated that HuR binds to the mRNAs encoding the Th2 transcription factor *trans*-acting T-cell–specific transcription factor (GATA-3) and Th2 cytokines interleukin (IL)-4 and IL-13, thereby regulating their expression. By using a novel conditional HuR knockout (KO) mouse in which HuR is deleted in activated T cells, we show that Th2-polarized cells from heterozygous HuR conditional (OX40-*Cre* $HuR^{fl/+}$) KO mice had decreased steady-state levels of *Gata3*, *Il4* and *Il13* mRNAs with little changes at the protein level. Surprisingly, Th2-polarized cells from homozygous HuR conditional (OX40-*Cre* $HuR^{fl/fl}$) KO mice showed increased *Il2*, *Il4* and *Il13* mRNA and protein via different mechanisms. Specifically, *Il4* was transcriptionally upregulated in HuR KO T cells, whereas *Il2* and *Il13* mRNA stabilities increased. Additionally, when using the standard ovalbumin model of allergic airway inflammation, HuR conditional KO mice mounted a robust inflammatory response similar to mice with wild-type HuR levels. These results reveal a complex differential posttranscriptional regulation of cytokines by HuR in which gene dosage plays an important role. These findings may have significant implications in allergies and asthma, as well as autoimmune diseases and infection.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2013.00127

INTRODUCTION

The process of naive T-cell activation and differentiation toward polarized effector T cells tailors specific immunity against pathogens, while allowing tolerance against harmless allergens, commensal organisms or self-antigens in a healthy individual (1). However, certain diseases arise as a direct consequence of aberrant effector T-cell cytokine production or alterations in immune homeostasis (2). Therefore, understanding the mechanisms involved in T-cell differentiation is critical. During T-cell activation, the cell undergoes rapid and dynamic changes with up to 50% of the changes in cell-specific gene expression occurring at the posttranscriptional level (3,4). Studies within the past decade have identified several *cis*-elements and *trans*-factors that mediate these processes in T cells (5). Chromatin remodeling and transcriptional activation are highly integrated with posttranscriptional mechanisms, which specifically regulate the rate of mRNA transport, turnover and translation (6,7). Several *trans*-acting factors regulating these changes have been identified, but the functional mechanisms of their actions are not well understood. Such factors include RNA binding proteins (RBPs), which determine mRNA maturation, localization, stability and translation. mRNAs that possess adenylate and uridylate–rich elements (AREs) or U-rich elements are subject to control by RBPs (8,9).

Upon activation, naive $CD4^+$ T cells can differentiate (also known as polarization) in several different lineages, such as Th1, Th2, Th17, Treg and Tfh subsets. Each of these subsets is characterized by relevant signature transcription factors and cytokines. For example, Th1 lineage is characterized by the expression of T-bet transcription factor and production of interferon (IFN)-γ. In contrast, Th2 lineage is characterized by the transcription factor *trans*-acting T-cell–specific transcription factor (GATA-3) and by IL-4. A fuller

**MMG and PT contributed equally to this work.*

Address correspondence to *Ulus Atasoy, One Hospital Drive, M610C, Columbia, MO 65212. Phone: 573-882-6512; Fax: 573-882-4287. E-mail: atasoyu@missouri.edu.*

Submitted October 11, 2013; Accepted for publication January 23, 2014; Epub (www.molmed.org) ahead of print January 24, 2014.

The Feinstein Institute for Medical Research North Shore LIJ

discussion of $CD4^+$ T-cell differentiation can be found in the review by Bill Paul and associates (1).

Early-response genes including growth factors, signaling molecules and oncogenes frequently contain AREs. Interestingly, 90% of chemokines and cytokines contain AREs, illustrating the importance of posttranscriptional gene regulation during an immune response (10). Within the selective set of RBPs recognizing AREs, HuR has emerged as a key regulatory factor modulating mRNA stability, localization and translation (11). HuR contains three highly conserved recognition motifs, which generally bind to AREs or U-rich elements usually found in the 3′ untranslated region (UTR) of its targets. In resting T cells, HuR is 90% nuclear but is upregulated and translocated to the cytoplasm upon activation (12). In the cytoplasm, HuR can affect mRNA stability and/or translation via interplay with other RBPs, including T-cell intracellular antigen-1 (TIA-1), tristetraprolin (TTP), transcription factor IIIB 90 kDa subunit (BRF1), heterogeneous nuclear ribonucleoprotein D (hnRNPD; AUF1) and KH-type splicing regulatory protein (KSRP), as well as microRNAs (miRNAs) such as *let-7* (13–18). HuR regulates multiple transcripts involved in adaptive immunity including CD247 (CD3ζ), IL-3, IL-6, CD95/Fas, CD40L, cytochrome c oxidase subunit II (COX-2), granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α (10,19,20). Additionally, we and others have previously demonstrated that the Th2 master transcription factor GATA-3 and Th2 cytokines IL-4 and IL-13 are positively regulated at the posttranscriptional level by HuR (21–23). Therefore, we hypothesized that HuR is coordinately regulating Th2 polarization and cytokine secretion, implicating its involvement in Th2-mediated diseases, such as allergic airway inflammation (24,25).

Despite recent advances in our understanding of posttranscriptional gene regulation, most of the information currently available has been derived from cell lines. To study disease processes such as allergic airway inflammation, transgenic and knockout mice are needed. A limitation that has arisen in knocking out HuR in all tissues is that this scenario results in embryonic lethality, thus preventing the assessment of its involvement in immune processes and diseases (26). Therefore, we decided to create a novel HuR conditional knockout mouse using the Cre-loxP strategy to knockout HuR in activated T cells. Previous studies using the Cre-loxP system deleting HuR early in thymocyte development during the double-negative stage implicated the involvement of HuR in thymocyte maturation, selection and egress from the thymus. These mice had defects in thymocyte-positive and -negative selection, and thymic T cells lacked the ability to traffic normally from the thymus to the periphery (27). To circumvent these problems, we used a model that allowed for the deletion of HuR after T cells have undergone thymic selection, exited the thymus and become activated.

In our study, using this novel knockout mouse, we demonstrate that deleting HuR in activated T cells during Th2 polarization leads to paradoxical increases in IL-2, IL-4 and IL-13 cytokine production, targets that HuR normally positively regulates. Furthermore, we show that *Il2* and *Il13* mRNA stabilities surprisingly increased, whereas *Il4* was transcriptionally upregulated in Th2 cells from HuR conditional knockout mice. When an allergic airway inflammation model was used, HuR knockout mice mounted a robust response and displayed similar inflammation and airway hyperreponsiveness as control mice, indicating that the regulation of Th2 polarization is complex and may differ in animal models compared with cell lines.

MATERIALS AND METHODS

Generation of Conditional HuR Knockout Mouse

A vector was designed for homologous recombination in which the *HuR* gene was floxed with the insertion of loxP sites, which will result in the targeted deletion of exons 1 and 2 and a portion of the promoter region, additionally introducing a frame shift mutation upon Cre-mediated recombination. The *Neo* gene was used as a selection marker, and FRT sites were used to delete the *Neo* gene after selection was completed. An ~11.6-kb region used to construct the targeting vector was first subcloned from a positively identified C57BL/6 bacterial artificial chromosome (BAC) clone (RPCI23) by using a homologous recombination-based technique. The region was designed such that the short homology arm (SA) extends 2.4 kb to 5′ of the lox P/FRT-flanked Neo cassette. The long homology arm (LA) ends on the 3′ side of the lox P/FRT-flanked Neo cassette and is ~9.2 kb long. The single lox P site is inserted upstream of exon 1, and the lox P/FRT-flanked Neo cassette is inserted downstream of exon 1. The target region is 1.8 kb including exon 1.

The targeting vector was confirmed by restriction analysis after each modification step and by sequencing using primers designed to read from the selection cassette into the 3′ end of the LA (N2) and the 5′ end of the SA (N1), or from primers that anneal to the vector sequence, P6 and T7, and read into the 5′ and 3′ ends of the BAC subclone. The BAC was subcloned into an ~2.4-kb pSP72 backbone vector containing an ampicillin selection cassette for retransformation of the construct before electroporation. A pGK-gb2 loxP/FRT-flanked neomycin cassette was inserted into the gene. The targeting construct was linearized by using *Not*I and electroporated into embryonic stem cells. The founder mouse was then bred with OX40-*Cre*, which was provided by Nigel Killeen (University of Califor, San Francisco). All mice used were on the C57BL/6 background. All animal experiments and procedures were conducted in accordance with the guidelines set forth by the University of Missouri Animal Care and Use Committee.

Murine T-Cell Polarization *In Vitro*

Naive splenocytes were isolated from 6- to 8-wk-old C57BL/6 OX40-*Cre* $HuR^{fl/fl}$ knockout mice or C57BL/6 $HuR^{fl/fl}$ control mice. $CD4^+$ T cells were isolated using CD4 (L3T4) MicroBeads (Miltenyi Biotec, San Diego, CA, USA) following the manufacturer's protocol. Cells were activated with anti-CD3 and anti-CD28 (5 μg/mL each) for 5 d in T-cell media (Dulbecco's modified Eagle medium, 10% fetal calf serum, gentamicin, Na pyruvate, L-glutamine and β-mercaptoethanol) under Th1-polarizing (20 ng/mL rIL-12 and 20 μg/mL anti–IL-4 antibody), Th2-polarizing (100 U/mL rIL-4, 50 U/mL rIL-2 and 10 μg/mL anti–IFN-γ antibody) or nonpolarizing (no cytokines and blocking Abs) conditions. Anti-CD3, anti-CD28, anti–IL-4 and anti–IFN-γ antibodies were purchased from eBioscience (San Diego, CA, USA). Cytokines were all purchased from PeproTech (Rocky Hill, NJ, USA). Tissue culture reagents were all purchased from Gibco/Life Technologies (Carlsbad, CA, USA).

Proliferation Assay

On d 0, 1×10^6 naive $CD4^+$ T cells were isolated from the spleens of mice and stimulated with plate-bound anti-CD3 and anti-CD28 in one well of a 24-well plate. Cells were cultured in media containing rIL-2. On d 5 after activation, wells were harvested and cells were counted by using a hemocytometer. Cell viability was assessed via trypan blue dead cell exclusion dye. Triplicate wells were done for each group, and counting was done three independent times for each well. Experiments were repeated at least three times, and data was expressed as the mean number of total cells on d 5 from each group.

Western Blot Analysis

For Western blotting to detect HuR and GATA-3, activated or polarized T cells were washed three times with phosphate-buffered saline (PBS) and lysed in triple-detergent radioimmunoprecipitation assay buffer, consisting of 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1% sodium dodecyl sulfate (SDS), 1 mmol/L EDTA (ethylenediaminetetraacetic acid), and Complete Proteinase Inhibitor Mixture Tablets (Roche Applied Science, Indianapolis, IN, USA). Whole-cell protein lysates (100 μg) were loaded on a 12% SDS-PAGE (polyacrylamide gel electrophoresis) gel and transferred onto nitrocellulose membrane. The membrane was probed with anti–GATA-3 (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) anti–HuR clone 3A2 (1 μg/mL) and reprobed with anti–β-tubulin (1 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA). The secondary antibody used was a sheep anti–mouse immunoglobulin (Ig) conjugated with horseradish peroxidase (1:4,000) (GE Healthcare, Pittburgh, PA, USA). HuR and GATA-3 levels were determined by using Quantity One software (Bio-Rad, Hercules, CA, USA) after normalization to β-tubulin. Anti–HuR 3A2 hybridomas were provided by Joan Steitz (Yale School of Medicine). Anti-AUF1, anti-TTP and anti-CUGBP (anti–CUG triplet repeat, RNA binding protein 1 [Elav-like family member 1]) were provided by Gary Brewer (Rutgers University), Perry Blackshear (National Institute of Health [NIH]) and Paul Bohjanen (University of Minnesota), respectively.

