Regulation of T cell Receptor CD3ζ Chain Expression by L-Arginine

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Running title: Regulation of CD3ζ by L-Arginine
Summary

L-Arginine (L-Arg) plays a central role in the normal function of several organ systems including the immune system. L-Arg can be depleted by arginase I produced by macrophages and hepatocytes in several disease states such as trauma, sepsis and following liver transplantation. The decrease in L-Arg levels induces a profound decrease in T cell function through mechanisms that have remained unclear. The data presented here demonstrate that Jurkat T cells cultured in media without L-Arg (L-Arg-free RPMI) have a rapid decrease in the expression of the T cell antigen receptor ζ chain (CD3ζ), the principal signal transduction element in this receptor, and a decrease in T cell proliferation. This phenomenon is completely reversed by the replenishment of L-Arg but not other amino acids. These changes are not caused by cell apoptosis, instead, the diminished expression of CD3ζ protein is paralleled by a decrease in CD3ζ mRNA. This change in CD3ζ mRNA expression is not caused by a decrease in the transcription rate, but rather by a significantly shorter CD3ζ mRNA half-life. This mechanism is sensitive to cycloheximide. Therefore, the regulation of L-Arg concentration in the microenvironment could represent an important mechanism to modulate the expression of CD3ζ and the T cell receptor, and consequently of T cell function.
**Introduction**

L-Arginine (L-Arg) plays a central role in several functions of the immune system\(^1\text{-}\(^3\)). It is metabolized in macrophages by two independent enzymatic pathways\(^4\), the inducible nitric oxide synthetase (iNOS) and arginase I, leading to different effects on the immune system\(^4\text{-}\(^11\)). L-Arg is metabolized by iNOS to produce nitric oxide, one of the principal cytotoxic mechanisms in macrophages\(^12\text{-}\(^15\)). Alternatively, arginase I metabolizes L-Arg to L-Ornithine and urea, the first being the precursor for the production of polyamines that are essential for cell proliferation and fibroblast function\(^5\text{-}\(^6\);\(^11\)). The depletion of L-Arg by an increased production of arginase I following liver transplantation\(^16\text{-}\(^18\)), severe trauma\(^19\text{-}\(^20\) or sepsis\(^21\) coincides with a major decrease in T cell proliferation. Furthermore, the infusion of high doses of L-Arg results in a recovery of T cell function and an increase in the number of CD4\(^{+}\) cells\(^22\text{-}\(^24\)), suggesting that L-Arg may play an important role in regulating the T cell function by mechanisms that have remained unclear.

The T cell receptor ζ chain (CD3\(^{ζ}\)) is the principal signal-transduction element of the T cell antigen receptor (TCR)\(^25\text{-}\(^27\)). A decreased expression of CD3\(^{ζ}\) has been described in T cells from patients with cancer\(^28\text{-}\(^32\)), lupus\(^33\) and chronic infectious diseases such as leprosy\(^34\) and tuberculosis\(^35\). The mechanisms mediating the CD3\(^{ζ}\) decrease are poorly understood. We tested the effect of the absence of L-Arg on T cell signal transduction. The results show that Jurkat T cells cultured in tissue culture media without L-Arg had a rapid decrease in the expression of CD3\(^{ζ}\), but not of other chains of the TCR such as CD3\(^{ε}\)\(^36\). The absence of L-Arg did not impair the up-regulation of the IL2 receptor chains nor the production of IL-2 after antigen stimulation\(^36\). This effect was specific of L-Arg since the depletion of other amino acids such as L-glutamine or L-leucine did not change the expression of CD3\(^{ζ}\)\(^36\). In addition, the work presented here suggests that the CD3\(^{ζ}\) down-regulation induced by L-Arg starvation is not
caused by apoptosis, but rather through post-transcriptional mechanisms that decrease the half-life of the CD3ζ mRNA. This process is completely reversible by the replenishment of L-Arg.
Experimental procedures

*Tissue Culture media:* Tissue culture media included complete RPMI 1640 (C-RPMI), which contains 1140 μM L-Arg (Gibco-BRL/Life technologies, Grand Island, or BioWhitaker, Walkersville, MD), L-Arg-free RPMI and L-glutamine-free RPMI (L-Gln-free RPMI) (Gibco BRL/Life Technologies, Grand Island, NY). All media was supplemented with 10% Fetal calf serum (Hyclone, Road Logan, UT), 25mM HEPES (Gibco BRL/Life technologies), 4mM L-Glutamine (Biowhitaker) and 100U/mL of Penicillin/Streptomycin (Gibco BRL/Life technologies). RPMI from Gibco-BRL or RPMI from BioWhitaker showed similar effects in Jurkat CD3ζ expression.

