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L-arginine availability regulates T-lymphocyte cell-cycle progression

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L-arginine (L-Arg) plays a central role in several biologic systems including the regulation of T-cell function. L-Arg depletion by myeloid-derived suppressor cells producing arginase I is seen in patients with cancer inducing T-cell anergy. We studied how L-Arg starvation could regulate T-cell–cycle progression. Stimulated T cells cultured in the absence of L-Arg are arrested in the G_0-G_1 phase of the cell cycle. This was associated with an inability of T cells to up-regulate cyclin D3 and cyclin-dependent kinase 4 (cdk4), but not cdk6, resulting in an impaired downstream signaling with a decreased phosphorylation of Rb protein and a low expression and binding of E2F1. Silencing of cyclin D3 reproduced the cell cycle arrest caused by L-Arg starvation. The regulation of cyclin D3 and cdk4 by L-Arg starvation occurs at transcriptional and posttranscriptional levels. Signaling through GCN2 kinase is triggered during amino acid starvation. Experiments demonstrated that T cells from GCN2 knockout mice did not show a decreased proliferation and were able to up-regulate cyclin D3 when cultured in the absence of L-Arg. These results contribute to the understanding of a central mechanism by which cancer and other diseases characterized by high arginase I production may cause T-cell dysfunction. (Blood. 2007;109:1568-1573)

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Introduction

L-arginine (L-Arg) is a nonessential amino acid that plays a central role in regulating the immune response.[1] In mammalian cells, L-Arg can be catabolized by 4 enzymatic pathways, namely nitric oxide synthase, arginases I and II, arginine:glycine amidinotransferase, and arginine decarboxylase. L-Arg is profoundly reduced in cancer patients,[2] following liver transplantation,[3] or in severe trauma[4] by an increased production of arginase I. This results in a decreased T-cell proliferation and an impaired T-cell function. This effect can be reversed in trauma by the enteral or parenteral supplementation of L-Arg.[5] We demonstrated that activated T cells cultured in medium without L-Arg or cocultured with myeloid-derived suppressor cells (MDSCs) isolated from tumors and producing arginase I have a decreased proliferation, a low expression of T-cell receptor CD3ζ chain, and an impaired production of cytokines.[2,6,7] However, the mechanisms by which L-Arg starvation blocks T-cell proliferation have not been determined.

Signaling through the T-cell receptor, as shown by calcium flux and tyrosine phosphorylation, was not affected for the first 12 hours of culture in the absence of L-Arg and therefore could not completely explain the low proliferation of T cells.[8,9] Furthermore, certain T-cell functions such as up-regulation of IL-2 receptor alpha and production of IL-2 were maintained even in the absence of L-Arg.[8,9] Therefore, we explored whether changes in proteins regulating cell cycle could explain the loss of proliferation in T cells cultured without L-Arg. Cyclin-dependent kinase 4 (cdk4) and cyclin-dependent kinase 6 (cdk6) associate with the D-type cyclins, including cyclin D3, to regulate the progression through early G_1 and into the S phase of cell cycle. This regulation requires inactivation of cyclin D/cdk complex inhibitors and phosphorylation of the Rb protein family. Phosphorylation of Rb by cyclin/cdk complexes induces the subsequent release and nuclear translocation of E2F transcription factors, inducing the expression of genes that promote cell-cycle progression into late G_1 and S phases.[10]

The effects of amino acid starvation have been well studied in yeast and some tumor cell lines; however, their role in regulating cell cycle in T cells is unknown. The results shown here demonstrate that L-Arg depletion selectively impairs the expression of cyclin D3 and cdk4, blocking the downstream signaling. GCN2, a kinase involved in amino acid starvation, plays a central role in regulating the cell-cycle arrest induced by L-Arg starvation. These results may provide a new understanding of the impairment of the immune response in various diseases where myeloid-derived suppressor cells, producing high levels of arginase, deplete L-Arg.

Materials and methods

Cells, cultures, and chemicals

Human peripheral blood mononuclear cells 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 CD3ε expression and ranged between 94% and 98%. Jurkat cells were obtained from ATCC (Manassas, VA). RPMI-1640 containing 1040 μM L-Arg (Cambrex Biosciences, Walkersville, MD) or L-Arg–free RPMI (Invitrogen, Life Technologies, Grand Island, NY) was supplemented with 5% fetal bovine serum (Hyclone, Logan, UT), 25 mM HEPES (Gibco, Grand Island, NY), 4 mM L-glutamine (Cambrex Biosciences), and 100 U/mL penicillin-streptomycin (Gibco). Stimulation of T lymphocytes was done with immunoimmobilized anti-CD3 plus anti-CD28. Briefly, 10 μg/mL purified goat antibody to mouse IgG was bound to polystyrene culture plates for 2 hours at 37°C. T

