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L-Arginine Deprivation Regulates Cyclin D3 mRNA Stability in Human T Cells by Controlling HuR Expression

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Myeloid-derived suppressor cells are a major mechanism of tumor-induced immune suppression in cancer. Arginase I-producing myeloid-derived suppressor cells deplete L-arginine (L-Arg) from the microenvironment, which arrests T cells in the G₀–G₁ phase of the cell cycle. This cell cycle arrest correlated with an inability to increase cyclin D3 expression resulting from a decreased mRNA stability and an impaired translation. We sought to determine the mechanisms leading to a decreased cyclin D3 mRNA stability in activated T cells cultured in medium deprived of L-Arg. Results show that cyclin D3 mRNA instability induced by L-Arg deprivation is dependent on response elements found in its 3'-untranslated region (UTR). RNA-binding protein HuR was found to be increased in T cells cultured in medium with L-Arg and bound to the 3'-untranslated region of cyclin D3 mRNA *in vitro* and endogenously in activated T cells. Silencing of HuR expression significantly impaired cyclin D3 mRNA stability. L-Arg deprivation inhibited the expression of HuR through a global arrest in *de novo* protein synthesis, but it did not affect its mRNA expression. This alteration is dependent on the expression of the amino acid starvation sensor general control nonderepressible 2 kinase. These data contribute to an understanding of a central mechanism by which diseases characterized by increased arginase I production may cause T cell dysfunction. *The Journal of Immunology*, 2010, 185: 5198–5204.

L-arginine (L-Arg) is a nonessential amino acid that plays a central role in regulating several biological systems, including the immune response (1, 2). L-Arg levels are profoundly reduced in patients with cancer or in severe trauma by the excess production of arginase I in myeloid-derived suppressor cells (MDSCs) (3–6). This results in an impaired cytokine production and an arrest in T cell proliferation, which leads to T cell anergy (5, 7). We recently showed that activated primary T cells cultured in medium deprived of L-Arg were arrested in the G₀–G₁ phase of the cell cycle (8). The G₀–G₁ arrest in the cell cycle observed in T cells cultured in L-Arg–deprived medium correlated with an inability to upregulate the expression of cyclin D3 (8). This was the result of a decreased cyclin D3 mRNA stability and a diminished cyclin D3 translation. The arrest in cyclin D3 protein synthesis by L-Arg deprivation was triggered by the general control nonderepressible 2 (GCN2) kinase (8) and the subsequent phosphorylation of eukaryotic translation initiation factor 2 (eIF2) α (9). However, the mechanisms to explain the decrease in cyclin D3 mRNA stability induced by L-Arg starvation are still unknown.

The posttranscriptional regulation of mRNAs in T cells may account as much as 50% of all changes in gene expression (10, 11). The posttranscriptional fate of a given mRNA is governed in part by its interaction with specific *trans*-acting factors such as RNA-binding proteins (RBPs) (12). Several RBPs have been identified to promote AU-rich element (ARE) mRNA decay, including AUF1 (also named hnRNP D) and tristetraprolin (TTP) (13–15). In contrast, some other RBPs, including HuR protein, also known as ELAV-like 1 or HuA, promote mRNA stability (16, 17). Recombinant HuR has been shown to stabilize ARE-containing transcripts *in vitro* (18) and *in vivo* (19).

Results shown in this study demonstrate that the decrease in cyclin D3 mRNA stability induced by L-Arg deprivation is dependent on elements within the 3'-UTR of the cyclin D3 mRNA. The RBP HuR binds to the cyclin D3 mRNA *in vitro* and endogenously in activated T cells cultured with L-Arg, but not in L-Arg–deprived T cells. Silencing of HuR expression significantly impaired cyclin D3 mRNA stability. Interestingly, L-Arg deprivation impaired the expression of HuR through mechanisms that arrest global protein synthesis. These mechanisms are triggered by the expression of GCN2 kinase and are associated with the phosphorylation of eIF2 α . Therefore, these results contribute to an understanding of the central mechanism by which cancer and other diseases characterized by high arginase I production and low levels of L-Arg may cause T cell dysfunction.

Materials and Methods

Cells, cultures, and chemicals

Human PBMCs were obtained from healthy donor buffy coats. T cells were purified using human T cell-enrichment columns (R&D Systems, Minneapolis, MN), following the vendor's recommendations. T cell purity was tested by CD3 ϵ expression and ranged from 95 to 98%. RPMI 1640 or L-Arg–free RPMI (Invitrogen Life Technologies Grand Island, NY) were supplemented with 5% FBS (HyClone Laboratories, Logan, UT), 25 mM HEPES (Invitrogen Life Technologies), 4 mM L-glutamine (Cambrex, East Rutherford, NJ), and 100 U/ml penicillin-streptomycin (Invitrogen Life Technologies). The final concentration of L-Arg in the FBS-supplemented standard RPMI 1640 is 1040 μ M, while in the FBS-supplemented L-Arg–

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Abbreviations used in this paper: ARE, AU-rich element; Cyto, cytoplasmic protein extracts; eIF2, eukaryotic translation initiation factor 2; GCN2, general control nonderepressible 2; L-Arg, L-arginine; MDSC, myeloid-derived suppressor cell; ORF, open reading frame; RBP, RNA-binding protein; siRNA, small interfering RNA; TTP, tristetraprolin; UTR, untranslated region.