*Cell Lines:* Jurkat T cells, a CD4+ cell line (Clone E6-1) (ATCC, Manassas, VA), was used to test the role of L-Arg starvation on CD3ζ expression. Cells were counted and re-cultured every 2 days to 5 x 10^5 cells/ml in fresh C-RPMI (Bio Whitaker, Walkersville, MD). In each experiment CD3ζ was tested by flow cytometry at time 0 (at the time of passage) and its value ranged between 55-63 mean fluorescence intensity (MFI). The flow cytometry parameters were kept constant, so data could be compared from one experiment to the next. COS-7 L african green monkey kidney cells (Gibco-BRL) were used for transfection experiments. These cells do not express detectable levels of CD3ζ mRNA or protein before the transfection.

*Antibodies and Probes:* Anti-CD3ε-FITC, anti-CD3ζ-PE and anti-APO2.7-FITC (Beckman-Coulter, Miami, FL) were used for flow cytometry. Mouse IgG1-FITC and mouse IgG-PE (Beckman-Coulter) were used as isotype controls. Human cDNA for glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (1.6 Kb) (Clontech, Palo Alto, CA) and CD3ζ (1.7 Kb) (a kind gift
from Dr Cox Terhorst and Dr Allan Weissman) were used to detect CD3ζ and GAPDH by Northern Blot.

**Flow cytometry:** Flow cytometry analysis was performed as previously described\(^{36}\). Briefly, 5 X 10^5 Jurkat cells were washed once with Dulbecco phosphate-buffered saline (D-PBS) and resuspended in 200 µL of D-PBS containing 1 µg of anti-CD3ε or isotype control. Cells were incubated for 15 min at 4°C, washed with D-PBS and resuspended in 200 µl D-PBS containing 500 µg/ml of digitonin plus 1 µg of anti-CD3ζ or 1 µg of isotypic control. To detect apoptosis 1 µg of anti-APO2.7 was added. Cells were incubated for 8 minutes after which they were washed and resuspended in 400 µl of D-PBS. Fluorescence acquisition and analysis were done in a Coulter-EPICS XL flow cytometer (Beckman-Coulter, Miami, FL) with a 488 nm argon laser. The data are expressed as mean channel fluorescence intensity (MFI).

**Northern blot:** Ten million Jurkat cells were used for RNA extraction. Total RNA was extracted by lysis with TRIzol (Gibco BRL/Life technologies) and purified according to the manufacturer’s specifications. Northern blot analysis was performed as previously described\(^{37}\). Briefly, 10 micrograms of total RNA from each sample were electrophoresed under denaturing conditions, blotted onto nytran membranes (Schleicher & Schuell Inc, Keene, NH) and cross-linked by UV irradiation. Membranes were pre-hybridized at 42°C in ULTRAhyb buffer (Ambion, Austin, TX) and hybridized overnight with 1x10^6 cpm/mL of \(^{32}\)P-labeled probe. Human cDNA for GAPDH (Clontech, Palo Alto, CA) and CD3ζ were labeled by random priming using a RediPrime Kit (Amersham, Arlington Heights, IL) and (\(\alpha\)-\(^{32}\) P) dCTP (3,000 Ci/mmol; NEN Life science products, Boston, MA). Membranes were washed three times, once
at 65°C for 30 min, using a buffer containing 2X SSC and 0.1% sodium-dodecyl sulfate (SDS) and twice at 65°C for 30 minutes in 0.1 X SSC and 0.1% SDS. Membranes were subjected to autoradiography at -70°C using Kodak Biomax-MR (Eastman Kodak Company, Rochester, NY) films and intensifying screens. To test mRNA transcription inhibition, cells were cultured in tissue culture media containing actinomycin D (Act D) (Sigma, St Louis, MO) at a final concentration of 5 µg/ml. Cycloheximide (10 µg/ml) (Sigma) was used to study the role of de novo proteins in the mRNA stability. To determine the half-life of RNA, the CD3ζ /GAPDH ratio at time 0 was considered as 100% of expression and was used to calculate the half-life of CD3ζ mRNA at all other time points. A densitometer, alphalImager 2000 (Alpha Innotech Corporation, San Leandro, CA) was used to analyze the band intensities. All signal intensities were normalized to GAPDH.