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cells were stimulated with 1 μg/mL anti-CD3 (OKT-3; Ortho Biotech Products, Raritan, NJ) and 0.1 μg/mL anti-CD28 (BD Biosciences, San Jose, CA) in media that did or did not contain L-Arg. T cells isolated from GCN2 knock-out mice (kindly provided by Dr David Munn, Medical College of Georgia, Augusta, GA) were purified by negative selection (R&D systems) and activated with immobilized anti-CD3 plus anti-CD28 (BD Biosciences) as previously reported.[7]

Cell-cycle measurement by DNA content

Analysis of nuclear DNA content of stimulated T cells was done using CycleTEST PLUS DNA reagent kit (BD Biosciences), following the vendor's recommendations. T-cell proliferation was tested by CFSE (Molecular Probes–Invitrogen, Eugene, OR) following the vendor's recommendations or by [^{3}H]-thymidine incorporation. Data are expressed as counts per minute (CPM) (mean ± SD) of at least triplicate experiments.

Antibodies, Western blotting, immunoprecipitation, and immunodepletion

Mouse monoclonal antibodies against human cyclin D1, cyclin D2, cyclin D3, and $p15^{INK4}$, $p16^{INK}$, $p18^{INK4}$, $p19^{INK4}$, $p21^{Kip}$, and $p27^{Kip}$ were purchased from BD Biosciences; mouse monoclonal anti-cdk6 (B-10) and anti–E2F-1 (KH-95), and rabbit polyclonal antibodies against cyclin D3 (C-16), cdk4 (C-22), Rb (M-153), and $p57^{Kip}$ were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-eIF2 alpha, phospho-eIF2 alpha, and GCN2 were purchased from Biosource (Carlsbad, CA) and cell signaling technologies (Danvers, MA). Whole-cell lysates from T cells were prepared by resuspending cells in lysis buffer containing 50 mM HEPES, 250 mM NaCl, 5 mM EDTA, 0.5 mM DTT, 0.1% nonidet P-40, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 100 μg/mL trypsin-chymotrypsin inhibitor. For Western blotting experiments, 25 μg whole-cell extract was electrophoresed on 8%, 10%, or 12% Tris-Gly gels (Invitrogen, Life Technologies, Carlsbad, CA) and transferred to PVDF membranes (Invitrogen, Carlsbad, CA). Membrane-bound immune complexes were detected by using enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Biosciences, Arlington Heights, IL), followed by exposure to BioMax MR films (Kodak, Rochester, NY).

Immunoprecipitation and immunodepletion assays were done using 100 μg T-cell whole-cell lysates. Agarose-conjugated antibodies against cyclin D3 (D7), cdk4 (H-22), cdk6 (C-21), and $P27^{Kip}$ (F-8) were purchased from Santa Cruz Biotechnology. Extracts were precleared by 2-hour incubation at 4°C with protein G plus agarose. Supernatants were harvested for immunodepletion and/or immunoprecipitation, which were performed by overnight incubation with 2 μg anti–cyclin D3 antibody at 4°C.

Rb phosphorylation

After immunoprecipitation with anti–cyclin D3, immune complexes were washed twice with 500 μL ice-cold PBS, twice with 500 μL washing buffer (50 mM HEPES [pH 7.5], 1 mM DTT), and twice with 500 μL kinase assay buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 2.5 mM EGTA [pH 8.0], 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM DTT, 10 mM β-glycerophosphate, and 20 μM ATP). Agarose beads bound to the targeted enzymatic complexes were resuspended in 30 μL reaction cocktail containing 0.2 μg GST-Rb (Santa Cruz Biotechnology) and 10 μCi (0.37 MBq) [γ-^{32}P] ATP, and incubated at 30°C for 45 minutes. Enzymatic reactions were stopped by adding 5 μL 6X protein loading buffer followed by heating at 95°C for 5 minutes. Samples were loaded onto 10% Tris-glycine gels (Invitrogen, Carlsbad, CA), dried, and autoradiographed.

Cyclin D3 silencing in Jurkat cells

The constitutive expression of cyclin D3 in Jurkat cells was silenced using RNAi technology. Briefly, $CD4^+$ T-cell line Jurkat was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with a plasmid coding for sh–cyclin D3 (Origene, Rockville, MD), containing a puromycin-resistance gene. Controls included transfection with an empty plasmid or a plasmid coding for sh-GFP (Origene), which has no homology to any human sequence. Transfections were established following the manufacturer's protocol. Briefly, a premixed 500-μL solution containing 4 μg of the respective plasmid and 10 μL LipoFECTAMINE 2000 reagent (Invitrogen, Carlsbad, CA) was added to 1×10^6 Jurkat cells previously plated in 2 mL antibiotic-free RPMI containing 10% FBS. Transfected cells were cultured for 24 hours, washed, and cultured in 10% FBS RPMI containing 0.5 μg/mL puromycin.