RNA Isolation and Analysis

Total RNA was extracted by using TRIzol (Invitrogen/Life Technologies). One microgram of RNA was reverse transcribed, and real-time polymerase chain reaction (PCR) was performed by using cDNA (in triplicate) for using SuperScript III two-step qRT-PCR with SYBR green (Invitrogen/Life Technologies). PCRs were performed by using an Applied Biosystems StepOne PCR system. Results were analyzed using a comparative CT method with glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as an endogenous reference control. Primers for specific murine targets were as follows (forward/reverse):

Il2: 5′-CCCAAGCAGGCCACAGAATT GAAA-3′/5′-AGTCAAATCCAGAAC ATGCCGCAG-3′;

Il4: 5′-AGATGGATGTGCCAAACGTC CTCA-3′/5′-AATATGCGAAGCACC TTGGAAGCC-3′;

Il13: 5′-TGAGGAGCTGAGCAACATCA CACA-3′/5′-TGCGGTTACAGAGGC CATGCAATA-3′;

Ifng: 5′-GGCCATCAGCAACAACATAA GCGT-3′/5′-TGGGTTGTTGACCTC AAACTTGGC-3′;

Gata3: 5′-TTTACCCTCCGGCTT CATCCTCCT-3′/5′-TGCACCTGAT ACTTGAGGCACTCT-3′;

HuR (*Elave1*): 5′-ATGAAGACCA CATGGCCGAAGACT-3′/5′-AGTTC ACAAAGCCATAGCCCAAGC-3′;

Gapdh: 5′-TCAACAGCAACTCCC ACTCTTCCA-3′/5′-ACCCTGTTGCTGTAG CCGTATTCA-3′.

Intracellular Staining and Flow Cytometry

For detection of HuR by intracellular staining, cells were fixed and permeabilized with a Fix/Perm buffer set (BD Biosciences, San Jose, CA, USA), stained with 2 μg anti-HuR 3A2 and then incubated with phycoerythrin-conjugated anti–mouse immunoglobulin G (IgG) to detect either HA or HuR. Cells were analyzed on a FACScan flow cytometer (BD Biosciences) by using CellQuest software (BD Biosciences).

Transcriptional Analysis

Transcriptional activity was measured by using the Click-iT Nascent RNA Capture Kit (Invitrogen/Life Technologies) following the manufacturer's protocol. Briefly, activated Th2 cells were pulse labeled with EU for 0.5 h or 1 h and harvested. RNA was extracted by using TRIzol reagent by following the manufacturer's protocol. A total of 0.5 μg RNA was used for the click reaction. Purified nascent RNA was reverse-transcribed, and real-time PCR was performed by using SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen/Life Technologies) and analyzed on the Step-One Real-time

PCR system (Applied Biosystems/Life Technologies).

Cytokine Assays

On d 5, Th2-polarized cells were washed and restimulated with 50 ng/mL PMA and 500 ng/mL ionomycin for 6 h. Supernatants were collected and used for cytokine detection by using mIL-2, IL-4, IL-13 or IFN-γ enzyme-linked immunosorbent assay (ELISA) Ready-SET-Go kit (eBioscience).

Experimental Asthma Induction

Experimental asthma was induced by intraperitoneal injection with 50 or 200 μg chicken ovalbumin (OVA) grade V (Sigma-Aldrich) and 0.5 or 2 mg aluminum hydroxide (Thermo Scientific [Thermo Fisher Scientific Inc., Waltham, MA USA]) diluted in sterile PBS (Gibco/Life Technologies) on d 0 and 7. Mice were given 1% or 2% OVA by intranasal challenge for 30 min/d on d 12–15. Mice were harvested on d 17.

RNA Immunoprecipitation

NF90 and AUF1 immunoprecipitation was performed as previously described. Briefly, Th2-polarized cell lysates were prepared from OX40-*Cre* $HuR^{fl/fl}$ knockout and $HuR^{fl/fl}$ control after 6 d of *in vitro* polarization. Cell lysates were prepared in NT-2 buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L $NaCl_2$, 1 mmol/L $MgCl_2$ and 0.05% NP-40) supplemented with 1 mmol/L dithiothreitol, 0.25 U/mL RNAse out and 1× protease inhibitor and incubated on ice for 10 min. Preclear cell lysates were obtained from supernatant of cells after high-speed centrifugation at 7,558*g* for 30 min at 4°C. Equal amounts of lysates were used for immunoprecipitation with NF90, AUF1 and IgG1 control. NF90, AUF1 or IgG1 were precoated onto protein A Sepharose beads and incubated with cell lysates. Protein A Sepharose beads were then washed with NT-2 buffer and incubated with 20 U RNase-free DNase I (15 min, 30°C) and further incubated in 100 μL NT-2 buffer containing 0.1% SDS and 0.5 mg/mL proteinase K (30 min, 55°C). The isolated RNAs were reverse-transcribed, and quantitative PCR (qPCR) was performed to measure GAPDH, IL-2, IL-4 and IL-13.

Bronchoalveolar Lavage

Bronchoalveolar lavage was performed by instilling lungs with 1 mL sterile PBS and removing PBS. A total of 10 μL was then removed for cell count by using a hemocytometer. The remaining volume was centrifuged and supernatants were collected and stored at –20°C. Pelleted cells were resuspended in 400 μL PBS, and cytospin was performed. Wright stain was then performed to differentiate cells.

Serum Collection

Blood was collected by using retro-orbital bleeding on fully anesthetized mice. Blood was centrifuged and serum was collected and stored at –20°C.

Cytokine Assays

Supernatants from bronchoalveolar lavage fluid (BALF) were used for cytokine detection by using a mIL-4 or mIL-13 ELISA Ready-SET-Go kit (eBioscience). Serum (diluted 1:10) was used for IgE detection by using a BD OptEIA mouse IgE ELISA Set (BD Biosciences).

Polysome Analysis

Polysome analysis was performed as previously described (28).

Lung Histology

Lungs from immunized and nonimmunized mice were removed from mice and inflated with zinc-buffered formalin. Lungs were sectioned and stained with hematoxylin and eosin by using a standard procedure.

Airway Physiology

Anesthetized mice were given neuromuscular blockade (0.8 mL/kg pancuronium bromide) and ventilated with a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, QC, Canada) with a tidal volume of 7.5 mL/kg and a positive end-expiratory pressure of 3 cm H_2O. Mice were anesthetized, given a neuromuscular blockade and ventilated with a computer-controlled ventilator. Measurements of respiratory mechanics were made by the forced oscillation technique. Response to aerosolized methacholine (Sigma-Aldrich) (0, 10, 25 and 100 mg/mL) was determined by resistance measurements every 30 s for 3 min to ensure airway resistance had peaked. The lungs were inflated to total lung capacity after each exposure to methacholine, maintaining open airways and returning the measurements back to baseline. The resistance measurements were then averaged at each dose and graphed.

Statistical Analysis

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

All supplementary materials are available online at www.molmed.org.

RESULTS

Conditional Deletion of HuR in Activated T Cells

To more fully investigate the impact of HuR expression on the Th2 and allergic airway inflammation phenotype, we generated a conditional knockout mouse on the C57BL/6 background by using the Cre-loxP strategy, as described in Materials and Methods and shown in Supplementary Figures S1A–D. To obviate potential interference with T-cell ontogeny, we crossed the $HuR^{fl/fl}$ mouse with an OX40-*Cre* transgenic mouse to obtain a mouse that had a deletion of either one HuR allele (OX40-*Cre* $HuR^{fl/+}$) or both alleles (OX40-*Cre* $HuR^{fl/fl}$) after activation. OX40 (CD134) is a tumor necrosis factor receptor primarily expressed on activated T cells upon T-cell receptor engagement but not in naive T cells. OX40 is highly expressed 24–72 h after activation (29).Thus, when OX40 is expressed during T-cell activation in OX40-*Cre* transgenic mice, Cre recombinase expression and subsequent genetic

ablation of HuR in HuR$^{fl/fl}$ KO mice results. Under these conditions, only activated T cells will display decreased levels of HuR compared with controls. Therefore, most developing thymocytes will have wild-type levels of HuR, since only a small proportion of thymocytes express OX40 (30). Deletion of a single HuR allele (HuR$^{fl/+}$) in activated T cells reduces HuR protein levels by ~24% (Figures 1A, B). In addition, deletion of both HuR alleles further reduced HuR protein levels by ~93% in activated T cells (Figures 1C, D). Because of the described role of HuR in regulating cell growth and proliferation, we performed counting assays to assess the proliferative capabilities of HuR-depleted T cells and found that the absence of HuR had no effect on the ability of T cells to proliferate (Figure 1E). Additionally, histological analysis of major organs and both percent and number of B and T cells were analyzed and found to be similar between the HuR knockout and control mice (data not shown).

To determine timing of HuR knockout during Th2 polarization, we performed serial HuR protein analysis by Western blotting in Th2-polarized cells on d 0 (unactivated) and on d 1, 2, 3, 4 and 6, respectively. Our data showed that it takes several days to achieve a significant knockout of HuR during Th2 polarization (d 3, >50% reduction in HuR protein levels) (Supplementary Figures S3A, B). This result confirmed that, in our system, HuR can be genetically ablated and thus knocked out under Th2 polarization conditions in OX40-Cre HuR$^{fl/fl}$ mice.