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free RPMI it is 7.5 μ M. For simplicity, we will use the term L-Arg–deprived RPMI to describe the medium containing the very little amounts of L-Arg. Stimulation of T lymphocytes was done with immuno-immobilized anti-CD3 plus anti-CD28 as previously described (8). T cells isolated from GCN2 knockout mice (provided by Dr. David Munn, Medical College of Georgia, Augusta, GA) were purified by negative selection (R&D Systems) and activated with plate-bound anti-CD3 (2 μ g/ml) plus anti-CD28 (1 μ g/ml) (BD Biosciences, San Jose, CA).

Abs and immunoprecipitation

Abs against HuR (Santa Cruz Biotechnology, Santa Cruz, CA, or Zymed Laboratories/Invitrogen, Carlsbad, CA), TTP (BioSource International/Invitrogen, Carlsbad, CA), AUF1, cyclin D3, lamin B1 (Santa Cruz Biotechnology), actin (Sigma-Aldrich, St. Louis, MO), phospho-eIF2 α , eIF2 α , phospho-GCN2, GCN2 (Cell Signaling Technology, Beverly, MA), and GAPDH (Fitzgerald Industries, Concord, MA) were used in this study. Whole-cell extracts were prepared using lysis buffer (50 mM HEPES [pH 7.2], 250 mM NaCl, 5 mM EDTA, 0.5 mM DTT 0.5, 0.1% Nonidet P-40) and a mix of protease inhibitors including 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 100 μ g/ml trypsin-chymotrypsin inhibitor. Cytoplasmic cell lysates from T cells were prepared by resuspending the cells in cytoplasmic lysis buffer containing 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 0.5% Triton X-100, 2 mM PMSF, and the protease inhibitors mixture. To obtain nuclear extracts, pellets from cytoplasmic isolations were resuspended in nuclear lysis buffer containing 0.5 M KCl, 25 mM HEPES (pH 7.2), 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, and the protease inhibitors mixture. For Western blotting experiments, 25 μ g of whole-cell extract was electrophoresed on 8, 10, or 12% Tris-Gly gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes (Invitrogen). Membrane-bound immune complexes were detected by using an ECL Western blotting detection system (Amersham Biosciences, Piscataway, NJ), followed by exposure to BioMax MR films (Kodak, Rochester, NY).

Immunoprecipitation assays were done using 300 μ g of T cell lysates, as previously described (8). For RNA binding assays, mRNA was isolated after immunoprecipitation, treated with DNase I (Invitrogen), and converted into cDNA using SuperScript II reverse transcriptase (Invitrogen). As input controls, mRNA was also isolated from whole cells. PCR reactions using recombinant Taq polymerase (Invitrogen) were done to determine the expression of cyclin D3 mRNA expression by RT-PCR (PCR product, 348 bases) using the following primers: cyclin D3, 5'-AAGG-TGTTGTCCTTCTAGG and 3'-AACAGCAACCACCAGGGTTA; Actin 5'-TGACGGGGTACCCACACTGTGCCCA and 3'-CTAGAAGCATTT-GCGGTGGAC GATG.

Transfection of cells with cyclin D3 open reading frame or cyclin D3 cDNA

Cyclin D3-negative cell line EBV-Em, generated postinfection of mononuclear cells with EBV, was transfected with either a plasmid coding for the cyclin D3 cDNA (NM_001760) or another coding only for the cyclin D3 open reading frame (ORF) (DQ891748). Sequences coding for cyclin D3 cDNA and cyclin D3 ORF were excised from the parental plasmids (Open Biosystems, Huntsville, AL), ligated to XhoI and HindIII oligonucleotides, and subcloned into linearized phospho-enhanced GFP plasmid (Clontech, Mountain View, CA). Direction and sequence of the cyclin D3 inserts into the pEGFP plasmid vector were confirmed by sequencing and by restriction endonuclease map analysis. Cells were transfected using Lipofectamine 2000 (Invitrogen) and 5 μ g of the corresponding plasmid, following the vendor's recommendations. Stable transfected cells were obtained by culturing the cells in the presence of 500 μ g/ml Geneticin (Invitrogen). Individual cyclin D3-expressing clones were further obtained by limiting dilutions and were tested for cyclin D3. Clones expressing similar levels of cyclin D3 were selected for the experiments. To determine mRNA stability, transfected cells were cultured for 12 h in the presence or the absence of L-Arg. The transcription inhibitor actinomycin D (5 μ g/ml) (Sigma-Aldrich) was then added and mRNAs were collected after 2, 4, and 6 h and tested for cyclin D3 mRNA expression using Northern blot.