Northern blots

Total RNA (5 μg) was electrophoresed under denaturing conditions, blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH), and cross-linked by UV irradiation. Membranes were prehybridized at 42°C in ULTRAHyb buffer (Ambion, Austin, TX) and hybridized overnight with 1×10^6 cpm/mL ^{32}P-labeled probe. Probes for cyclin D3, cdk4, cdk6, and GAPDH were generated by reverse transcriptase–polymerase chain reaction (RT-PCR) and labeled by using the RediPrime-II random primer labeling kit (Amersham Biosciences) and [α-^{32}P] dCTP. Membranes were washed once at 42°C for 30 minutes using a 2X SSPE and 0.1% SDS buffer, then twice at 65°C for 30 minutes with 0.2X SSPE and 0.1% SDS buffer. Autoradiography of the membranes was done by exposure of BioMax MR films at −70°C.

Electrophoretic mobility shift assays

T-cell nuclear lysate (5 μg) was incubated for 20 minutes with gel shift binding buffer (Promega, Madison, WI) and the [α-^{32}P] dCTP radiolabeled E2F-1 consensus sequence (Santa Cruz Biotechnology). Samples were electrophoresed in 5% polyacrylamide gels and vacuum-dried. The blots were exposed overnight to BioMax MR films at −70°C in intensifying screens.

[^{35}S] methionine pulse analysis

Primary T lymphocytes were isolated and activated in the presence or absence of L-Arg. After 48 hours of activation, the cells were washed 4 times in L-methionine–free RPMI 1640. The cells were then seeded at a density of 2×10^6 cells/mL in L-methionine–free RPMI 1640 that did or did not contain L-Arg, incubated for 20 minutes, and then pulsed with 0.25 mCi (9.25 MBq) [^{35}S] methionine for 2 hours. Cells were washed twice with PBS, and the protein extraction and immunoprecipitations with anti–cyclin D3 antibody were performed as described. The immunoprecipitates were separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), which was subsequently transferred to a membrane and exposed to photographic film.

Nuclear run-on

Nuclear run-on experiments were performed as previously described.[6] Briefly, nuclei from 2×10^8 cells/sample were isolated by lysing cells in 4 mL lysis buffer (10 mM Tris-HCl [pH 7.4], 3 mM $MgCl_2$, 10 mM NaCl, 150 mM sucrose, and 0.5% nonidet P-40) for 5 minutes on ice. Nuclei were centrifuged at 2000 rpm (425 *g*) for 5 minutes at 4°C, and pellets were resuspended in lysis buffer without nonidet P-40. Nuclei were pelleted and resuspended in 150 μL freezing buffer (50 mM Tris-HCl [pH 8.3], 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). Run-on assays were performed by adding 150 μL 2X transcription buffer (20 mM Tris-HCl [pH 8.0], 300 mM KCl, 10 mM $MgCl_2$, 200 mM sucrose, 20% glycerol, 1 mM dithiotreitol, 0.5 mM adenosine triphosphate [ATP], guanosine triphosphate [GTP], cytidine triphosphate [CTP]) and 100 μCi (3.7 MBq) of 800 Ci (29600.0 GBq)/mmol [α-^{32}P] UTP (NEN, Boston, MA). Samples were incubated at 29°C for 30 minutes. Labeled transcripts were isolated using TRIzol (Invitrogen, Carlsbad, CA) as described before and purified according to the manufacturer's specifications. Equal amounts of radioactivity (2×10^6 cpm labeled RNA) were added in 2 mL ULTRAhyb buffer (Ambion) to nytran membranes onto which 500 ng denatured full-length human cyclin D3, cdk4, and cdk6 cDNA were immobilized using a slot blot apparatus (GIBCO-BRL, Gaithersburg, MD) and a UV cross-linker (Fisher Scientific, Pittsburgh, PA). Hybridization was performed at 42°C for 48 hours. Filters were washed 3 times at 42°C for 15 minutes with 2X SSC/0.1% SDS and twice at 65°C for 20 minutes with 0.2X SSC/0.1% SDS. Filters were then autoradiographed at −70°C.

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Human subjects

Human studies were approved by the institutional review board of the Louisiana State University Health Sciences Center, according to the Declaration of Helsinki. Informed consent was obtained from all subjects. All experiments using normal T cells were done using blood purchased from the blood center (New Orleans, LA).

Statistical analysis

Comparison of values was done using a one-way ANOVA test and *t* test using the Graph Pad statistical program (Graph Pad, San Diego, CA).