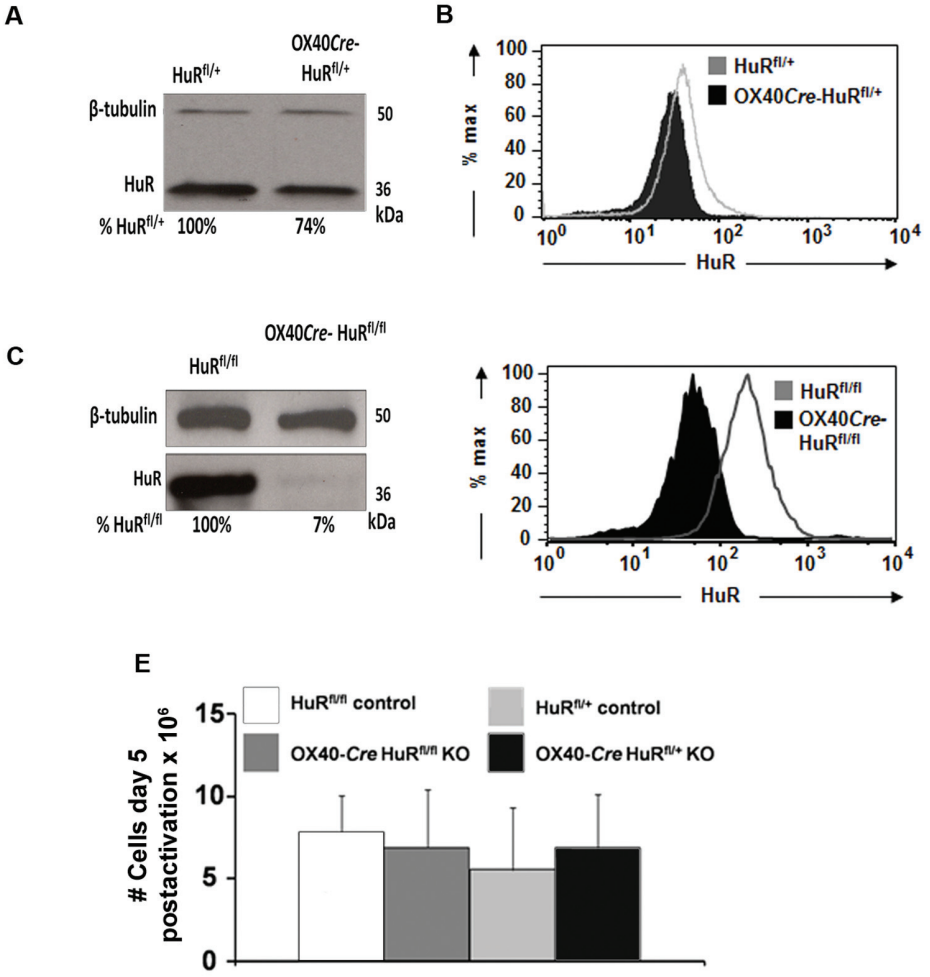

Figure 1. OX40-*Cre* HuR$^{fl/+}$ and OX40-*Cre* HuR$^{fl/fl}$ knockout mice display significant decreases in HuR protein levels. (A) Western blot analysis of HuR expression (and β-tubulin as loading control) in activated T cells from OX40-*Cre* HuR$^{fl/+}$ compared with HuR$^{fl/+}$ control mice and (C) in activated T cells from OX40-*Cre* HuR$^{fl/fl}$ compared with HuR$^{fl/fl}$ control mice. (B) Intracellular HuR staining assessed by fluorescence-activated cell sorter analysis in activated T cells from OX40-*Cre* HuR$^{fl/+}$ and HuR$^{fl/+}$ control mice and (D) from OX40-*Cre* HuR$^{fl/fl}$ and HuR$^{fl/fl}$ control mice. (E) Proliferation assay reveals HuR knockout does not affect T-cell proliferation (mean ± standard error of the mean (SEM); n = 3). Western blot and intracellular HuR staining representative of n = 3.

Th2-Polarized Cells from Heterozygous Conditional Knockout Mice Have Decreases in the Levels of *Gata3*, *Il4* and *Il13* mRNAs

To assess the role of HuR in Th1 or Th2 polarization, we isolated naive CD4$^+$ T cells from both heterozygous and homozygous OX40-*Cre* HuR$^{fl/fl}$ mice and respective control mice and polarized them under either Th1 or Th2 conditions. For controls, we used HuR$^{fl/fl}$ mice lacking the OX40-*Cre* transgene. Surprisingly, the effects on cytokine profiles and GATA-3 expression were different between the hetero- and homozygous models. The mRNAs encoding Th2 transcription factor GATA-3 and cytokines IL-4 and IL-13 are known HuR targets. Additionally, IL-2 plays an important role in Th2 polarization and contains putative HuR binding sites, although how it is regulated by HuR is unclear (30–32). Therefore, we decided to measure the mRNA and protein levels of GATA-3, IL-2, IL-4 and IL-13 in HuR knockout mice.

Besides reduced HuR levels, Th2-polarized CD4$^+$ T cells from heterozygous OX40-*Cre* HuR$^{fl/+}$ knockout mice showed modest but significant reductions in steady-state levels of *Gata3*, *Il4* and *Il13* mRNAs, whereas *Il2* mRNA levels in Th2-skewed cells were not affected (Figure 2A). Because we had previously shown that HuR regulates

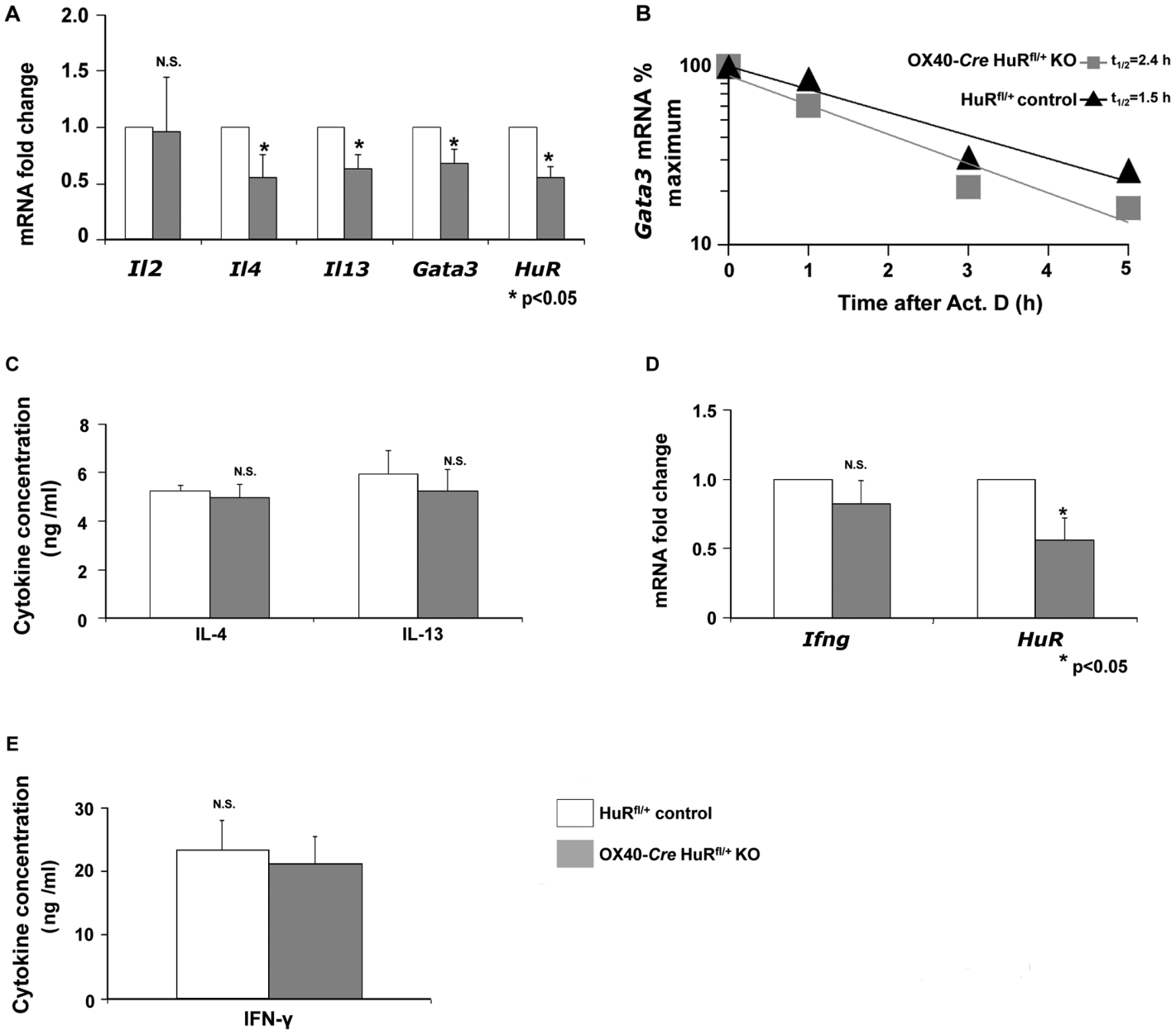

Figure 2. Th2-polarized cells from OX40-*Cre* HuR$^{fl/+}$ heterozygous knockout mice display decreases in *Il4, Il13* and *Gata3* steady-state mRNA levels. (A) Real-time PCR analysis of *Gata3, Il2, Il4* and *Il13* mRNA in Th2-polarized cells. (B) *Gata3* mRNA decay as assessed by real-time PCR in OX40-*Cre* HuR$^{fl/+}$ and HuR$^{fl/+}$ T cells (n = 3 for half-life). (C) ELISA of supernatants from Th2-polarized OX40-*Cre* HuR$^{fl/+}$ mice. (D) Real-time PCR analysis of *Ifng* in Th1-polarized cells from OX40-*Cre* HuR$^{fl/+}$ compared with HuR$^{fl/+}$ control mice. (E) IFN-γ ELISA of supernatants from Th1-polarized OX40-*Cre* HuR$^{fl/+}$ mice (error bars represent mean ± SEM; n = 3; *$p < 0.05$). Act. D, actinomycin D; N.S., not significant.

Gata-3, the signature transcription factor for Th2 differentiation, we investigated whether HuR ablation altered *Gata-3* mRNA stability (21). In Figure 2B, Th2-polarized OX40-*Cre* HuR$^{fl/+}$ T cells treated with actinomycin D, a transcriptional inhibitor, showed a small but consistent reduction (2.4 versus 1.5 h) in *Gata3* mRNA stability compared with HuR$^{fl/+}$ controls, similar to what has been previously observed in human systems. We performed ELISAs on supernatants from restimulated Th1- or Th2-polarized cells. Surprisingly, there were no statistically significant alterations in the IL-4 or IL-13 protein amounts secreted by Th2-polarized cells from the heterozygous OX40-*Cre* HuR$^{fl/+}$ mice compared with HuR$^{fl/+}$ control mice (Figure 2C). Additionally, IFN-γ mRNA and protein levels were unaltered in

Th1-polarized CD4$^+$ T cells from heterozygous OX40-*Cre* HuR$^{fl/+}$ mice (Figures 2D, E).

Th2-Polarized Cells from Homozygous HuR Knockout Mice Have Increases in *Il2*, *Il4* and *Il13* mRNA and Protein Expression

In stark contrast to the heterozygous mice, Th2-polarized CD4$^+$ T cells from homozygous OX40-*Cre* HuR$^{fl/fl}$ knockout mice showed statistically significant increases in steady-state levels of *Il4* and *Il13* mRNAs, respectively, compared with the Th2-polarized cells from the control mice. Furthermore, we also observed a more dramatic (~3.5-fold) increase in *Il2* mRNA (Figure 3A). *Gata3* steady-state mRNA levels from Th2 cells and *Ifng* steady-state mRNA levels in Th1-skewed cells did not change significantly (Figures 3A, D). Surprisingly, *Gata3* mRNA stability was unaltered in Th2 cells from HuR knockout mice compared with control mice (Figure 3B). To determine whether increases in Th2 cytokine mRNA correlated with protein production, we performed ELISA on supernatants from restimulated Th1- or Th2-polarized CD4$^+$ T cells. This analysis revealed there were slightly higher levels of IL-4 and IL-13, but not of IFN-γ (Th1-skewed), in the supernatants of Th2-skewed cells from HuR knockout mice compared with control Th2 cells (Figures 3C, E). Consistent with the mRNA data, supernatants from Th2 cells from HuR knockout mice revealed a greater increase (more than twofold) in IL-2 compared with IL-4 and IL-13 (Figure 3C). We further performed kinetic studies to ascertain whether there was a temporal relationship between HuR ablation and cytokine secretion. As seen in Supplementary Figures S3C–F, there appears to be a correlation with IL-2 expression but not as much with IL-13.