Real-time PCR

Two micrograms of total RNA isolated from activated T cells cultured in the presence or the absence of L-Arg was treated with DNase I (Invitrogen) and converted into cDNA using a SuperScript kit (Invitrogen). cDNA was used for the amplification of the human HuR and GCN2 mRNA using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). Housekeeping controls included the human large ribosomal protein and the human cyclophilin A (Applied Biosystems). The real-time PCR reaction was

conducted in a 7900HT real-time PCR machine (Applied Biosystems). The results of the PCR were analyzed with the 7900HT sequence detection software version 2.2 (Applied Biosystems) and are expressed as the ratio of the HuR or GCN2 cycle threshold (C_T) to the C_T of the housekeeping controls.

In vitro transcription

A fragment coding for the 3'-UTR of cyclin D3 mRNA (bases 1419–1768), which contains the three cyclin D3 AREs (1470, 1603 and 1679), was amplified by PCR using primers 5'-AAGGTGTTGCCCTTCTAGG and 3'-AACAGCAACCACCAGGGTTA. Additionally, a control sequence coding for a fragment of GAPDH ORF (bases 115–557) was amplified by PCR using primers 5'-AAGGTCGGAGTCAACGGATT and 3'-CAGGAGGCA-TTGCTGATGAT. PCR amplification was performed using as template plasmids containing the cyclin D3 or the GAPDH cDNA. The PCR product was then cloned into pGEM-T Easy plasmid (Promega, Madison, WI) downstream of the T7 promoter. Plasmid containing the coding sequence for cyclin D3 3'-UTR was linearized with SpeI, electrophoresed in a 1% agarose gel, and purified using StrataPrep isolating kit (Stratagene, La Jolla, CA). The isolated linearized fragment was used to synthesize the radiolabeled or unlabeled RNA in vitro by using a T7 Riboprobe in vitro transcription kit (Promega) with or without 50 μ Ci [α - 32 P]rCTP, following the vendor's recommendations. The integrity of the mRNA was confirmed by running the reaction product in a denaturing 5% PAGE gel (8 M urea).

In vitro RNA mobility shift assays

RNA-protein binding reactions were carried out using a variation of the method described by Leibold and Munro (20). Binding reactions were performed using 10 μ g of cytoplasmic extract and 15 pg of [32 P]-labeled RNA probe in 30 μ l of buffer D (10 mM HEPES [pH 7.6], 3 mM MgCl₂, 40 mM KCl, 2% glycerol, 1 mM DTT, and 5 mg/ml heparin). Reactions were incubated for 20 min at 30°C and immediately run in 5% non-denaturing PAGE gels. Autoradiography was performed at -80°C. Specificity of the reaction was determined by adding excess of unlabeled in vitro transcribed cyclin D3 or GAPDH mRNA (15 or 150 pg). Blocking assays were done using 4 μ g of the Abs against HuR or TTP.

[35 S]methionine pulse analysis

Primary T lymphocytes were isolated and activated in the presence or the absence of L-Arg. After 48 h of activation, the cells were washed four times in L-methionine-free RPMI 1640. Cells were then seeded at a density of 2×10^6 cells/ml in L-methionine-free RPMI 1640 or L-Arg/L-methionine-free RPMI 1640, incubated for 20 min, and then pulsed with 0.3 mCi of [35 S]methionine for 2 h. Cells were washed twice with PBS, and immunoprecipitations with HuR Ab were performed as described above.

Isolation of polysomes

Cytoplasmic extracts harvested from activated T cells cultured in the presence or the absence of L-Arg were layered onto 10–50% sucrose gradients prepared on polysome lysis buffer (50 mM Tris-HCl [pH 7.2], 150 mM KCl, 5 mM MgCl₂, 1 mM DTT) containing 100 μ g/ml cycloheximide. Gradients were spun at 35,000 rpm for 160 min at 4°C using an SW 41Ti rotor (Beckman Coulter, Palo Alto, CA), as previously described (21). Gradients of 1 ml were fractionated using a density gradient fractionator, and the polysome profile was monitored by absorbance at 260 nm (22). RNA from the different fractions was obtained using phenol/chloroform, quantified, and tested for the presence of HuR mRNA by Northern blot.

HuR silencing in primary T cells

HuR was silenced in primary T cells using small interfering RNA (siRNA), and cells were transfected by electroporation, as previously described (23). Briefly, 300 nmol of a pool of three different siRNAs directed against HuR labeled with FAM (sense I, 5'-GGAUGAGUUACGAAGCCUGtt-3', anti-sense I, 5'-CAGGCUUCGUAACUCAUCctg-3'; sense II, 5'-GGAGUU-GAAACUUUUCUUGtt-3', anti-sense, II 5'-CAAGAAAAGUUUACAACU-Cctt-3'; sense III, 5'-CGUAGAAGACAUGUUCUCUtt-3', anti-sense III, 5'-AGAGAACAUGUCUUCUACGtc-3') purchased from Ambion (Austin, TX) were added to prechilled 0.4-cm electrode gap cuvettes (Bio-Rad, Hercules, CA). T cells were resuspended using Opti-MEM I to 3×10^6 cells/ml, added to the cuvettes, mixed, and pulsed once at 300 mV, 975 μ F with a Gene Pulser electroporator II (Bio-Rad). Transfections using an irrelevant FAM-labeled siRNA (Ambion) were used as controls. Cell viability immediately after electroporation was typically ~90%. Cells were plated into 6-well culture plates and incubated at 37°C in a humidified 5% CO₂ chamber overnight. FAM-positive T cells were sorted next day by flow