Nuclear run-on: Nuclear run-on experiments were performed as previously described38. Briefly, nuclei from 50 x 10⁷ cells/sample were isolated by lysing cells in 4 mL of lysis buffer (10 mM Tris-HCl pH 7.4, 3 mM MgCl2, 10 mM NaCl, 150 mM sucrose, and 0.5% nonidet P-40 (Sigma Chemical Co) for 5 minutes on ice. Nuclei were centrifuged at 2000 rpm for 5 minutes at 4°C and pellets were resuspended in lysis buffer without nonidet P-40. Nuclei were pelleted again as described above and resuspended in 150 µL of freezing buffer (50 mM Tris-HCl pH 8.3, 40% glycerol, 5 mM MgCl2, 0.1 mM EDTA). Run-on assays were performed by adding 150 µL of 2 X transcription buffer (20 mM Tris-HCl pH 8.0, 300 mM KCl, 10 mM MgCl2, 200 mM sucrose 20% glycerol, 1 mM dithiotreitol, 0.5 mM adenosine triphosphate [ATP], guanosine triphosphate [GTP], cytidine triphosphate [CTP]) and 100 µCi of 800 Ci/mmol [α ³²P] uridine triphosphate (NEN, Boston, MA). Samples were incubated at 29°C for 30 minutes. Labeled transcripts were
isolated using TRIzol (GIBCO-BRL) as described before and purified according to the manufacturer’s specifications. Equal amounts of radioactivity (2 x 10⁶ cpm of labeled RNA) or the same number of nuclei were added in 2 mL of ULTRAhyb buffer (Ambion) to nytran membranes onto which 500 ng of denatured full-length human CD3ζ cDNA and Human GAPDH cDNA were immobilized using a slot blot apparatus (GIBCO-BRL) and a UV crosslinker (Fisher Scientific, Pittsburgh, PA). Hybridization was performed at 42°C for 48 hours. Filters were washed three times at 42°C for 15 minutes with 2 X SSC/0.1% SDS and twice at 65°C for 20 minutes with 0.2 X SSC/0.1% SDS. Filters were then autoradiographed at -70°C. Data were normalized for the content of GAPDH present in each sample using an alphaImager 2000 (Alpha Innotech Corporation).

Transfection of COS 7 L cells with CD3ζ cDNA: Coding-region CD3ζ cDNA (1.5 Kb) was excised from pGEM/zeta and joined by T4 DNA ligase (Promega, Madison, WI) to pCI mammalian expression vector (CMV promoter) (Promega), which had been previously linearized with Xbal and EcoRI. COS-7 L african green monkey kidney cells (Gibco-BRL) were cultured to 90% confluence according to the manufacturer’s specifications, after which they were transfected with PCI/CD3ζ or empty vector using lipofectAMINE 2000 reagent (Gibco-BRL), following the manufacture’s recommendations. The cells were then cultured and studied for mRNA stability. Briefly, COS-7 L cells were cultured for 48 hours, after which 3 x 10⁶ cells were cultured in C-RPMI or L-Arg-free RPMI in presence of Act D (5 µg/ml) for 2, 4 and 8 hours. Northern blot was used to study the CD3ζ and GAPDH half-life, as described above.
Statistical analysis: Comparison in CD3ζ expression between Jurkat cells cultured in C-RPMI and L-Arg-free RPMI was done by Students t test using the Graph Pad statistical program (Graph Pad, San Diego, CA).
Results