Knockout of HuR in Th2-Polarized Cells Increases the Transcription of *Il4* and the Stability of *Il2* and *Il13* mRNAs

To more fully investigate the putative mechanism of altered mRNA and protein expression in the HuR knockout mice, we reasoned that the increases in steady-state *Il2*, *Il4* and *Il13* mRNA levels are most likely due to increased transcription, increased mRNA stability or both. Therefore, we performed transcriptional assays and mRNA stability assays to uncover the mechanisms by which a deletion of HuR in Th2-polarized cells increases the expression of IL-2, IL-4 and IL-13. Transcriptional assays using a nascent mRNA capture kit and real-time PCR analysis revealed a small but statistically significant increase in *Il4* transcription in Th2-polarized T cells from the HuR knockout mice and no alteration in either *Il2* or *Il13* transcription (Figure 4A). Actinomycin D mRNA stability assays revealed an increase in the *Il2* (2.5 versus 0.94 h) and the *Il13* mRNA stability (1.6 versus 1.1 h) in the HuR knockout mice with no change in the *Il4* mRNA stability (Figures 4B–D). Therefore, we conclude that knocking out HuR in Th2-polarized cells surprisingly increases the stabilities of *Il2* and *Il13* mRNAs and the transcription of *Il4* by modest amounts.

Knockout of HuR in Th2-Polarized Cells Does Not Alter the Polysomal Distribution of *Il2* or *Il13* mRNAs

To ascertain whether translational recruitment to polysomes played a role in altered cytokine protein expression, we performed experiments to determine if a HuR deletion in Th2-polarized cells had an effect on translation, specifically whether the distribution of *Il2* and *Il13* mRNAs was altered between the high-molecular-weight polysomes and the low-molecular-weight polysomes in Th2-polarized cells from HuR knockout mice. Recruitment to the high-molecular-weight polysomes is one potential mechanism to explain increased protein production (33). As shown in Figure 5, the absorbance profile for RNA separated by velocity sedimentation through a sucrose gradient indicates which fractions contain the low-molecular-weight and high-molecular-weight polysomes (Figure 5A). Reverse transcriptase (RT)-qPCR was performed on RNA extracted from each fraction to determine the distribution of *Il2* and *Il13* mRNAs. *Il13* mRNA distribution was similar between HuR knockout and control mice, although there was a slight shift in the mRNA peak in the HuR knockout group (Figure 5B). However, these observations were not specific to *Il13* mRNA, since there appeared to also be a similar shift in the endogenous control, *Gapdh* mRNA (Figures 5B, D). *Il2* mRNA distribution was likewise unaffected by a knockout of HuR (Figure 5C). Taken together, we conclude that deletion of HuR in Th2-polarized cells had little effect on the translation efficiency of *Il2* and *Il13* mRNAs and thus this is unlikely to be a mechanism to explain increased protein production.

HuR Ablation in Th2 Cells Does Not Affect Global Expression of Other RNA Binding Proteins but May Interfere with Cytokine Transcript Milieu

The collection of *trans*-acting factors, such as RBPs and microRNAs, which interact with cytokine 3′ UTR, is commonly called transcript milieu. Because HuR is normally a positive regulator of its targets, including IL-4 and IL-13, it was surprising to find these mRNA transcripts and proteins are increased in the absence of HuR. The unexpected results led us to speculate that perhaps knocking out HuR in Th2 cells was affecting the expression of other RBPs, since there is significant feedback and autoregulation among RBPs. Therefore, we analyzed the expression of several well-known ARE-binding proteins that are known to be regulators of mRNA stability and protein expression. By using real-time PCR and Western blotting, we analyzed the expression of AUF1, BRF1, TTP, CUGBP, TIA-1, TIA1 cytotoxic granule-associated RNA binding protein-like 1 (TIAR), KSRP and NF-90 in Th2-polarized cells from HuR knockout and control mice. We found no significant differences at the mRNA level of *AUF1*, *BRF1*, *CUGBP*, *TIA-1*, *TIAR*, *KSRP* or *NF90* between knockout and control mice (Supplementary Figure S2A). Addi-

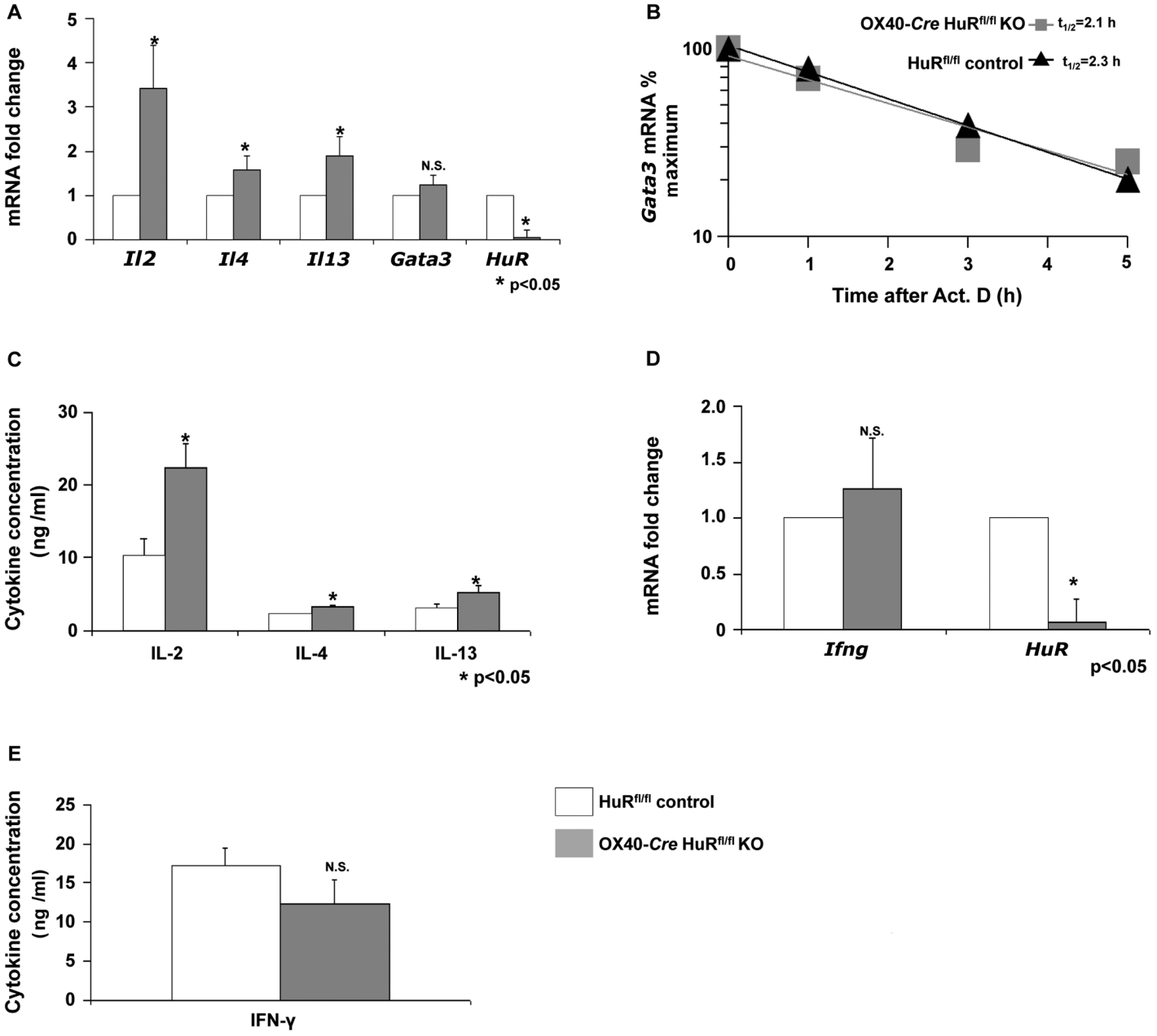

Figure 3. Th2-polarized cells from OX40-*Cre* HuR$^{fl/fl}$ homozygous knockout mice display increases in *Il2, Il4* and *Il13* steady-state mRNA and protein levels. Real-time PCR analysis of *Gata3, Il2, Il4* and *Il13* mRNA in Th2-polarized cells. (B) *Gata3* mRNA decay as assessed by real-time PCR in OX40-*Cre* HuR$^{fl/fl}$ and HuR$^{fl/fl}$ T cells (n = 3 for half-life). (C) ELISA of supernatants from Th2-polarized OX40-*Cre* HuR$^{fl/fl}$ mice. (D) Real-time PCR analysis of *Ifng* in Th1-polarized cells from OX40-*Cre* HuR$^{fl/fl}$ compared with HuR$^{fl/fl}$ control mice. (E) IFN-γ ELISA of supernatants from Th1-polarized OX40-*Cre* HuR$^{fl/fl}$ mice (error bars represent mean ± SEM; n = 3; *$p < 0.05$). N.S., not significant.

tionally, TTP, CUGBP and AUF1 protein levels were unaltered (Supplementary Figure S2B).

The stark differences between heterozygous and homozygous knockout mice led us to speculate that the dosage of HuR was playing a major role in the regulation of Th2 polarization and cytokine production. A similar gene dosage effect was described in other models of single- versus double-allele gene knockouts and may involve the occurrence of indirect effects because of the loss of control of other regulatory genes targeted by the ablated gene. This phenotype was described for the cell cycle inhibitor *p27^{Kip1}* gene. Because p27^{Kip1} is an important negative regulator of Th2 polarization and is also a target of HuR, we measured the levels of *p27^{Kip1}* mRNA and protein in the OX40-*Cre* HuR$^{fl/fl}$ T cells