cytometry (FACSria cell sorting system, BD Biosciences) and then activated with anti-CD3 plus anti-CD28. The percentage of FAM-positive cells ranged from 35 to 41% before sorting and from 95 to 97% after sorting.

Results

3'-UTR cyclin D3 mRNA is responsible for the low cyclin D3 mRNA half-life seen in L-Arg-deprived T cells

To determine the role of the cyclin D3 3'-UTR on the cyclin D3 mRNA instability induced by the L-Arg deprivation, the cyclin D3-negative cell line EBV-Em was transfected with plasmids coding for the cyclin D3 cDNA (contains the ORF and the 3'-UTR) or coding only for the cyclin D3 ORF (which lacks 3'-UTR). Clones of EBV-Em cells transfected with the cyclin D3 cDNA or the cyclin D3 ORF showing similar increased expression of cyclin D3 protein were used for the experiments (Fig. 1A). The EBV cells transfected with the cyclin D3 cDNA showed a decreased cyclin D3 mRNA stability when cultured in the absence of L-Arg, as compared with the same cells cultured in the presence of L-Arg (Fig. 1B). In contrast, cyclin D3 mRNA half-life was not affected by L-Arg deprivation in cells transfected with the cyclin D3 ORF, suggesting that the 3'-UTR of the cyclin D3 mRNA is responsible for the cyclin D3 instability induced by the deprivation of L-Arg.

HuR binds to the cyclin D3 mRNA in the presence of L-Arg

We hypothesized that the reason why cyclin D3 mRNA is more stable in T cells cultured in the presence of L-Arg is because a RBP is binding to the 3'-UTR of the cyclin D3 mRNA. An *in vitro*-transcribed [α - 32 P]rCTP-labeled cyclin D3 3'-UTR mRNA was incubated with cytoplasmic extracts harvested from activated T cells cultured in the presence or the absence of L-Arg, and RNA-protein complexes were analyzed by electrophoresis. A major retardation in the migration of the radiolabeled cyclin D3 3'-UTR mRNA was observed after mixture with protein extracts from activated T cells cultured in the presence of L-Arg (Fig. 2A). In

contrast, the complex was absent when radiolabeled cyclin D3 3'-UTR mRNA was mixed with extracts obtained from nonactivated T cells or activated T cells cultured in the L-Arg-deprived medium. The specificity of the binding was confirmed by the addition of excess nonradioactive cyclin D3 3'-UTR mRNA as competitor, which completely blocked the binding of the extract to the radiolabeled mRNA. In contrast, the addition of excess of an unrelated transcript did not block the binding of the protein to the radiolabeled cyclin D3 3'-UTR mRNA.

RBP HuR has been identified to promote mRNA stability (16, 17), whereas several other RBPs, including AUF1 and TTP, promote mRNA decay (13–15). Activated T cells cultured in the presence of L-Arg display an increased expression of HuR and TTP in whole-cell protein extracts as compared with L-Arg-deprived T cells (Fig. 2B). In contrast, AUF1 expression did not change in T cells cultured with or without L-Arg. The binding of HuR and TTP to the cyclin D3 mRNA *in vitro* was then tested. The addition of a blocking Ab against HuR, but not against TTP, completely prevented the formation of the protein-RNA complex (Fig. 2C), suggesting that HuR, but not TTP, was binding to the cyclin D3 mRNA *in vitro*. To determine whether HuR was binding to the cyclin D3 mRNA endogenously in T cells, HuR was immunoprecipitated from cytoplasmic extracts harvested from T cells cultured in the presence or absence of L-Arg, the mRNA was isolated, and cyclin D3 mRNA tested by RT-PCR. Cyclin D3 mRNA expression was detected in cytoplasmic extracts and in HuR immunoprecipitates from T cells cultured in the presence of L-Arg (Fig. 2D). Conversely, we detected cyclin D3 mRNA in cytoplasmic extracts from cells cultured in medium deprived of L-Arg, but this was not found in HuR immunoprecipitates, suggesting that L-Arg deprivation prevents the formation of HuR-cyclin D3 mRNA complex endogenously in activated T cells. Furthermore, TTP immunoprecipitates from both conditions do not contain cyclin D3 mRNA, suggesting that TTP is not binding to the cyclin D3 mRNA endogenously in activated T cells.

After activation of T cells, HuR ubiquitously expressed in the nucleus binds to the target mRNA and shuttles it to the cytoplasm (24). Activated T cells cultured in the presence of L-Arg had a higher expression of HuR in both cytoplasm and nucleus, as compared with activated T cells cultured in medium deprived of L-Arg (Fig. 3).