The absence of L-Arginine induces a decrease of CD3ζ expression in Jurkat cells

Jurkat cells cultured in RPMI-1640 without L-Arg (L-Arg-free RPMI) have a rapid decrease in the expression of CD3ζ by 24 hours that becomes more pronounced after 48 hours (Fig 1A). A careful study of the kinetics of this phenomenon shows a gradual decrease of CD3ζ detectable after 2 hours of culture in L-Arg-free RPMI, followed by a plateau of 24 hours and a final and more pronounced decrease by 48 hours. In contrast, cells cultured in C-RPMI or L-Glutamine-free RPMI (L-Gln-free RPMI) do not show changes in the expression of CD3ζ (Fig 1B). The flow cytometry data was confirmed using western blots (data not shown). We previously reported that the down-regulation of the CD3ζ induced by L-Arg starvation was specific, since the depletion of other amino acids such as L-glutamine and L-leucine did not change the expression of this protein36. In addition, we showed that the absence of L-Arg does not produce a decrease in other TCR proteins such as CD3ε. Furthermore, it does not prevent the up-regulation of other receptors such as the IL2R nor the production of IL236. The decrease in CD3ζ induced by the absence of L-Arg was completely reversed by replenishment of L-Arg (Fig 2). Jurkat cells cultured in L-Arg-free RPMI for 48 hours and transferred to RPMI with 100 µM L-Arg recover the CD3ζ expression to normal levels within 24 hours, demonstrating the role of L-Arg in this phenomenon. In contrast, cells kept in L-Arg-free RPMI transferred to fresh L-Arg-free RPMI continued to have a decreased expression of CD3ζ (Fig 2).

An increase in T cell apoptosis has been suggested as one of the mechanisms that could cause a decreased expression of CD3ζ39:40. However, Jurkat cells cultured in L-Arg free media did not
show any increase in the percentage of APO 2.7 positive cells in the first 72 hours of culture, compared to cells cultured in C-RPMI (Table 1). Similar results were observed using annexin V and propidium iodide as markers of apoptosis/necrosis (data not shown).

The Absence of L-Arg leads to a decrease in CD3ζ mRNA expression

We studied the expression of mRNA encoding for CD3ζ as a possible explanation for the decreased expression on CD3ζ protein. As shown in Figure 3A, Jurkat cells cultured in L-Arg-free RPMI for 24 and 48 hours showed a marked decrease in CD3ζ mRNA expression (2.3 and 4.2 fold decrease, respectively), compared to control cells cultured in C-RPMI. The kinetics of the phenomena showed a gradual decrease in CD3ζ mRNA expression starting at 4 hours of culture in L-Arg-free RPMI, which continued throughout the 48 hours of culture (Fig 3B and 3C). In contrast, cells cultured in C-RPMI had a constant expression of the CD3ζ RNA.

We then tested whether the decrease in CD3ζ mRNA caused by L-Arg starvation was due to a decrease in the transcription rate of the CD3ζ mRNA. Run-on experiments were done using the nuclei from Jurkat cells cultured for 24 hours in the presence or absence of L-Arg. Results consistently showed lower α−32P-UTP incorporation (data not shown) and lower transcription rates for both CD3ζ and GAPDH in the nuclei of Jurkat cells cultured in L-Arg-free RPMI (Fig 4A), suggesting a general decrease in RNA synthesis in the absence of L-Arg. However, there were not significant differences in the CD3ζ/GAPDH ratio when compared with cells cultured in C-RPMI (Fig 4A). In addition, when run-on experiments were normalized based on the amount of radiolabeled RNA (2 x 10^6 cpm), there were no measurable differences in either CD3ζ or GAPDH mRNA expression in Jurkat cells cultured in C-RPMI and L-Arg-free RPMI (Fig 4B).
Therefore, although the absence of L-Arg induced a general decrease in the transcriptional rate, but it was not specific for CD3ζ mRNA.

_L-Arg starvation diminishes the half-life of CD3ζ mRNA in Jurkat cells_

The diminished expression of CD3ζ mRNA could also be explained by a decrease in RNA stability. It has been previously reported that the absence of L-Arg induces changes in post-transcriptional mechanisms in yeast\(^1\). Furthermore, L-Arg starvation induced changes in the expression of genes associated with its own metabolic pathway\(^4\). To test whether L-Arg starvation induced changes in the CD3ζ RNA stability Jurkat cells were cultured in C-RPMI and L-Arg-free RPMI in the presence of Act D (5 µg/ml), an inhibitor of transcription, followed by the measurement of the expression CD3ζ mRNA at various time points. Jurkat cells cultured in C-RPMI displayed a constant expression of CD3ζ mRNA for at least 8 hours. In contrast, cells cultured in L-Arg-free RPMI had a significantly lower CD3ζ mRNA stability (P<0.0001), which was seen as early as 4 hours after culture (Fig 5A). The half-life of CD3ζ mRNA cultured in L-Arg-free RPMI was 3.8 hours as compared to 11.2 hours of Jurkat cells cultured in C-RPMI (Fig 5B). Therefore the data suggest that the decreased half-life of CD3ζ mRNA could be induced by post-transcriptional mechanisms.