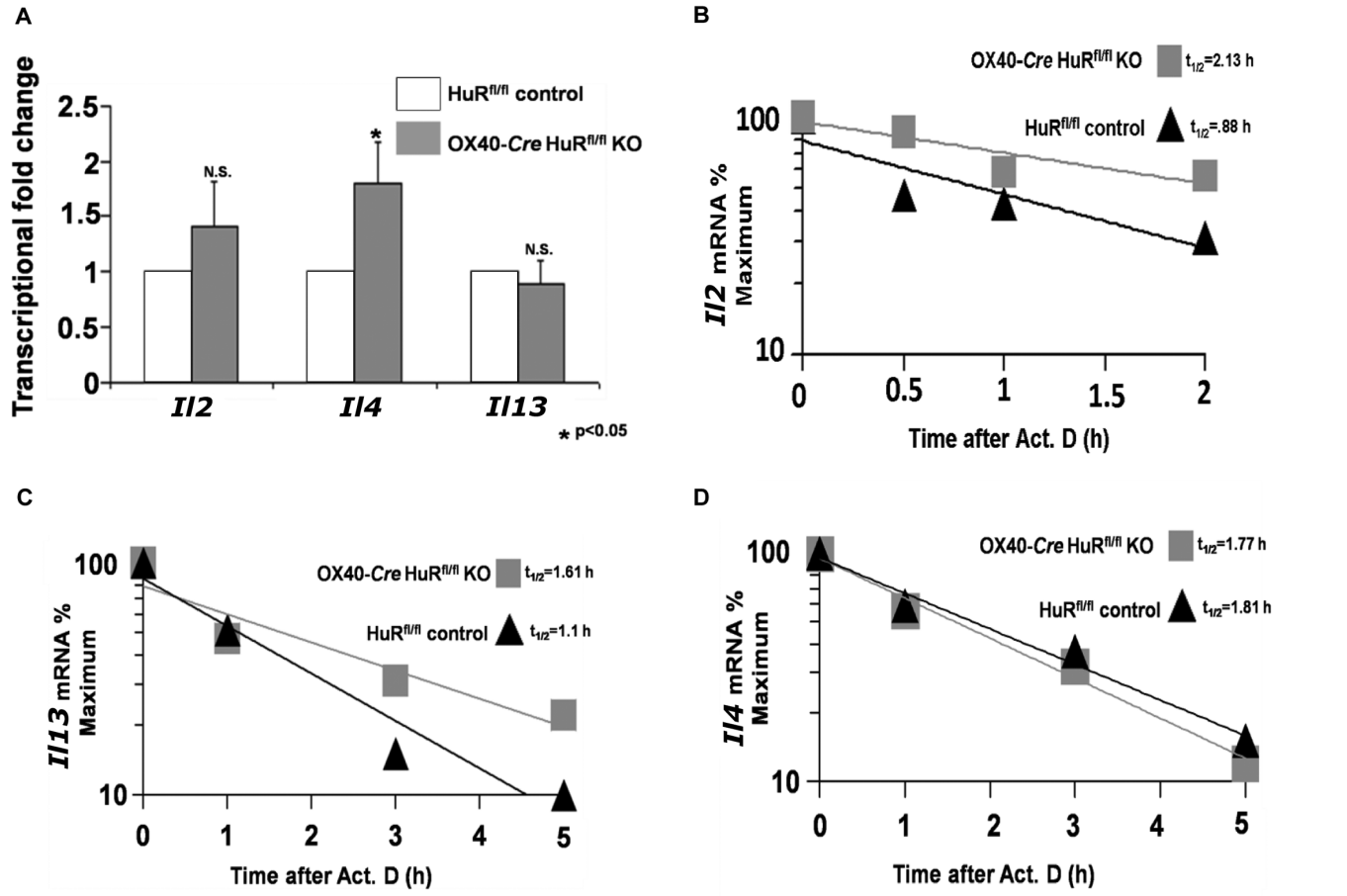

Figure 4. *Il4* is transcriptionally upregulated in Th2-polarized cells from OX40-*Cre* HuR$^{fl/fl}$ homozygous knockout mice while *Il13* and *Il2* mRNA stabilities are increased. (A) Transcriptional assay using nascent RNA capture assay and real-time PCR analysis of *Il13, Il4* and *Il2* mRNA in Th2-polarized cells from OX40-*Cre* HuR$^{fl/fl}$ and HuR$^{fl/fl}$ mice. (B) *Il2* mRNA decay as assessed by real-time PCR in OX40-*Cre* HuR$^{fl/fl}$ and HuR$^{fl/fl}$ T cells. (C) *Il13* mRNA decay as assessed by real-time PCR in OX40-*Cre* HuR$^{fl/fl}$ and HuR$^{fl/fl}$ T cells. (D) *Il4* mRNA decay as assessed by real-time PCR in OX40-*Cre* HuR$^{fl/fl}$ and HuR$^{fl/fl}$ T cells (error bars represent mean ± SEM; n = 3; *$p < 0.05$; n = 3 for half-life).

compared with control cells and found no differences in $p27^{Kip1}$ mRNA levels and a small decrease in protein by Western blot analysis (data not shown). Therefore, we concluded that HuR is not dramatically affecting $p27^{kip1}$ expression.

The previous experiments indicated that there were no alterations in global expression of the previously mentioned RBPs. However, distribution of other *trans*-acting factors that interact with 3′ UTR of mRNA transcripts can be altered when HuR is absent. We asked whether recruitment of NF90 and AUF1 was changed under reduced HuR conditions in HuR KO T cells. NF90 had previously been described to act both as a transcriptional factor and as RBPs, which stabilized *Il2* mRNA. We performed RIP and measured relative amounts of mRNA, which interacted with either NF90 or AUF1. AUF1 did not appreciably bind to *Il2*, *Il4* or *Il13* mRNAs (data not shown). NF90 did bind to *Il2* mRNA equally well under wild-type or reduced HuR conditions. Surprisingly, when HuR is genetically ablated in $CD4^+$ Th2 cells, NF90 did not bind as efficiently to the *Il13* transcript (Supplementary Figure S2). There was no appreciable binding to *Il4* mRNA, regardless of HuR cellular levels. Taken together, these results indicate that distribution of *trans*-acting factors, such as RBPs, which interact with cytokine mRNA, can dramatically change depending on ambient cellular HuR levels.

Mice Lacking HuR in Activated T Cells Display Robust Allergic Airway Inflammation

Although the exact mechanism of regulation of HuR in Th2 polarization is unclear, increases in IL-2, IL-4 and IL-13 protein expression in HuR KO T cells implicated HuR in the regulation of allergic airway inflammation. Therefore, we induced allergic airway inflammation to assess the disease phenotype in HuR KO mice. Initially we chose to analyze homozygous HuR$^{fl/fl}$ KO mice, since Th2 cells from these animals displayed alterations in cytokine protein production, whereas the Th2 cells from heterozygous mice had no alterations in cytokine protein secretion. We used a standard

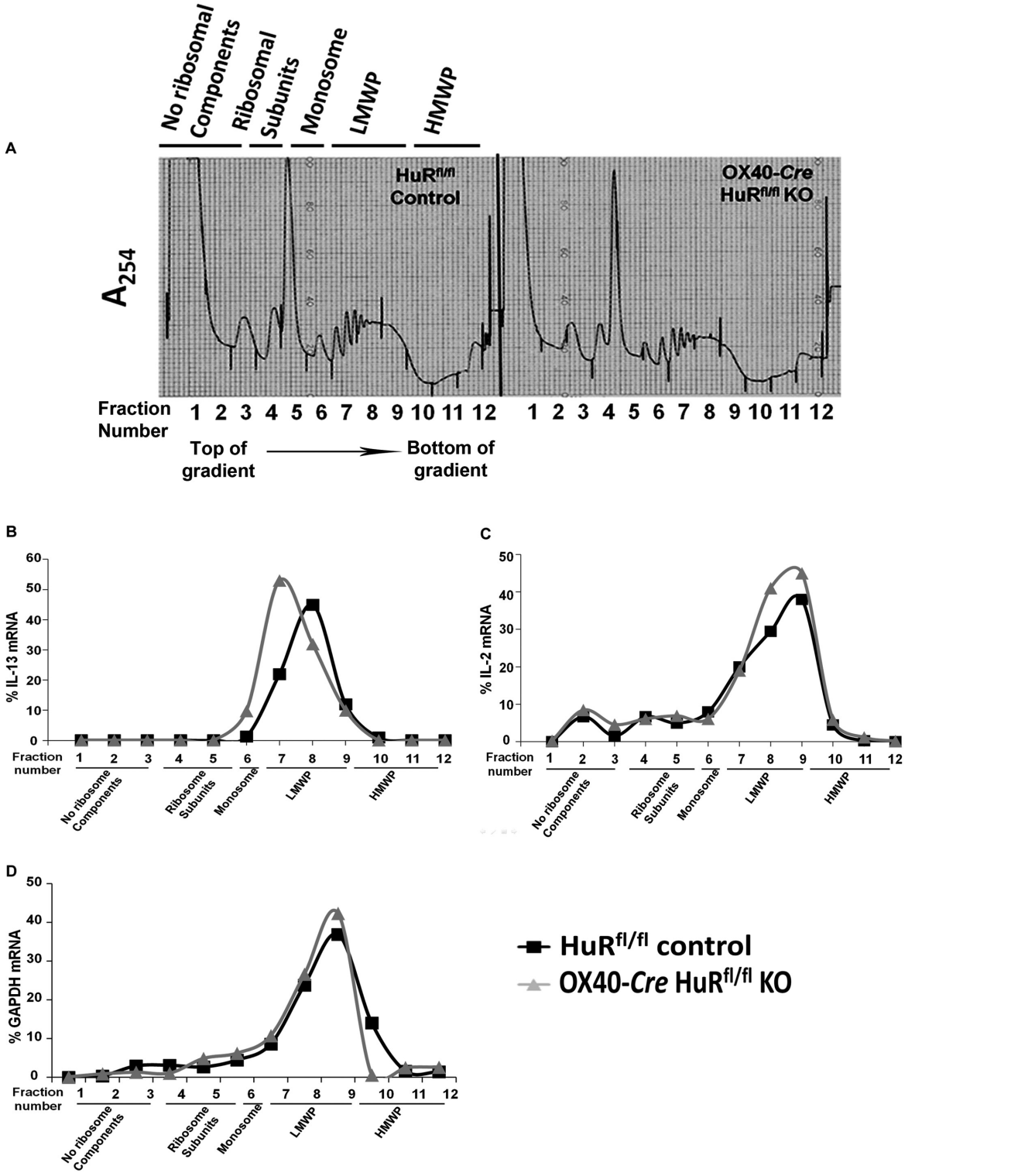

Figure 5. Polysomal distribution of *Il2* and *Il13* mRNA is unaltered in OX40-*Cre* HuR$^{fl/fl}$ homozygous knockout mice. (A) Absorbance profile for RNA separated by velocity sedimentation through a sucrose gradient. RNA was extracted from each fraction (B), and the levels of *Il13* and *Il2* mRNA (C) in each fraction from each population (OX40-*Cre* HuR$^{fl/fl}$ or HuR$^{fl/fl}$ control) was measured by RT-qPCR. (D) The levels of *Gapdh* mRNA were also measured in each fraction. The data shown are representative of two independent experiments. LMWP, low-molecular-weight polysomes; HMWP, heavy-molecular-weight polysomes.