Results

L-Arg starvation induces arrest of T-cell cycle in G_0-G_1 and blocks up-regulation of cyclin D3 and cdk4

Normal T cells isolated from healthy donors were stimulated and cultured in media with and without L-Arg. Stimulated T cells cultured in the absence of L-Arg have a significant decrease in proliferation (Figure 1A-B), which was not caused by an increase in apoptosis, as described previously.[9,11] The addition of exogenous L-Arg (2 mM) or citrulline (2 mM), but not other amino acids such as L-lysine and L-glutamine, completely recovered T-cell proliferation (Figure 1C). Cell-cycle analysis using propidium iodide showed that stimulated T cells cultured in the absence of L-Arg were arrested in the G_0-G_1 phase, while those cultured in the presence of L-Arg progressed into S and G_2-M phases after 48 and 72 hours of culture (Figure 2A-B).

We then tested the expression of cyclin D and cdk proteins in T cells cultured in the presence and the absence of L-Arg. Unstimulated T cells showed a low expression for cyclin D1, cyclin D2, cyclin D3, cdk4, and cdk6 when cultured with and without L-Arg (data not shown). Stimulation did not change the expression of cyclin D1 and cyclin D2 (Figure 3A). However, cyclin D3 (Figure 3A) and cdk4 (Figure 3B) expression rapidly increased in T cells activated and cultured in L-Arg–containing medium, but failed to

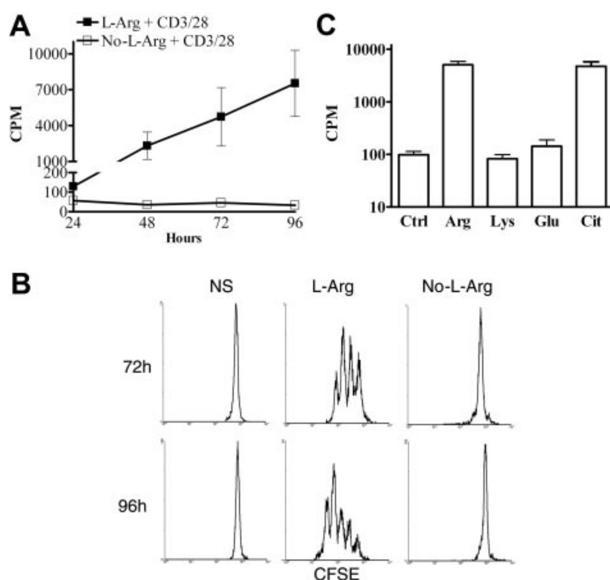

Figure 1. Decreased proliferation in stimulated T cells cultured in the absence of L-Arg. (A) Freshly isolated human T cells (5×10^5) stimulated with anti-CD3 plus anti-CD28 were cultured in the presence or the absence of L-Arg, and uptake of [^{3}H]-thymidine was measured at 24, 48, 72, and 96 hours. (B) Stimulated T cells (5×10^5) were labeled with 1 μM CFSE and cultured in the presence or the absence of L-Arg; fluorescence was measured at 72 and 96 hours. Unstimulated (NS) control cells were CFSE-labeled T cells cultured in medium containing L-Arg. (C) Stimulated T cells (5×10^5) cultured in the absence of L-Arg for 24 hours were replenished with 2 mM L-lysine, L-glutamine, L-citrulline, or L-Arg, and incorporation of [^{3}H]-thymidine was measured at 72 hours.

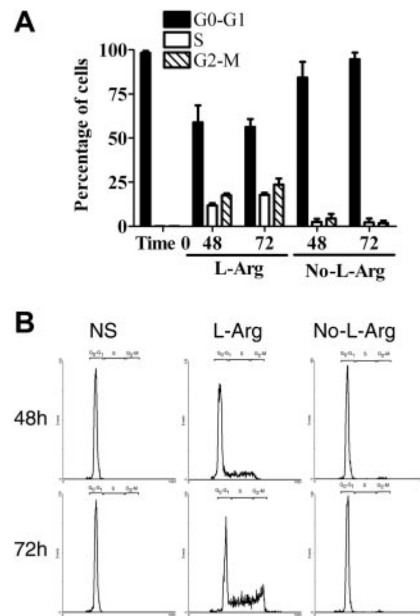

Figure 2. L-Arg starvation arrests T cells in G_0-G_1 phase of cell cycle. (A) Human T cells (5×10^5) were stimulated with anti-CD3 plus anti-CD28 in the presence or the absence of L-Arg, and cell cycle was assessed at 48 and 72 hours by flow cytometry using propidium iodide. Percentages of T cells at each cell-cycle phase from 5 different experiments were determined. (B) Representative experiments from panel A. Unstimulated control T cells (NS, left row) were in medium containing L-Arg.