To test this possibility, COS-7 L african green monkey kidney cells were transfected with a mammalian expression vector containing the coding-region for CD3ζ. The transfected COS-7 L cells were then cultured in C-RPMI or L-Arg-free RPMI and tested for mRNA stability. As shown in Figure 6, CD3ζ mRNA was detected in cells COS-7 L cells transfected with the plasmid containing the coding-region CD3ζ cDNA (lane 3), but it was not detectable in cells not
transfected or transfected with the empty vector (lanes 1 and 2). CD3ζ mRNA expression was similar in transfected COS-7 L cells cultured in presence or absence of L-Arg for 2, 4 and 8 hours (lanes 4-9). However, when cells were cultured in the presence of Act D, an inhibitor of transcription, COS-7 L cultured in L-Arg-free RPMI had a decreased CD3ζ mRNA half-life (lanes 13-15) when compared to cells cultured in C-RPMI (lanes 10-12). These results confirm that the absence of L-Arg induces a decrease in CD3ζ mRNA stability by post-transcriptional mechanisms.

*Decrease in mRNA half-life is associated with de novo protein synthesis*

Because little is known about the mechanisms regulating CD3ζ mRNA under these conditions, we tested whether the decreased in RNA stability was associated with the *de novo* synthesis of proteins. Jurkat cells were cultured in C-RPMI or L-Arg-free RPMI in the presence cycloheximide (a protein synthesis inhibitor) and Act D (an inhibitor of RNA synthesis). As shown in Figure 7, cells cultured in C-RPMI had a longer CD3ζ mRNA half-life than cells cultured in L-Arg-free RPMI (lanes 2 and 3 vs lanes 4 and 5). There were no differences in CD3ζ mRNA when cycloheximide was added to cells cultured with and without L-Arg (lanes 6-9), since, as previously shown, there were no differences in transcriptional rate. In contrast, when cells were cultured in the presence of Act D and cycloheximide, there was a significant increase in the CD3ζ mRNA level in cells cultured in L-Arg-free RPMI (lanes 4-5 vs lanes 12-13). This suggests that the decrease in CD3ζ mRNA stability could be associated with the synthesis of a new protein such as an RNAase or other protein controlling the CD3ζ mRNA stability.
Discussion

Activation of T cells is initiated by the binding of antigen to the αβ chains of the TCR in the context of major histocompatibility complex (MHC) and the delivery of co-stimulatory signals. The TCR is a multiple subunit complex made of eight polypeptide subunits including TCR αβ, CD3 εγ, εδ and ζζ. CD3ζ generally exists within the TCR/CD3 complex as a disulfide-linked homodimer which is mostly intracellular and plays a central role in initiating the signal-transduction cascade that leads to T-cell activation. CD3ζ is also the rate limiting step in the assembly and membrane expression of the TCR. Regulation of CD3ζ expression is mostly mediated by antigen stimulation. Binding of antigen to the TCR αβ chains leads to the phosphorylation of the CD3ζ immuno-receptor tyrosine-based activation motifs (ITAMS), which starts T cell activation. It also triggers TCR internalization and the subsequent degradation of CD3ζ in lysosomes or proteasomes. T cell activation eventually leads to the up-regulation of CD3ζ mRNA, resulting in the recovery of CD3ζ expression to normal levels by 24 to 48 hours and an increase in the total number of TCR expressed on the cell membrane.