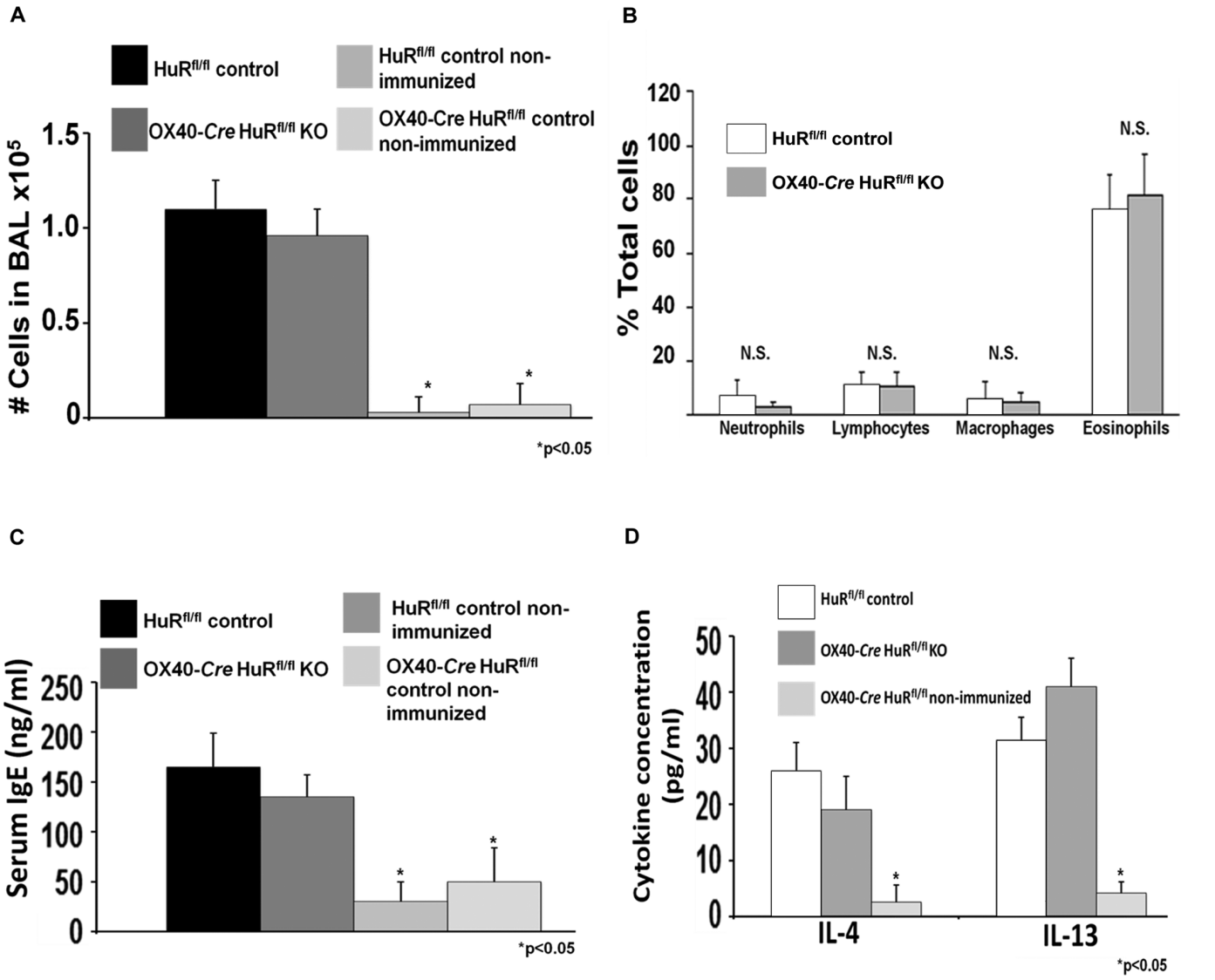

Figure 6. BALF cellularity and serum IgE titers in HuR knockout mice with allergic airway inflammation. (A) Number of cells recovered from the BALF of immunized HuR knockout and control mice and nonimmunized mice. (B) Cellular composition represented as a percent of total cells recovered from the BALF from immunized HuR knockout mice and control mice. (C) Serum IgE levels as measured by ELISA from immunized HuR knockout or control mice and nonimmunized knockout mice. (D) BALF IL-4 and IL-13 levels were measured by using ELISA comparing immunized HuR knockout and control mice with nonimmunized knockout mice (mean ± SEM of n = 8 mice in each group; three independent experiments; *$p < 0.05$). N.S., not significant.

chicken OVA, aluminum hydroxide (alum) model of allergic airway inflammation. To assess the asthma phenotype, we measured a variety of parameters, including number of cells and the type of cells recovered from the BALF, as well as serum IgE. As shown in Figure 6, the number of cells in the BALF from both immunized knockout and control mice was significantly greater than the number of cells recovered from the BALF of nonimmunized mice control or HuR KO animals, which validates the induction of allergic airway inflammation. However, there were no significant differences in the number of cells recovered from the BALF when comparing immunized knockout mice with immunized control mice (Figure 6A). Additionally, cell differentials indicated that ~80% of the cells recovered from the BALF were eosinophils, consistent with an OVA/alum model of asthma in C57BL/6 mice (34). However, the percent of eosinophils, neutrophils, macrophages and lymphocytes recovered from the knockout mice was similar to control mice (Figure 6B). Serum IgE levels were higher in the immunized knockout and control mice compared with nonimmunized controls

and HuR KO mice. However, there were no significant differences in the serum IgE levels in immunized knockout and control mice (Figure 6C).

The *ex vivo* polarization experiments indicated that HuR KO Th2 cells had increased production of IL-13 and to a lesser extent IL-4. Therefore, we sought to determine whether Th2 cytokine levels would be higher in the BALF from HuR knockout mice. ELISA was performed on BALF, and we found similar amounts of cytokine secretion between immunized HuR KO mice and control mice (Figure 6D). Nonimmunized HuR KO mice expressed low levels of IL-4 and IL-13.

Although the results thus far indicated that HuR knockout mice have similar allergic airway inflammation as control mice, it is possible that there are differences histologically or, more importantly, there could be differences in airway hyperresponsiveness. To determine if there were histological changes in the lungs, we performed hematoxylin and eosin staining on formalin-fixed lungs from immunized HuR knockout and control mice, along with nonimmunized control mice. As expected, lungs from nonimmunized mice had no significant lesions in the lungs, whereas immunized HuR knockout and control mice displayed significant inflammation consistent with allergic airway inflammation. However, there were no discernable differences between immunized HuR knockout and control mice (Figures 7A–D).

Finally, we measured airway hyperresponsiveness in response to methacholine with the flexiVent by using invasive measurements. Both humans and mice with asthma display increases in lung resistance (the level of constriction in the lungs) in response to methacholine, although in C57BL/6 mice, the airway changes are more subtle than in other strains (34–36). As shown in Figure 7, immunized HuR knockout and control mice displayed significant increases in airway resistance compared with nonimmunized control mice at methacholine doses of 50 and 100 mg/mL. However, both knockout and control mice had similar airway hyperresponsiveness (Figure 7E). Lung elastance (elastic rigidity of the lungs), compliance (the ease with which the lungs can be extended) and Newton resistance were measured and were similar between the knockout and control mice (data not shown).

Experiments were also performed on heterozygous $HuR^{fl/+}$ KO mice. Similar

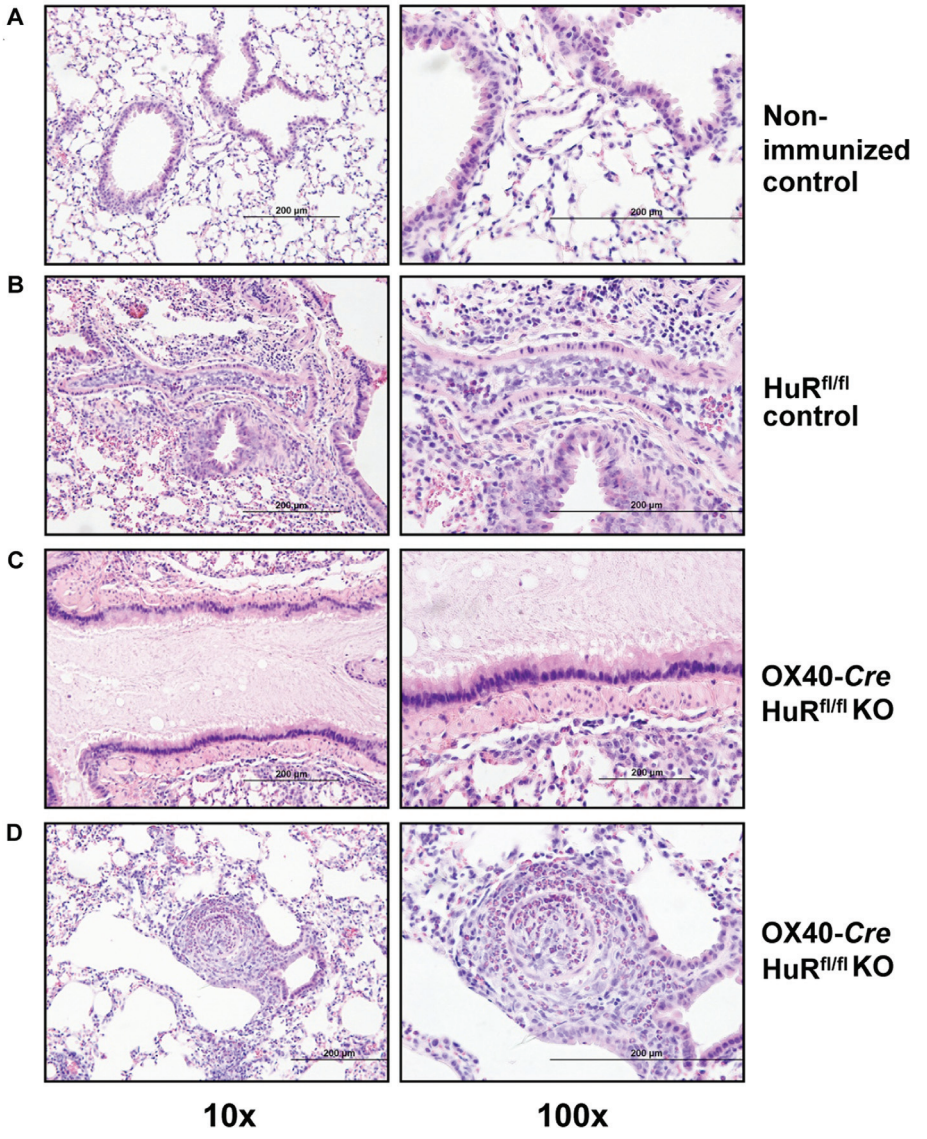

Figure 7. Lung histology and airway hyperresponsiveness. (A) Lungs recovered from nonimmunized $HuR^{fl/fl}$ control mice display normal lung architecture with little inflammation. (B) Lungs from immunized $HuR^{fl/fl}$ control mice display significant inflammation consistent with allergic airway inflammation. (C, D) Lungs from immunized HuR knockout mice have significant lung inflammation consistent with an allergic airway inflammatory response (representative hematoxylin and eosin staining of lungs; n = 3 nonimmunized control mice, n = 5 immunized knockout, and n = 5 immunized control mice). (E) Lung airway resistance in response to methacholine exposure (mean ± SEM of n = 6 mice in each group; two independent experiments; $*p < 0.05$). N.S., not significant. Scale bars, left panels, 200 μm × 10; right panels; 200 μm × 100.

Continued on next page

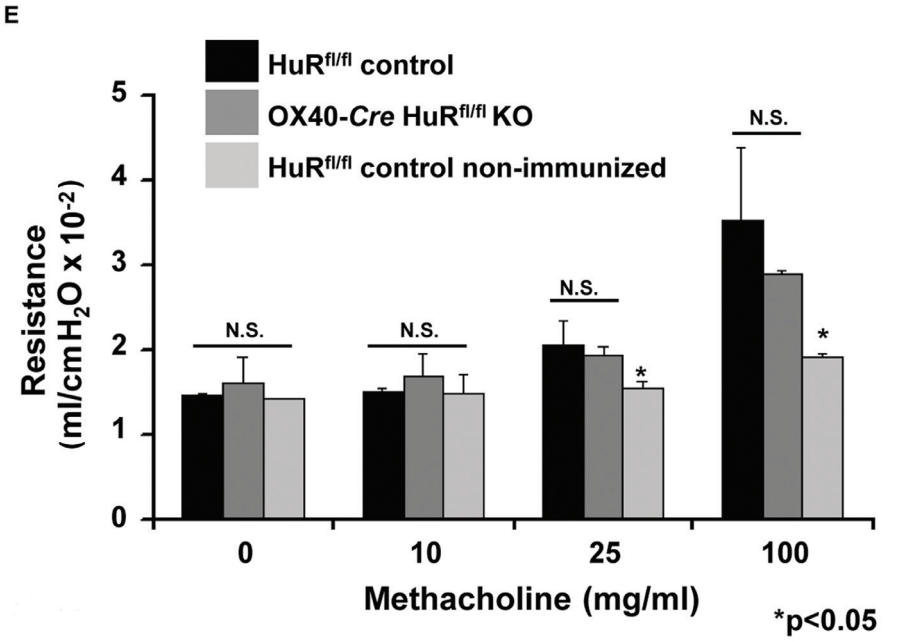

Figure 7. *Continued.*

to the homozygous knockout mice, BALF from the heterozygous mice revealed similar numbers and composition of cells as the control mice. Serum IgE levels were also unaltered in the heterozygous HuR knockout mice (Supplementary Figures S4A–C). In summary, HuR knockout mice have similar total cellular infiltration, lymphocyte and eosinophil counts in BALF. Surprisingly, HuR knockout mice had similar BALF cytokine levels of IL-4 and IL-13 along with similar serum IgE levels. In addition, despite the absence of HuR in activated T cells, knockout mice displayed robust airway and lung inflammation and hyperresponsiveness similar to that observed in control mice. These findings are consistent with our unexpected *ex vivo* data in that Th2 cells from HuR KO mice produce higher cytokine levels.