HuR silencing impairs cyclin D3 mRNA stability

If HuR is controlling the cyclin D3 mRNA stability in T cells cultured in media containing L-Arg, then silencing of HuR mRNA expression should induce a decrease in cyclin D3 mRNA stability. Activated T cells transfected with siRNA against HuR display a shorter cyclin D3 mRNA stability, as compared with the same cells transfected with a nonrelevant siRNA or untransfected cells (Fig. 4A). Additionally, silencing of HuR expression leads to a decreased expression of cyclin D3 protein (Fig. 4B).

L-Arg deprivation impairs cyclin D3 mRNA stability in a GCN2 kinase-dependent manner

We then investigated the mechanisms by which the deprivation of L-Arg impaired the expression of HuR in primary T cells. A similar increase in HuR mRNA expression was found in activated T cells cultured with and without L-Arg (Fig. 5A). However, HuR protein synthesis, tested by pulse-chase analysis, was significantly impaired in L-Arg-deprived activated T cells, as compared with cells cultured with L-Arg (Fig. 5B). Localization of mRNAs in heavy polysomes is a characteristic of active translation. Accordingly, HuR mRNA was located in heavy polysomes of activated T cells cultured in the presence of L-Arg, whereas it was located in lighter

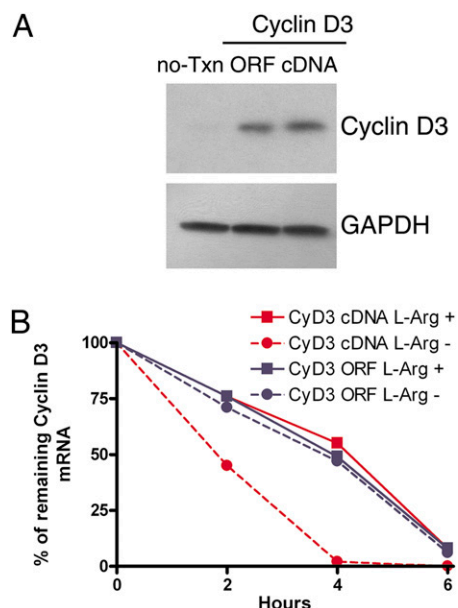


FIGURE 1. Cyclin D3 3'-UTR is responsible for the low cyclin D3 mRNA stability found in L-Arg-deprived T cells. *A*, Similar expression of cyclin D3 in selected clones of EBV cells transfected with cyclin D3 ORF or cyclin D3 cDNA plasmids. *B*, EBV cells stably transfected with cyclin D3 ORF or cyclin D3 cDNA plasmids were cultured for 12 h in medium containing or deprived of L-Arg, after which actinomycin D was added, and cyclin D3 mRNA half-life was tested by Northern blot. Values are from three similar experiments.