CD3ζ expression has been found decreased in T cells and NK cells of patients with cancer, autoimmune diseases and chronic infectious diseases. The mechanisms that can lead to a down-regulation of CD3ζ include T cell apoptosis, the production of peroxide by macrophages and polymorphonuclear cells and chronic T cell stimulation. Here we describe a new mechanism by which the expression of CD3ζ may be modulated in several diseases by low levels of L-Arg. Several mechanisms including transport, synthesis and recycling help maintain serum
concentrations of L-Arg between 80 and 120 µM. L-Arg is metabolized by nitric oxide synthase to produce NO and citrulline and by arginase I and II to produce urea and ornithine. High concentrations of arginase I and the resulting low levels of L-Arg have been reported in the serum of patients undergoing liver transplantation, trauma, and patients with certain tumors, who also have a significant impairment in T cell function. We therefore studied whether L-Arg could modulate the expression of CD3ζ. Recently we showed that the absence of L-Arg induced a decrease in T cell proliferation and down-regulated the expression of CD3ζ, but did not alter the production of IL-2 nor the up-regulation of the IL-2 receptor chains. Thus the deficiency of L-Arg selectively alters the expression of certain proteins essential to T cell activation. In this report we have elucidated some of the mechanisms by which the absence of L-Arg induces the down-regulation of CD3ζ in a T cell line. The decrease of CD3ζ expression is seen as early as 2 hours, followed by a more significant drop after 24 hours of L-Arg starvation. The mechanisms mediating the initial decrease on CD3ζ (first 2 hours of L-Arg starvation) are unclear, but do not appear to be associated with a decrease in RNA expression, since the initial changes in the CD3ζ mRNA levels are only seen at 4 hours. This consistent decrease in CD3ζ mRNA is more likely associated with the second and more profound decrease in CD3ζ protein seen by 24 hours, since the mean transcription time for CD3ζ is around 16 hours.

The molecular mechanisms involved in the control of gene expression by amino acid deprivation have been extensively studied in yeast. In mammalian cells, the effect of starvation of different amino acids is less clear. The lack of L-Arg has been associated with the induction of certain genes regulating L-Arg metabolism such as arginosuccinate synthase, probably as a pathway for the synthesis of arginine from citrulline. Gazzola et al and Hyatt et al have reported...
that the absence of L-Arg induces the transcription of genes encoding for the several amino acid transport system, including CAT-1\textsuperscript{57,58}, which transport cationic amino acids such as L-Arg from the extracellular space into the cytoplasm. Moreover, Diah et al\textsuperscript{60} recently identified the TA1/LAT-1/CD98 light chain gene encoding a protein associated to lymphocyte activation, integrin signaling and amino acid transport including L-Arg, which is also increased by L-Arg starvation. The absence of leucine and L-Arg have also been associated with an increase in the amount and stability of mRNA for the \textit{CHOP} gene\textsuperscript{61-64}. This gene encodes a transcription factor that blocks the action of the CCAAT/enhancer-binding protein \(\beta\), which in turn inhibits the normal proliferation of cells\textsuperscript{62}. Therefore there is clear evidence of amino acids causing changes in the expression of specific genes.

In the model presented here, a diminished expression of CD3\(\zeta\) appears to be clearly related to a decrease in mRNA. CD3\(\zeta\) gene is directed by a TATA-less promoter that extends from +58 to –307. Rellahan et al\textsuperscript{65} have shown that the nuclear transcription factor Elf-1 is associated with CD3\(\zeta\) transcription. Elf-1 binds two sequences in the CD3\(\zeta\) promoter (-35 and -135) maintaining the constitutive expression of CD3\(\zeta\) mRNA. A decrease in cytoplasmic Elf-1 has been described in the T cells of patients with systemic lupus erythematosus, who also have a concomitant decrease in CD3\(\zeta\) expression\textsuperscript{66}. In our model, we found that Jurkat cells cultured without L-Arg displayed a decrease in nuclear Elf-1 expression and a decrease in the Elf-1 binding to ets-1 binding site in CD3\(\zeta\) promoter (data not shown). However, we did not find a specific down-regulation in the transcriptional rate of CD3\(\zeta\) (Fig 4). It is possible however that this gene is regulated by multiple nuclear transcription factors, which could maintain the CD3\(\zeta\) transcription in the absence of Elf-1. More recently the consensus amino acid response element
(ATTGCATCA) has been described in several genes\textsuperscript{41}. Several reports have suggested that amino acids such as methionine, histidine, asparagine, cysteine and arginine function as nuclear transcription factors increasing the transcription and stability of several genes\textsuperscript{41}. However, the promoter of CD3ζ does not contain this particular amino acid response element and lack of L-Arg did not induce a specific decrease in the transcription level of CD3ζ, although, it induced a general decrease in transcriptional rate.