We further investigated these results repeating the OVA challenge experiment with drastically lowered (75%) amounts of OVA, reasoning that perhaps a subtle phenotype would be masked by high antigen sensitization and challenge. We still obtained robust airway inflammation, but, as before, there were no significant differences between control and HuR KO mice (Supplementary Figures S4D–F). Together, our data suggest that mice with genetic ablation of HuR in activated T cells develop allergic airway inflammation in response to OVA challenge.

DISCUSSION

The role of posttranscriptional gene regulation in controlling Th2 cytokine gene expression and allergic airway inflammation is not completely understood (37–43). HuR plays an important role in regulation of many genes involved in Th2-driven inflammatory diseases, including allergic asthma in humans (21–23). Interestingly, up to 50% of changes in mRNAs and proteins during T-cell activation occur at the posttranscriptional level (4).

By using combinations of cellular and animal models, our lab and others have identified *Il4* and *Il13* as HuR-regulated genes (21–23). In addition, we recently demonstrated HuR binds to the *Gata3* 3′UTR and regulates its expression by increasing its mRNA stability (21). Furthermore, the *Il2* mRNA contains AREs, and it was previously suggested that it may be regulated by HuR (21–23). Therefore, we hypothesized that HuR is coordinately regulating Th2 polarization and cytokine expression and, thus, may be playing a central role in allergic airway inflammation.

The results obtained in our murine model of conditional HuR ablation in T cells are complex, showing a gene dosage effect on *Il4*, *Il13* and *Gata3* mRNA turnover and a decoupling of the effect of HuR on mRNA metabolism from that of protein translation. In fact, a limited reduction in HuR expression (by ~26%) in $CD4^+$ T cells from heterozygous OX40-*Cre* $HuR^{fl/+}$ KO animals was sufficient to decrease steady-state levels of *Gata3*, *Il4* and *Il13* mRNAs, as well as modestly reducing *Gata3* mRNA stability, reproducing closely the effects seen in our previous studies, but without concomitant changes in protein levels. However, whereas the changes in *Il4*, *Il13* and *Gata3* mRNA levels are statistically significant, they are nevertheless modest, which may partially explain the discordance between mRNA steady-state levels and protein expression. In the Th2-polarized cells from the homozygous OX40-*Cre* $HuR^{fl/fl}$ KO animals, the effect on *Gata3* mRNA steady-state levels and stability was lost. $HuR^{fl/fl}$ KO Th2 cells also displayed paradoxical but modest increases in mRNA and protein of other HuR targets IL-4 and IL-13, as well as IL-2.

Coupled examination of transcriptional activation and mRNA turnover for *Il2*, *Il4* and *Il13* in homozygous $HuR^{fl/fl}$ KO and control cells revealed that an increase in transcription was mainly responsible for the altered *Il4* mRNA levels, whereas a small increase in *Il13* mRNA and a greater increase in *Il2* mRNA occurred in the absence of further transcriptional activation, since the *Il2* and *Il13* mRNAs were more stable in the HuR KO Th2 cells. Although the mechanisms of these responses are unclear, they underscore the pleiotropic role of HuR in posttranscriptional control of gene expression and suggest the potentially critical role of additional cofactors, miRNA or other RBPs in shaping HuR function. For example, under spe-

cific conditions, HuR binding to a target mRNA has been shown to lead, through association with other RBPs, to destabilization of target mRNAs (44). Furthermore, new data indicate that miRNAs regulate HuR levels and that, conversely, HuR interacts with miRNAs to conduct its regulation, adding an additional layer of complexity to the interpretation of results from our loss-of-function mouse model (45,46). Along these lines, Leung and Sharp (47,48) proposed that miRNAs form complexes with different RBPs such as HuR in different cellular conditions to alter mRNA survival and translatability. Therefore, another possible, though yet unproven, mechanism underlying the results obtained in HuR KO Th2 cells may involve altered interactions of microRNA, and perhaps other RBPs, with *Gata3* and Th2 cytokine 3′UTR. Consistent with this hypothesis, it was recently shown that HuR can repress *c-myc* expression by mediating miRNA *let-7* binding. In the absence of HuR, *let-7* cannot bind to *c-myc* and thus *c-myc* expression is increased (17). Interestingly, recent reports identified *Il13* as a target of *let-7* (49). Therefore, it is possible that HuR recruits miRNAs to target certain genes, and, in the absence of HuR, the miRNAs cannot bind. Overall, these data may also indicate that specific levels of HuR are critical for normal T-cell homeostasis.

The surprising results of the opposite regulation of Th2 cytokines in heterozygous versus homozygous T-cell HuR-deficient mice is not the first case of an unusual gene dosage effect in animal models. A similar effect was reported after ablation of the cell cycle inhibitor $p27^{Kip1}$ in a mouse model of prostate cancer and a mouse model with *CFTR* ablation (50–52). $p27^{Kip1}$ is relevant, since this cell cycle inhibitor is a key negative regulator of Th2 polarization and T-cell proliferation, and its expression is regulated posttranscriptionally by HuR (53–55). We found no significant alterations in $p27^{Kip1}$ expression in Th2 cells from HuR KO mice. More global expression profiling analysis of the single- or double-allele knockout and of control T cells will be needed to identify novel HuR-regulated genes, besides $p27^{Kip1}$, which might be responsible for this phenomenon.

We also explored the possibility that deletion of HuR leads to alterations in expression of other RBPs. Many RBPs contain AREs, which implicate cross-regulation between the RBPs (56). We measured mRNA levels of several RBPs, including *BRF1*, *TTP*, *AUF1*, *CUGBP*, *NF90* and *KSRP* (9). We found no differences in the mRNA expression of any of these transcripts. Additionally, we found no difference in protein expression of CUGBP, AUF1 or TTP; however, we cannot rule out the possibility that the binding of the RBP to its target may be altered in the absence of HuR. Consistent with this idea was the finding that NF90 does not associate as efficiently with *Il13* mRNA under HuR KO conditions. An earlier report showed that when HuR was overexpressed in myeloid cells, proinflammatory cytokine expression was suppressed (57). This result was a puzzling finding, since many of these cytokines are normally positively regulated by HuR. It was discovered that HuR overexpression lead to increases in TIA-1, a known translational repressor. TIA-1 suppressed the translation of many proinflammatory cytokines, including TNF-α (57). We assessed the mRNA levels of *TIA-1* and its related protein, TIAR, and found no differences in expression between knockout mice and control mice. A recent report by the same group supported the notion that HuR can repress the expression of proinflammatory cytokines. By using a myeloid-specific HuR knockout mouse, the authors showed that macrophages deficient in HuR had increased expression of many cytokines, including those that are regulated by HuR. Additionally, these mice developed colitis-associated cancer, further supporting the notion that normal HuR expression is required for homeostasis (58). Taken together, both our data and data from other labs indicate that the effect of a HuR knockout in complex animal models differs from cell lines. Additionally, the data obtained with our conditional knockout mouse model suggest that HuR-dependent and -independent control mechanisms of GATA-3 and Th2 cytokines may coexist. Consistent with this idea, we have recently demonstrated that HuR can positively or negatively control expression of the same genes in breast cancer cells depending on the cellular milieu (59,60).

Although the results obtained with the *ex vivo* polarization were surprising, they still implicate HuR as playing a role in regulating the expression of key genes involved in allergic airway inflammation. We induced allergic airway inflammation and assessed the disease phenotype. The BALF IL-4 and IL-13 levels were not significantly altered in the HuR KO mice, which could be due to several factors. About 80% of the cells infiltrating the lungs are eosinophils, which also make IL-13 (61,62). Therefore, increases in IL-13 expression in HuR-KO T cells may not contribute greatly to the overall IL-13 protein in the BALF. Reports indicate that IL-13 from both eosinophils and T cells is required for allergic airway inflammation, but the required contribution from each cell subset is unknown (63). Additionally, the recombination event that deletes HuR does not occur until after the process of polarization has begun (29,30). Therefore, it is possible that deletion of HuR earlier would have profoundly different effects. Indeed, our group has recently reported that using the identical OX40-*Cre* system to genetically ablate HuR does significantly impair Th17 differentiation and onset of neuroinflammation in an experimental autoimmune encephalitis model of multiple sclerosis (64). The mechanism is *Il17* mRNA destabilization in HuR KO T cells. In the future, use of other models that delete HuR at different stages of $CD4^+$ T-cell maturation will be important to determine if the timing of HuR deletion affects the phenotype.

Additionally, we histologically examined the lungs from immunized mice and found that the lungs displayed inflammation consistent with allergic airway in-

flammation. We also measured several parameters of lung function and found that in response to methacholine, HuR KO mice displayed significant airway hyperresponsiveness. These results were replicated when we immunized mice with substantially reduced amounts of OVA. In summary, mice with conditional deletion of HuR in activated T cells develop robust allergic airway inflammation.

CONCLUSION

In this study, we have uncovered a surprising and unexpected discovery whereby HuR negatively regulates the expression of IL-2, IL-4 and IL-13 in Th2 cells and in its absence leads to aberrant cytokine production. Furthermore, despite the absence of HuR in activated T cells, mice immunized and challenged with aerosolized OVA develop allergic airway inflammation, consistent with the *ex vivo* data, where T cells deficient in HuR do not have reductions in IL-4 or IL-13 as expected. Importantly, our results further support the notion that posttranscriptional gene regulation by HuR is complex and may be dose dependent. When HuR is removed from the cell, this alters binding of other *trans*-acting factors that affect transcript fate. Therefore, we propose that animal models of HuR deletion are critical for understanding the role of HuR in disease models, but underlying mechanisms are complex and further study is required.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants R01AI080870-01 and R21AI079341-01 (to U Atasoy). M Gorospe, K Abdelmohsen and JL Martindale were kindly supported by the National Institute on Aging Intramural Research Program, National Institutes of Health (NIA, IRP, NIH).