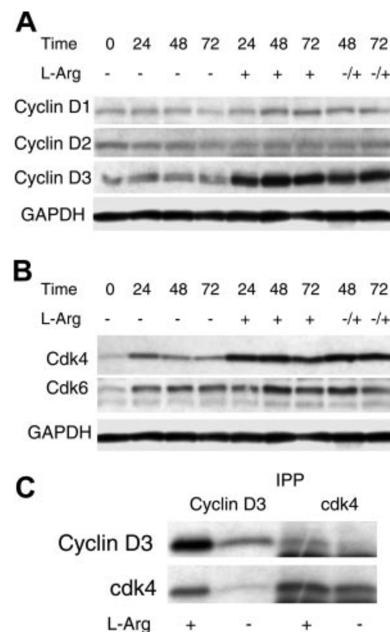

Figure 3. Impaired expression of cyclin D3 and cdk4 in stimulated T cells cultured in the absence of L-Arg. (A-B) Whole-cell lysates were obtained from 3×10^6 activated T cells cultured in the presence or the absence of L-Arg for various lengths of time (hours) or from activated T cells cultured in the absence of L-Arg for 24 hours and then replenished with L-Arg at 48 or 72 hours. The expression of cyclin and cdks was tested by Western blotting. (C) Lysates from stimulated T cells cultured for 24 hours were prepared and 100 μg was immunoprecipitated with agarose-conjugated anti–cyclin D3 and anti-cdk4. Immunoprecipitates were then tested for cyclin D3 and cdk4 expression by Western blotting.

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do so in the absence of L-Arg. In contrast, cdk6 increased in activated T cells cultured with or without L-Arg (Figure 3B). Replenishment of L-Arg at 24 hours of culture induced the re-expression of both cyclin D3 and cdk4 in cells initially cultured in the absence of L-Arg (Figure 3A-B, lanes 8-9). Furthermore, immunoprecipitation experiments using antibodies against cyclin D3 and cdk4 showed a decrease in cyclin D3 and cdk4 complexed in T cells cultured in the absence of L-Arg (Figure 3C).

Decreased phosphorylated Rb expression in L-Arg starvation conditions

Rb is the major substrate for the D-type cyclin/cdk enzymatic complex, and its phosphorylation is required for the release of the transcription factor E2F-1 and progression into late G_1 phase. We tested whether the decreased cyclin D3/cdk4 complexes had an impact in Rb phosphorylation. Consequent with our previous observations, activated T cells cultured in the absence of L-Arg had a significant decrease in Rb phosphorylation (Figure 4A). In addition, cyclin D3 immunoprecipitated only from T cells cultured with L-Arg, and not from the cells cultured in the absence of L-Arg, phosphorylated Rb-GST in vitro (Figure 4B). Furthermore, immunodepletion experiments showed that the depletion of cyclin D3 and cdk4, but not cdk6, decreased Rb phosphorylation, confirming the role of cyclin D3 and cdk4 (Figure 4C).

Translocation of the transcription factor E2F-1 into the nucleus is also required for the initiation of transcription of several genes involved in the transition to S phase. E2F-1 expression was decreased in T cells cultured in L-Arg–free medium (Figure S1A, available on the *Blood* website; see the Supplemental Figures link at the top of the online article). Furthermore, binding of E2F-1 to a DNA consensus sequence was observed only in nuclear extracts from T cells cultured in the presence of L-Arg (Figure S1B). Supershift analysis using a monoclonal antibody directed to E2F-1 and cold control probes confirmed a specific binding of E2F-1 to the consensus sequence.

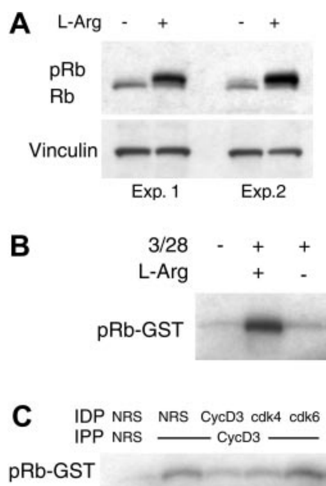

Figure 4. Activated T cells cultured in the absence of L-Arg have a decreased Rb phosphorylation and a decreased ability to phosphorylate Rb in vitro. (A) Whole-cell lysates obtained from stimulated T cells cultured for 24 hours in the presence or the absence of L-Arg were immunoblotted against Rb and vinculin. (B) Lysates from unstimulated or stimulated T cells cultured for 24 hours in the presence or the absence of L-Arg were prepared, and 100 μg was immunoprecipitated with agarose-conjugated anti–cyclin D3 and incubated with a reaction cocktail containing 0.2 μg GST-Rb and 10 μCi (0.37 MBq) [γ-^{32}P] ATP. (C) Similarly, 100 μg whole-cell lysates from stimulated T cells cultured for 24 hours in medium containing L-Arg was initially immunodepleted with an irrelevant protein G *(NRS),* or agarose-conjugated antibodies against cyclin D3, cdk4, or cdk6, and then immunoprecipitated with irrelevant protein G *(NRS)* or anti–cyclin D3 and tested for kinase activity.