We also studied whether post-transcriptional regulation was involved in the decrease of CD3ζ mRNA induced by L-Arg starvation. Cells cultured in the absence of L-Arg had a shorter half-life of CD3ζ mRNA compared to cells cultured in the presence of L-Arg (Fig 5), suggesting that L-Arg starvation induced a post-transcriptional regulation of the CD3ζ mRNA. The available information on mRNA stability in states of amino acid deprivation is complex and often contradictory. Guerrini et al\textsuperscript{67, 68} have described a differential post-transcriptional regulation of asparagine synthase mRNA induced by starvation of amino acids. Bruhat et al\textsuperscript{61,62} have found that mRNA extracted from HeLa cells cultured in the absence of leucine have a longer half-life than mRNA of cells cultured in the presence of leucine\textsuperscript{61,62}. However, the molecular mechanisms that affect the stability of mammalian genes in these settings remain to be characterized. Our data shows that a decrease in the CD3ζ mRNA half-life was associated with \textit{de novo} protein synthesis (Fig 7), suggesting a possible role of new proteins probably RNAses in mediating the post-transcriptional regulation of CD3ζ mRNA. This could occur by modification of the mRNA turnover or by degradation\textsuperscript{69-71}. Furthermore, transfection experiments using the coding-region CD3ζ cDNA under the control of a CMV promoter showed that COS-7 L cells cultured in the
absence of L-Arg displayed a decreased CD3ζ mRNA half-life, confirming the post-transcriptional regulation of CD3ζ gene in L-Arg starvation conditions.

In summary, our findings suggest that the L-Arg starvation for longer than 24 hours induces a decrease in CD3ζ expression in Jurkat cells, due in part to a decrease in mRNA stability. A similar decrease in CD3ζ expression can be observed in activated T cells cultured in the absence of L-Arg (Zea A, manuscript in preparation). These results could be a model to understand the decreased CD3ζ expression found in T cells from patients with cancer, chronic infections such as tuberculosis, or chronic inflammatory diseases such as lupus, where an increase in arginase I could result in a local or systemic decrease of L-Arg concentrations. Other diseases including trauma and sepsis also have an increased production of arginase I, low levels of L-Arg and a decreased T cell response, however, the expression of CD3ζ has not been studied in these patients.
LEGENDS

Figures

Figure 1. The Absence of L-Arg induces the down-regulation of the CD3ζ in Jurkat cells. A.

Cells were cultured in the presence (C-RPMI) or absence of L-Arg (L-Arg free RPMI) for 24 and 48 hours, during which changes in CD3ζ were measured using flow cytometry B. Jurkat T cells were cultured in C-RPMI, L-Arg-free RPMI and L-Gln-free RPMI. Changes in CD3ζ expression were measured at 2, 4, 8, 12, 24, 48 hours. All experiments were repeated at least 3 times. Error bars show the standard deviation.

Figure 2. Replenishment of L-Arg induces the recovery of the CD3ζ expression. Jurkat cells were cultured in L-Arg-free RPMI for 48 hours, after which they were washed and cultured in L-Arg-free RPMI plus 100 µM L-Arg (♦) or L-Arg-free RPMI (●) for an additional 24 hours. Cells were cultured in C-RPMI as control (■). CD3ζ expression was measured by flow cytometry.

Figure 3. The Absence of L-Arg induces a down-regulation of the CD3ζ mRNA in Jurkat cells. Cells were cultured in C-RPMI or L-Arg free RPMI for 24 and 48 hours (A) or during 2, 4, 8, 12, 24 hours (B and C). Ten micrograms of total RNA was used for Northern blot analysis. CD3ζ and GAPDH mRNA detection were always done on the same membrane. The band intensities were measured by densitometry and values are expressed as CD3ζ/GAPDH ratio (C). Data shown is from 1 representative experiment of 3 performed.
Figure 4. Down regulation of CD3ζ mRNA induced by L-Arg starvation is not due to a specific decreased in CD3ζ gene transcription.

Jurkat cells (5 X 10⁷) were cultured in C-RPMI or L-Arg-free RPMI for 24 hours. Nuclei were labeled and the rate of transcription of CD3ζ and GAPDH was assessed by nuclear run-on analysis. Data presented are from 2 experiments. The relative values are expressed as CD3ζ and GAPDH ratio.

Figure 5. The Absence of L-Arg reduces the half-life of the CD3ζ mRNA in Jurkat cells. A. Cells were cultured in C-RPMI or L-Arg-free RPMI plus Act D (5 µg/ml). Total RNA was extracted at 2, 4, 8, 12 and 24 hours, electrophoresed, transferred and hybridized with specific probes for CD3ζ and GAPDH. B. Kinetics of CD3ζ mRNA half-life was done based on the densitometry values. CD3ζ/GAPDH ratio at time 0 was considered as 100% expression and was used to calculate the mRNA half-life at all other time points. Data shown are from 1 of 3 experiments.