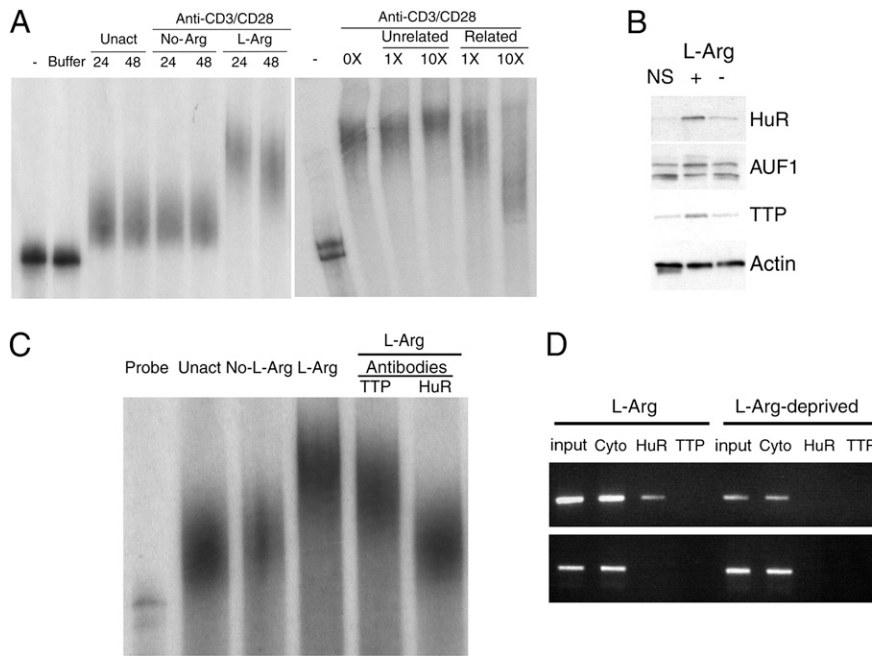


FIGURE 2. HuR binds to the 3'-UTR of cyclin D3 mRNA only in T cells cultured in the presence L-Arg. *A*, RNA-binding shift assay, where cell extracts from T cells cultured in medium with and without L-Arg for 24 and 48 h were mixed with an in vitro-transcribed radiolabeled 3'-UTR cyclin D3 mRNA. Specificity of the reaction was determined by adding an excess (15 or 150 pg) of unlabeled in vitro-transcribed cyclin D3 3'-UTR mRNA (related) or GAPDH mRNA (unrelated) to the protein extracts of activated T cells cultured with L-Arg and radiolabeled 3'-UTR cyclin D3 mRNA. *B*, Expression of HuR, AUF1, and TTP in whole-cell extracts from T cells cultured for 48 h in medium with or without L-Arg by Western blot. *C*, Blocking Abs against TTP and HuR were added before the mix with the in vitro-transcribed mRNA. *D*, Activated T cells were cultured in the presence or the absence of L-Arg for 48 h, after which mRNA was isolated from cells (input), cytoplasmic protein extracts (Cyto), and after immunoprecipitation of HuR or TTP. The mRNA was then tested for the expression of cyclin D3 mRNA using RT-PCR. Values are from three similar experiments.

polysomes of activated T cells deprived of L-Arg (Fig. 5C). We tested whether the inhibition of HuR translation by low levels of L-Arg was specific or part of a global arrest in translation. Measurements of total mRNA content in the different sucrose-polysome fractions showed that mRNAs accumulated in heavy polysomes in activated T cells cultured in normal medium, whereas they accumulated in lighter polysomes in L-Arg-deprived T cells (Fig. 5D). These data confirm that the decrease in HuR translation induced by the L-Arg deprivation is the result of a global arrest in the de novo protein synthesis.

Under starvation of amino acids conditions, an arrest in translation is initiated by the activation of GCN2 kinase, which phos-

phorylates translation initiation factor eIF2 α . Accordingly, activated T cells cultured in medium containing L-Arg show a rapid dephosphorylation of eIF2 α noticed as early as 2 h, whereas L-Arg-deprived T cells maintained high levels of phospho-eIF2 α during the culture times (Fig. 6A). Additionally, we found that L-Arg starvation induced the phosphorylation of GCN2 after 24 h of culture, but it did not impair GCN2 mRNA levels (Fig. 6B). To further test the role of GCN2 in the regulation of HuR expression and consequently in cyclin D3 mRNA stability, T cells from

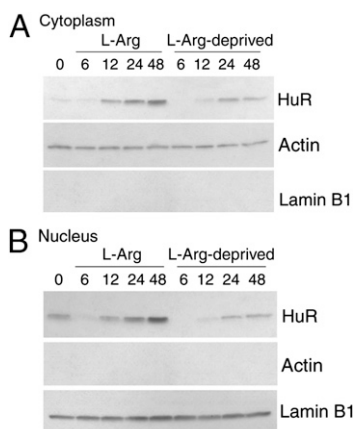


FIGURE 3. L-Arg deprivation impairs HuR expression in activated T cells. Representative experiment from three similar experiments showing the cytoplasmic (*A*) and nuclear (*B*) expression of HuR in activated T cells cultured with and without L-Arg by Western blot.

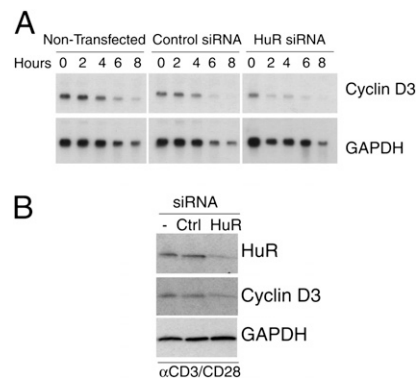


FIGURE 4. HuR silencing impairs cyclin D3 mRNA stability and expression. *A*, Primary T cells were transfected by electroporation with FAM-labeled siRNA against HuR or nonhomologous sequences (Control siRNA). FAM-positive cells were sorted, activated for 24 h in medium with or without L-Arg, and tested for cyclin mRNA stability using Northern blot. *B*, After 24 h of activation, transfected T cells were tested for the expression of HuR and cyclin D3 by Western blot. The experiment was repeated a minimum of three times, with similar results having been obtained.