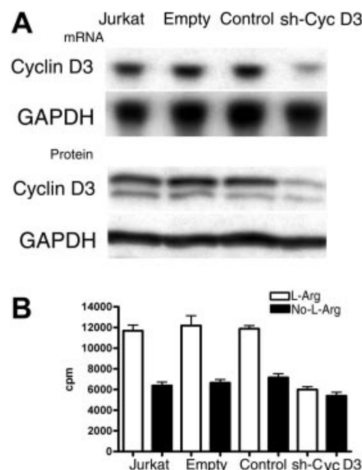

Figure 5. Cyclin D3 silencing mimics L-Arg starvation effects on cell cycle. (A) Wild-type Jurkat cells (Jurkat) and Jurkat cells transfected with empty vector, nonrelated human sequence, or sh–cyclin D3 plasmid were tested for the expression of cyclin D3 RNA by Northern blot and cyclin D3 protein by Western blot. (B) Wild-type Jurkat cells or transfected cells were cultured in the presence or the absence of L-Arg and tested for proliferation after 48 hours by [^{3}H]-thymidine incorporation.

The diminished Rb phosphorylation of cyclin D3/cdk4 complexes could also be explained by an increase in cyclin D/cdk inhibitors. Kinase inhibitory proteins (KIPs, with 3 described proteins: p21^{kip}, p27^{kip}, and p57^{kip}) bind cyclins D and E/cdk complexes inhibiting or stabilizing their catalytic action by sequestration.[12] Likewise cdk4- and cdk6-specific inhibitors, known as INK4 (with 4 members: p15^{Ink4}, p16^{Ink4}, p18^{Ink4}, and p19^{Ink4}), can also block the activity of cdk4/cdk6 or interfere with the binding of cdk to cyclin D. No differences in the expression of p27^{kip}, P57^{kip}, p16^{Ink4}, and p19^{Ink4} proteins were observed in T cells cultured in the absence or the presence of L-Arg, suggesting that this mechanism is unlikely to explain the decreased kinase activity of cyclin D3/cdk4-related complexes observed in L-Arg starvation (Figure S2A). No expression of p21^{kip}, p15^{Ink4}, or p18^{Ink4} was observed in T cells (data not shown). An increased complexing of p27^{kip} with cyclin D3 was observed only in cells cultured in the presence of L-Arg (Figure S2B).

Cyclin D3 silencing reproduces changes induced by L-Arg starvation

If L-arginine starvation targets cyclin D3 to block proliferation, then silencing of cyclin D3 should induce effects similar to those of L-Arg starvation. We tried to transfect primary T cells with plasmids coding for shRNA and siRNA cyclin D3 without success. We then tested the model using the human T-cell line Jurkat, in which we had previously demonstrated the same effects on cell proliferation caused by L-Arg starvation.[6] Similar to activated T cells, cyclin D3 was markedly decreased when Jurkat cells were cultured in the absence of L-Arg (data not shown). When cultured in the presence of L-Arg, sh–cyclin D3–silenced Jurkat cells (Figure 5A), but not cells transfected with a control or an empty vector, had a significant decrease in proliferation, similar to cells cultured in the absence of L-Arg (Figure 5B). Furthermore, cyclin D3–silenced Jurkat cells accumulated into G_0-G_1 phase of the cell cycle (data not shown). Silencing of cdk4 alone did not have an effect on Jurkat cell proliferation (data not shown).

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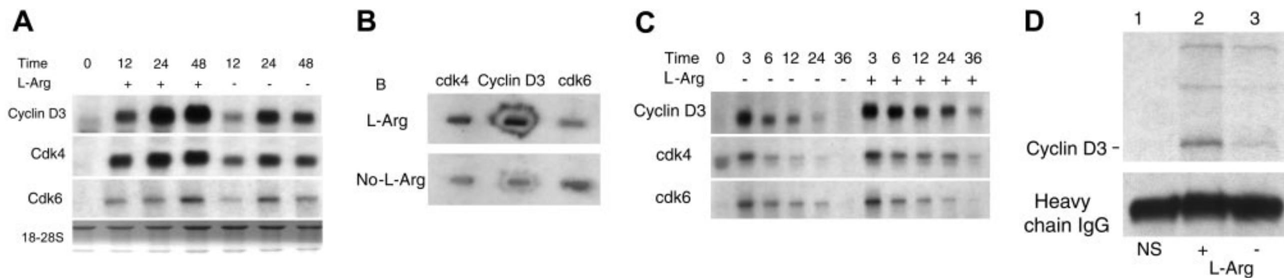