Figure 6. L-Arg starvation induces a down-regulation in the CD3ζ mRNA stability by post-transcriptional mechanisms. COS-7 L african green monkey kidney cells (Gibco-BRL) were cultured to 90% confluence. They were then transfected with an empty PCI mammalian expression vector (Promega) or PCI vector plus coding-region CD3ζ cDNA. Cells were then cultured in C-RPMI or L-Arg-free RPMI in the presence or the absence of Act D (5 µg/ml). Northern blot analysis was used to detect the CD3ζ mRNA stability.
Figure 7. The decrease in CD3ζ mRNA half-life is sensitive to Actinomycin-D. To determine whether a newly synthesized protein was associated with the decrease in CD3ζ RNA stability, Jurkat cells were cultured in C-RPMI or L-Arg-free RPMI for 2, 4 and 8 hours in the presence of 5 µg/ml Act D (Lanes 2-5, 10-13) and/or to 10 µg/ml cycloheximide (lanes 6-13). RNA was isolated and Northern blot analysis for the CD3ζ and GAPDH was done. B. Densitometric value analysis. Data shown are from 1 representative experiment of 2 performed.
We would like to thank Dr Cox Terhorst for providing the CD3ζ cDNA and Dr Allan Weissman (National Cancer Institute, Bethesda, MA), Dr Kevin Brown and Dr Ronald Luftig (Louisiana State University-Health Science Center, New Orleans, LA) for the review and constructive comments on the manuscript. We are also grateful to Sandra Lee for her assistance in the preparation of the manuscript.
Reference List


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Table 1. Percentage of apoptotic cells in Jurkat cells cultured in the presence or absence of L-Arg

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$^a$ Percentage of Apo 2.7 positive cells ± standard deviation
Figure 1

A

B

CD3ζ Mean Fluorescence Intensity (MFI)

CD3ζ Mean Fluorescence Intensity (MFI)

Hours

0 2 4 8 12 24 36 48

Isotype
C-RPMI 24h
L-Arg-free RPMI 24h
L-Arg-free RPMI 48h

C-RPMI
L-Arg-free RPMI
L-Gln-free RPMI

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Figure 2

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hours 0</th>
<th>Hours 24</th>
<th>Hours 48</th>
<th>Hours 72</th>
<th>Hours 96</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-RPMI</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>L-Arg-free RPMI</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>L-Arg-free RPMI + 100μM L-Arg</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
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</tr>
</tbody>
</table>
Figure 3

A

B

C

C-RPMI  | RPMI no L-Arg

<table>
<thead>
<tr>
<th>Hours</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
<th>48</th>
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<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>12</td>
<td>24</td>
<td>48</td>
</tr>
</tbody>
</table>

CD3ζ  | GAPDH

0  | 24h  | 48h
+A | +A   | -A
+A | +A   | -A

CD3ζ| GAPDH

GAPDH Ratio

0.3  | 0.2  | 0.1  | 0.0
0  | 2  | 4  | 8  | 12 | 24 | 44

Hours

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Figure 4

A

C-RPMI  L-Arg-free RPMI

CD3ζ  GAPDH

B

C-RPMI  L-Arg-free RPMI

CD3ζ  GAPDH
Figure 5

A

<table>
<thead>
<tr>
<th>C-RPMI</th>
<th>L-Arg-free RPMI</th>
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</thead>
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<tr>
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<td>4</td>
<td>8</td>
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<tr>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

Actinomycin-D (5 µg/mL)

CD3ζ

GAPDH

B

% CD3ζ mRNA remaining

0  25  50  75  100

0  2  4  8  12  24

Hours

- C-RPMI
- L-Arg-free RPMI
Table:

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<th>12</th>
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<td>4</td>
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<td>8</td>
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<td>PCI/CD3 ζ</td>
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</table>

Figure 6

CD3 ζ

GAPDH
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</table>

Figure 7

[Image of gel with bands labeled CD3ζ and GAPDH]
Regulation of T cell receptor CD3 chain expression by L-arginine

J. Biol. Chem. published online April 11, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M110675200

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