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. Zhu J, Yamane H, Paul WE. (2010) Differentiation of effector CD4 T cell populations. *Annu. Rev. Immunol.* 28:445–89.
2. Gutcher I, Becher B. (2007) APC-derived cytokines and T cell polarization in autoimmune inflammation. *J. Clin. Invest.* 117:1119–27.
3. Cheadle C, *et al.* (2005) Stability regulation of mRNA and the control of gene expression. *Ann. N. Y. Acad. Sci.* 1058:196–204.
4. Cheadle C, *et al.* (2005) Control of gene expression during T cell activation: alternate regulation of mRNA transcription and mRNA stability. *BMC Genomics.* 6:75.
5. Vavassori S, Covey LR. (2009) Post-transcriptional regulation in lymphocytes: the case of CD154. *RNA Biol.* 6:259–65.
6. Raghavan A, *et al.* (2004) Patterns of coordinate down-regulation of ARE-containing transcripts following immune cell activation. *Genomics.* 84:1002–13.
7. Raghavan A, *et al.* (2002) Genome-wide analysis of mRNA decay in resting and activated primary human T lymphocytes. *Nucleic Acids Res.* 30:5529–38.
8. Glisovic T, Bachorik JL, Yong J, Dreyfuss G. (2008) RNA-binding proteins and post-transcriptional gene regulation. *FEBS Lett.* 582:1977–86.
9. Pullmann R Jr, *et al.* (2007) Analysis of turnover and translation regulatory RNA-binding protein expression through binding to cognate mRNAs. *Mol. Cell. Biol.* 27:6265–78.
10. Anderson P. (2008) Post-transcriptional control of cytokine production. *Nat. Immunol.* 9:353–9.
11. Chen CY, Xu N, Shyu AB. (2002) Highly selective actions of HuR in antagonizing AU-rich element-mediated mRNA destabilization. *Mol. Cell. Biol.* 22:7268–78.
12. Atasoy U, Watson J, Patel D, Keene JD. (1998) ELAV protein HuA (HuR) can redistribute between nucleus and cytoplasm and is upregulated during serum stimulation and T cell activation. *J. Cell. Sci.* 111:3145–56.
13. Taylor GA, *et al.* (1996) A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity.* 4:445–54.
14. Bhattacharya S, Giordano T, Brewer G, Malter JS. (1999) Identification of AUF-1 ligands reveals vast diversity of early response gene mRNAs. *Nucleic Acids Res.* 27:1464–72.
15. Lai WS, Parker JS, Grissom SF, Stumpo DJ, Blackshear PJ. (2006) Novel mRNA targets for tristetraprolin (TTP) identified by global analysis of stabilized transcripts in TTP-deficient fibroblasts. *Mol. Cell. Biol.* 26:9196–208.
16. Meisner NC, Filipowicz W. (2011) Properties of the regulatory RNA-binding protein HuR and its role in controlling miRNA repression. *Adv. Exp. Med. Biol.* 700:106–23.
17. Kim HH, *et al.* (2009) HuR recruits let-7/RISC to repress c-Myc expression. *Genes Dev.* 23:1743–8.
18. Yamasaki S, Stoecklin G, Kedersha N, Simarro M, Anderson P. (2007) T-cell intracellular antigen-1 (TIA-1)-induced translational silencing promotes the decay of selected mRNAs. *J. Biol. Chem.* 282:30070–7.
19. Moulton VR, Kyttaris VC, Juang YT, Chowdhury B, Tsokos GC. (2008) The RNA-stabilizing protein HuR regulates the expression of zeta chain of the human T cell receptor-associated CD3 complex. *J. Biol. Chem.* 283:20037–44.
20. Ramgolam VS, *et al.* (2010) T cell LFA-1 engagement induces HuR-dependent cytokine mRNA stabilization through a Vav-1, Rac1/2, p38MAPK and MKK3 signaling cascade. *PLoS One.* 5:e14450.
21. Stellato C, *et al.* (2011) Coordinate regulation of GATA-3 and Th2 cytokine gene expression by the RNA-binding protein HuR. *J. Immunol.* 187:441–9.
22. Casolaro V, *et al.* (2008) Posttranscriptional regulation of IL-13 in T cells: role of the RNA-binding protein HuR. *J. Allergy Clin. Immunol.* 121:853–59, e854.
23. Yarovinsky TO, Butler NS, Monick MM, Hunninghake GW. (2006) Early exposure to IL-4 stabilizes IL-4 mRNA in CD4+ T cells via RNA-binding protein HuR. *J. Immunol.* 177:4426–35.
24. Bosnjak B, Stelzmueller B, Erb KJ, Epstein MM. (2011) Treatment of allergic asthma: modulation of Th2 cells and their responses. *Respir. Res.* 12:114.
25. Komai M, *et al.* (2003) Role of Th2 responses in the development of allergen-induced airway remodelling in a murine model of allergic asthma. *Br. J. Pharmacol.* 138:912–20.
26. Katsanou V, *et al.* (2009) The RNA-binding protein Elavl1/HuR is essential for placental branching morphogenesis and embryonic development. *Mol. Cell. Biol.* 29:2762–76.
27. Papadaki O, *et al.* (2009) Control of thymic T cell maturation, deletion and egress by the RNA-binding protein HuR. *J. Immunol.* 182:6779–88.
28. Galban S, *et al.* (2008) RNA-binding proteins HuR and PTB promote the translation of hypoxia-inducible factor 1alpha. *Mol. Cell. Biol.* 28:93–107.
29. Gutcher I, *et al.* (2011) Autocrine transforming growth factor-beta1 promotes in vivo Th17 cell differentiation. *Immunity.* 34:396–408.
30. Klinger M, *et al.* (2009) Thymic OX40 expression discriminates cells undergoing strong responses to selection ligands. *J. Immunol.* 182:4581–9.
31. Seko Y, Azmi H, Fariss R, Ragheb JA. (2004) Selective cytoplasmic translocation of HuR and site-specific binding to the interleukin-2 mRNA are not sufficient for CD28-mediated stabilization of the mRNA. *J. Biol. Chem.* 279:33359–67.
32. Meisner NC, *et al.* (2004) mRNA openers and closers: modulating AU-rich element-controlled mRNA stability by a molecular switch in mRNA secondary structure. *Chembiochemistry.* 5:1432–47.
33. Galban S, Gorospe M. (2009) Factors interacting with HIF-1alpha mRNA: novel therapeutic targets. *Curr. Pharm. Des.* 15:3853–60.
34. Whitehead GS, Walker JK, Berman KG, Foster WM, Schwartz DA. (2003) Allergen-induced airway disease is mouse strain dependent. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 285:L32–42.

35. Frush S, *et al.* (2011) The role of the extracellular matrix protein mindin in airway response to environmental airways injury. *Environ. Health Perspect.* 119:1403–8.
36. Li Z, Potts-Kant EN, Garantziotis S, Foster WM, Hollingsworth JW. (2011) Hyaluronan signaling during ozone-induced lung injury requires TLR4, MyD88, and TIRAP. *PLoS One.* 6:e27137.
37. Yamane H, Zhu J, Paul WE. (2005) Independent roles for IL-2 and GATA-3 in stimulating naive CD4+ T cells to generate a Th2-inducing cytokine environment. *J. Exp. Med.* 202:793–804.
38. Cote-Sierra J, *et al.* (2004) Interleukin 2 plays a central role in Th2 differentiation. *Proc. Natl. Acad. Sci. U. S. A.* 101:3880–5.
39. Zhu J, Cote-Sierra J, Guo L, Paul WE. (2003) Stat5 activation plays a critical role in Th2 differentiation. *Immunity.* 19:739–48.
40. Guo L, *et al.* (2002) In TH2 cells the Il4 gene has a series of accessibility states associated with distinctive probabilities of IL-4 production. *Proc. Natl. Acad. Sci. U. S. A.* 99:10623–8.
41. Zhu J, Guo L, Watson CJ, Hu-Li J, Paul WE. (2001) Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. *J. Immunol.* 166:7276–81.
42. Yagi R, Zhu J, Paul WE. (2011) An updated view on transcription factor GATA3-mediated regulation of Th1 and Th2 cell differentiation. *Int. Immunol.* 23:415–20.
43. Zhu J, Yamane H, Cote-Sierra J, Guo L, Paul WE. (2006) GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. *Cell Res.* 16:3–10.
44. Chang N, *et al.* (2010) HuR uses AUF1 as a cofactor to promote p16INK4 mRNA decay. *Mol. Cell. Biol.* 30:3875–86.
45. Meisner NC, Filipowicz W. (2010) Properties of the regulatory RNA-binding protein HuR and its role in controlling miRNA repression. *Adv. Exp. Med. Biol.* 700:106–23.
46. Guo X, Wu Y, HartleyRS. (2009) MicroRNA-125a represses cell growth by targeting HuR in breast cancer. *RNA Biol.* 6:575–83.
47. Leung AK, Sharp PA. (2007) microRNAs: a safeguard against turmoil? *Cell.* 130:581–5.
48. Leung AK, Sharp PA. (2006) Function and localization of microRNAs in mammalian cells. *Cold Spring Harb. Symp. Quant. Biol.* 71:29–38.
49. Kumar M, *et al.* (2011) Let-7 microRNA-mediated regulation of IL-13 and allergic airway inflammation. *J. Allergy Clin. Immunol.* 128:1077–1085.e10.
50. Abate-Shen C, Shen MM. (2005) An unusual gene dosage effect of p27kip1 in a mouse model of prostate cancer. *Cell Cycle.* 4:e45–7.
51. Gao H, *et al.* (2004) A critical role for p27kip1 gene dosage in a mouse model of prostate carcinogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 101:17204–9.
52. Cohen JC, Lundblad LK, Bates JH, Levitzky M, Larson JE. (2004) The "Goldilocks effect" in cystic fibrosis: identification of a lung phenotype in the cftr knockout and heterozygous mouse. *BMC Gen.* 5:21.
53. Shen R, Kaplan MH. (2002) The homeostasis but not the differentiation of T cells is regulated by p27(Kip1). *J. Immunol.* 169:714–21.
54. Kullmann M, Gopfert U, Siewe B, Hengst L. (2002) ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5'UTR. *Genes Dev.* 16:3087–99.
55. Ziegeler G, *et al.* (2010) Embryonic lethal abnormal vision-like HuR-dependent mRNA stability regulates post-transcriptional expression of cyclin-dependent kinase inhibitor p27Kip1. *J. Biol. Chem.* 285:15408–19.
56. Lopez de Silanes I, Zhan M, Lal A, Yang X, Gorospe M. (2004) Identification of a target RNA motif for RNA-binding protein HuR. *Proc. Natl. Acad. Sci. U. S. A.* 101:2987–992.
57. Katsanou V, *et al.* (2005) HuR as a negative posttranscriptional modulator in inflammation. *Mol. Cell.* 19:777–89.
58. Yiakouvaki A, *et al.* (2012) Myeloid cell expression of the RNA-binding protein HuR protects mice from pathologic inflammation and colorectal carcinogenesis. *J. Clin. Invest.* 122:48–61.
59. Calaluce R, *et al.* (2010) The RNA binding protein HuR differentially regulates unique subsets of mRNAs in estrogen receptor negative and estrogen receptor positive breast cancer. *BMC Cancer.* 10:126.
60. Gubin MM, *et al.* (2010) Overexpression of the RNA binding protein HuR impairs tumor growth in triple negative breast cancer associated with deficient angiogenesis. *Cell Cycle.* 9:3337–46.
61. Nials AT, Uddin S. (2008) Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis. Models Mech.* 1:213–20.
62. Jacobsen EA, *et al.* (2008) Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells. *J. Exp. Med.* 205:699–710.
63. Walsh ER, *et al.* (2011) Computational and experimental analysis reveals a requirement for eosinophil-derived IL-13 for the development of allergic airway responses in C57BL/6 mice. *J. Immunol.* 186:2936–49.
64. Chen J, *et al.* (2013) Posttranscriptional gene regulation of IL-17 by the RNA-binding protein HuR is required for initiation of experimental autoimmune encephalomyelitis. *J. Immunol.* 191:5441–50.