Figure 6. L-Arg starvation impairs expression of cyclin D3 and cdk4 through transcriptional, posttranscriptional, and translational mechanisms. (A) RNA (5 μg) from nonactivated human T cells or T cells activated with anti-CD3 plus anti-CD28 and cultured in the presence or the absence of L-Arg was tested for cyclin D3, cdk4, and cdk6 RNA expression by Northern blot. (B) Nuclei obtained from activated T cells cultured in the presence and the absence of L-Arg were tested for cyclin D3, cdk4, and cdk6 transcriptional rate by run-on analysis as described in "Materials and methods." (C) RNA stability was also tested in activated T cells cultured in the presence and the absence of L-Arg for 12 hours, after which Act D (5 μg/mL) was added and RNA collected after 3, 6, 12, 24, and 36 hours. RNA expression was then tested by Northern blot. (D) Nonactivated (NS, lane 1) and activated (lanes 2-3) T cells were cultured in the presence or the absence of L-Arg for 48 hours, after which cells were washed and pulsed with 250 μCi (9.25 MBq) [^{35}S] methionine for 3 hours. Lysates were immunoprecipitated using anti–cyclin D3 as described in "Materials and methods."

L-Arg starvation impairs expression of cyclin D3 and cdk4 through transcriptional, posttranscriptional, and translational mechanisms

We tested several mechanisms that could explain the decreased cyclin D3 and cdk4 expression caused by L-Arg starvation. Activated T cells cultured in the absence of L-Arg showed a decreased expression of mRNA for cyclin D3 and cdk4, but not for cdk6 (Figure 6A). The rate of transcription measured by run-on analysis showed lower transcriptional rate for cyclin D3 and cdk4, but not cdk6, in T cells cultured without L-Arg compared with the cells cultured with L-Arg (Figure 6B). Similarly, RNA stability experiments showed a lower stability of cyclin D3 and cdk4 RNA, but not cdk6, in the absence of L-Arg (Figure 6C). Furthermore, cyclin D3 protein synthesis was also impaired in the absence of L-Arg (Figure 6D). These results suggest that the L-Arg starvation regulates the expression of cyclin D3 and cdk4 through transcriptional, posttranscriptional, and translational mechanisms.

GCN2 mediates changes in cyclin D3 caused by L-Arg starvation

Previous reports have suggested that GCN2 controls responses in mammalian cells in response to nutrient starvation.[13] To test the role of GCN2 in our model, we used T cells isolated from spleens of GCN2 knock-out mice. Similar to human T cells, murine T lymphocytes do not proliferate in the absence of L-Arg.[7] However, T cells isolated from GCN2 knock-out mice cultured in the absence of L-Arg did not show a decreased proliferation (Figure 7A) and were able to up-regulate the expression of cyclin D3 and cdk4 (Figure 7B).

Discussion

L-Arg is a nonessential amino acid involved in several biologic systems including the immune response.[14-19] The role of L-Arg in the immune system has been suggested by the markedly diminished T-cell responses found in patients with trauma, liver transplantation, and some tumors where L-Arg is reduced by the excess production of arginase 1 in myeloid-derived suppressor cells (MDSCs).[2,3] Both in vitro and animal models have shown that the lack of L-Arg not only blocks T-cell proliferation, but also induces molecular changes including a low expression of CD3ζ chain and a decreased production of cytokines such IFNγ, but not IL-2.[2,6,7] The low expression of CD3ζ chain alone, however, cannot explain the inability of T cells to proliferate, since stimulation of T cells with PMA that bypass the T-cell receptor also failed to induce T-cell proliferation in the absence of L-Arg (data not shown). In addition, T cells cultured in the absence of L-Arg were able to up-regulate the expression of CD25 and CD69, and have similar patterns of calcium flux and tyrosine phosphorylation during the first 12 hours of culture as cells cultured with L-Arg.[9]

We therefore decided to study the mechanisms that could explain how the absence of L-Arg blocked T-cell proliferation. The absence of L-Arg arrests stimulated T cells in G_0-G_1 phase. Association and dissociation of key cell-cycle regulators control the transition between G_0-G_1 in mammalian cells. We found that the impaired T-cell proliferation caused by L-Arg starvation was associated with an inability to up-regulate the expression of cyclin D3 and cdk4, but not cyclin D1, cyclin D2, and cdk6. In addition, cyclin D3 immunoprecipitates from T cells cultured without L-Arg showed a lower expression of cyclin D3/cdk4 complexes and a decreased kinase activity. The decreased expression and kinase activity of cyclin D3/cdk4 complexes in the absence of L-Arg were not explained by an increased expression of KIP (p21^{kip}, p27^{kip}, and p57^{kip}) and INK4 (p15^{Ink4}, p16^{Ink4}, p18^{Ink4}, and p19^{Ink4}) inhibitors.