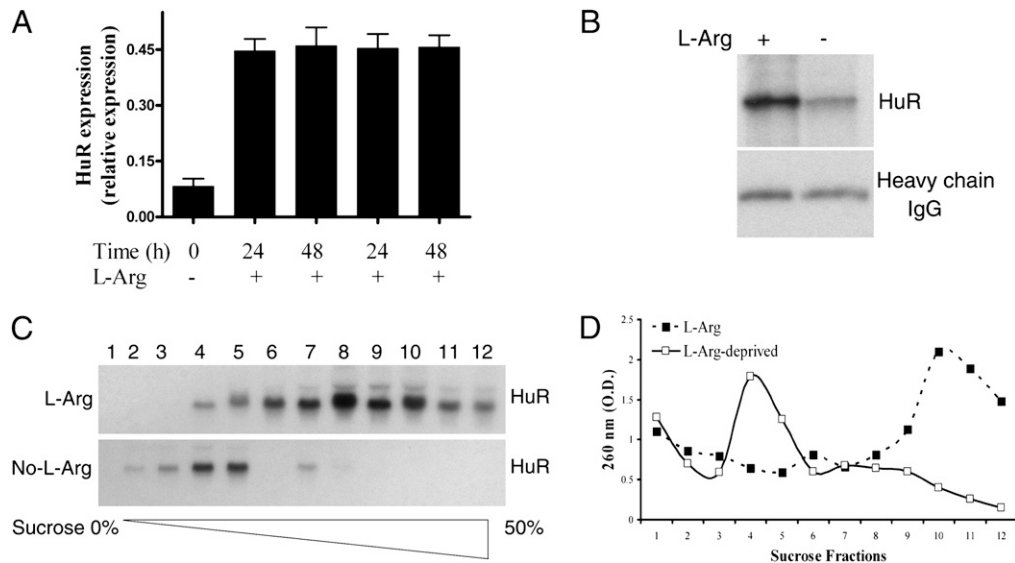


FIGURE 5. L-Arg deprivation inhibits HuR expression by impairing global translation. *A*, Detection of HuR mRNA levels using real-time PCR after culture of cells in medium with or without L-Arg for 24 and 48 h. *B*, Pulse-chase analysis for HuR in activated T cells cultured in the presence or absence of L-Arg for 48 h. *C* and *D*, HuR mRNA and total mRNA levels were tested in polysomes isolated using sucrose gradients from activated T cells cultured in the presence or absence of L-Arg for 48 h. The experiment was repeated a minimum of three times, with similar results having been obtained.

GCN2 knockout were activated, cultured in the L-Arg–deprived medium, and HuR expression and cyclin D3 mRNA stability were tested. Activated T cells from GCN2 knockout mice, but not from wild-type mice, increased cytoplasmic HuR expression when cultured in medium deprived of L-Arg (Fig. 6*C*). Subsequently, T cells from GCN2 knockout mice have a similar cyclin D3 mRNA half-life when cultured in the presence or absence of L-Arg (Fig. 6*D*). In contrast, cyclin D3 mRNA stability was significantly impaired when wild-type T cells were cultured in medium deprived of L-Arg.

Discussion

Metabolism of L-Arg by arginase I-producing MDSCs leads to a significant decrease in the extracellular levels of L-Arg in murine tumor models and in patients with cancer (5, 25). The decreased levels of L-Arg induced the prolonged loss in the expression of CD3 ζ (7, 26) and inhibited T cell proliferation (8). These

effects were not associated with the induction of apoptosis and were rapidly reversible after replenishment of L-Arg or citrulline (8). We recently showed that activated primary T cells cultured in the absence of L-Arg were arrested in the G₀–G₁ phase of the cell cycle (8). The G₀–G₁ arrest in the cell cycle observed in L-Arg–deprived T cells correlated with an inability to upregulate the expression of cyclin D3 (8). Results from cyclin D3 knockout mice had demonstrated that cyclin D3 is essential for the maturation of T cells in the thymus (27), and they suggested a potential and selective role in T cell proliferation. Additionally, silencing of cyclin D3 induced a similar inhibition of proliferation as that induced by L-Arg starvation (8).

The expression of cyclin D3 was impaired by limiting amounts of L-Arg through transcriptional, posttranscriptional, and translational mechanisms (8). How amino acid availability decreases cyclin D3 mRNA stability was still unclear. We found that cyclin D3 3'-UTR plays a relevant role in the instability of cyclin D3