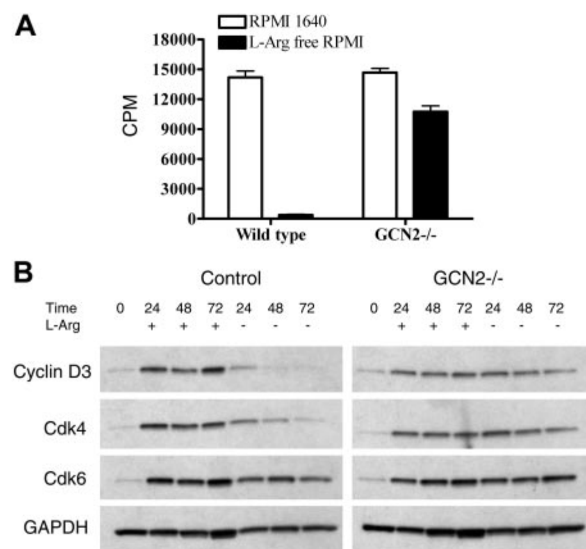

Figure 7. T cells from GCN2 KO mice proliferate in the absence of L-Arg. (A) T cells (2 × 10^5) were activated with bound anti-CD3 plus anti-CD28, and thymidine incorporation was tested after 48 hours (cpm ± SD). (B) Kinetics of expression of cyclin D3 and cdk4 in activated T cells (4 × 10^6) from GCN2 knock-out mice and congeneic wild-type mice.

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The molecular mechanisms involved in the control of gene expression following amino acid deprivation have been extensively studied in yeast,[20] but are poorly understood in mammalian cells. The lack of L-Arg has been associated with the induction of certain genes regulating L-Arg uptake and metabolism such as CAT-1 and arginosuccinate synthase.[20,21] The synthesis of L-Arg through alternative pathways may explain how the addition of citrulline recovered the proliferation of T cells cultured in the absence of L-Arg.[22] Gazzola et al[23] and Hyatt et al[24] also reported that the absence of L-Arg induces an increased transcription and stability of RNAs encoding a multi–amino acid transport system including CAT-1,[25,26] allowing an increase in the transport of cationic amino acids such as L-Arg from the extracellular space into the cytoplasm. Moreover, Diah et al[27] recently identified the TA1/LAT-1/CD98 light chain gene encoding a protein associated with lymphocyte activation, integrin signaling, and amino acid transport including L-Arg. This gene is also increased by L-Arg starvation.

How L-Arg starvation induces the multiple but discrete changes seen in T cells that lead to T-cell dysfunction is not yet completely understood. However, data shown here with cyclin D3, and previously with CD3 ζ chain,[6,9] suggest that starvation of L-Arg impaired the expression of these by transcriptional, posttranscriptional, and translational mechanisms. Amino acid deprivation and accumulation of empty tRNAs in eukaryotes activates the GCN2 kinase, which results in the phosphorylation of eIF2α. In turn, phosphorylated eIF2α suppresses the translation initiation and stability of some cellular mRNAs.[28] Munn et al have shown that T cells from GCN2 knock-out animals proliferate when cultured in the absence of tryptophan or cocultured with indoleamine 2,3-dioxygenase (IDO)–positive cells.[13] Similarly, T cells from GCN2 knock-out mice recovered CD3ζ and proliferate when cultured in the absence of L-Arg or when cocultured with MSCs producing arginase (data not shown). In addition, T cells cultured in the absence of L-Arg had an increased expression of the phosphorylated form of eIF2α (data not shown). Another possible mechanism of inhibition of the translation by amino acid starvation is through blocking of signaling through mammalian target of rapamycin (mTOR). However, its role in this model is unclear (data not shown).

In conclusion, accumulating evidence suggests that regulation of amino acid availability by tumor cells or infiltrating cells producing IDO or arginase is a potent mechanism by which tumor cells escape immune response. Whether these pathways act together in certain tumors or are preferentially expressed in different tumors is still unknown.

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Authorship

Contribution: P.C.R. designed and performed the experiments and wrote the paper; D.G.Q. performed experiments; and A.C.O. assisted in designing experiments and writing the paper.

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