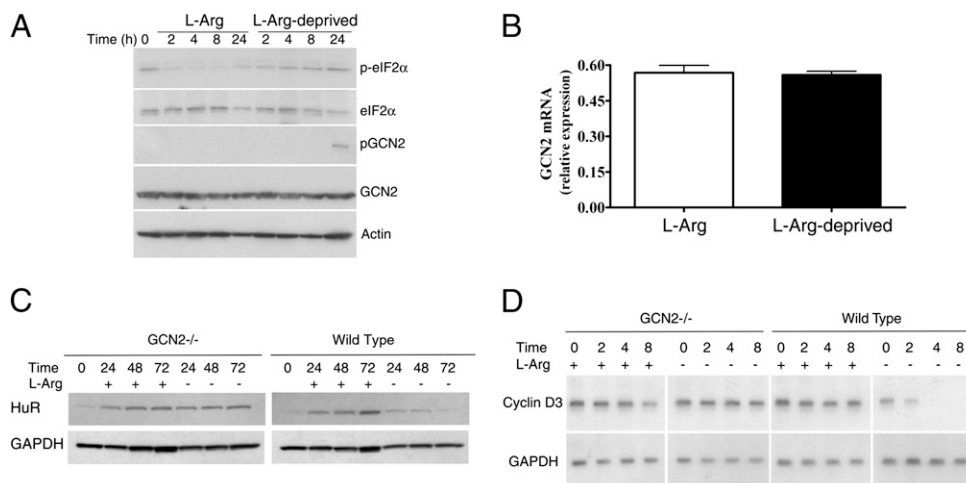


FIGURE 6. GCN2 mediates changes in cyclin D3 mRNA stability caused by L-Arg deprivation. *A*, Detection of phospho-eIF2 α and phospho-GCN2 in T cells cultured in medium with or without L-Arg. *B*, GCN2 mRNA levels in activated T cells cultured for 24 h in the presence or starvation of L-Arg. HuR expression (*C*) and cyclin D3 mRNA stability (*D*) were tested in activated T cells from GCN2 knockout and wild-type mice, cultured with or without L-Arg, by Western blot and Northern blot, respectively. The experiments were repeated three times, with similar results having been obtained.

mRNA induced by L-Arg deprivation. Human cyclin D3 3'-UTR contains several sites that could potentially mediate stability of the mRNA, including three different AREs starting in nucleotides 1470, 1603, and 1679. However, the role of these specific regions regulating cyclin D3 mRNA stability in the absence of L-Arg is still unknown. Among the cellular proteins that have been shown to bind and to modify mRNA stability are the RBPs HuR and TTP (17, 28). Increased expression of HuR has led to stabilization of ARE-containing reporter transcripts (19), whereas overexpression of TTP has led to destabilization of reporter constructs (29). We have found an increased expression of both RBPs in T cells cultured in the presence of L-Arg as compared with L-Arg-deprived T cells. However, our results indicate that HuR, but not TTP, binds to the cyclin D3 3'-UTR in vitro and endogenously in T cells cultured in the presence of L-Arg. Similar results were obtained by Raghavan et al. (28), who found that these proteins have different RNA binding specificities in T cells and may participate in opposite pathways by binding different target mRNAs. In this study, we have not determined the specific mechanisms by which the lack of binding of HuR to cyclin D3 mRNA impairs cyclin D3 mRNA stability. It is possible that the decreased binding of HuR to cyclin D3 3'-UTR makes cyclin D3 mRNA exposed to the mRNA decay machinery or allows the binding of cyclin D3 mRNA by destabilizing factors such as mRNA-binding proteins or micro-RNA that will facilitate its degradation by the mRNA decay processes (21).

The increase in cytoplasmic HuR levels could represent redistribution of HuR from the nucleus to the cytoplasm, new synthesis of HuR protein, or both. We found that the L-Arg deprivation impaired the expression of HuR through a global arrest in the protein synthesis. Therefore, the arrest in the translation of HuR induced by the absence of L-Arg may have a negative effect on the stability of multiple mRNAs. However, we cannot rule out that L-Arg has an effect on HuR redistribution on other cell types. In fact, glioma cells cultured in the absence of L-Arg have an initial shifting of HuR to the cytoplasm during the first 6 h of culture (30). Conversely, we have not found an increased cytoplasmic expression of HuR in T cells cultured in the absence of L-Arg even at early culture time points (data not shown).

Amino acid deprivation in eukaryotes has been shown to activate mechanisms that inhibit translation. The accumulation of empty aminoacyl tRNAs caused by amino acid starvation activates GCN2 kinase, which phosphorylates the translation initiation factor eIF2 α (9). Accordingly, we found that L-Arg deprivation maintained the phosphorylation of eIF2 α in activated T cells. The phosphorylated form of eIF2 α binds more tightly than usual to eIF2B, which exchanges GTP for GDP in the eIF2 complex. When eIF2B is bound to the phosphorylated eIF2 α , it is unable to exchange GDP for GTP, which inhibits the binding of methionine aminoacyl tRNA to the eIF2 complex and finally leads to inhibition in translation initiation. T cells from GCN2 knockout mice did not show an arrest in cell cycle or a decreased proliferation, and they were able to upregulate the expression of cyclin D3 and cdk4 when cultured in medium without L-Arg (8). Similarly, T cells from GCN2 knockout mice, but not from wild-type mice, upregulated HuR expression and did not have a decreased cyclin D3 mRNA stability when cultured in the absence of L-Arg. Although we found higher accumulation of phospho-eIF2 α in L-Arg-starved T cells, this did not correlate with an increased expression or phosphorylation of GCN2. Therefore, L-Arg deprivation activates GCN2 (independently of changes on expression or phosphorylation), which directly impairs cyclin D3 translation, but also inhibits cyclin D3 mRNA stability through HuR regulation.

The blocking of the GCN2 pathway could be potentially inhibited for therapies preventing T cell dysfunction in diseases characterized by a decrease in L-Arg levels such as trauma and cancer. Additionally, induction of the GCN2-triggered pathways could be used to block T cell proliferation in transplantations or malignant T cell proliferative disorders. Collectively, our results suggest that in T cells, GCN2 is the central mediator of the effects induced by the absence of L-Arg and may explain the arrest in translation and the decrease in cyclin D3 mRNA stability.

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Disclosures

The authors have no financial conflicts of interest.

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