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# L-Arginine Consumption by Macrophages Modulates the Expression of CD3ζ Chain in T Lymphocytes[1]

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**L-Arginine plays a central role in the normal function of several organs including the immune system. It is metabolized in macrophages by inducible nitric oxide synthase to produce nitric oxide, important in the cytotoxic mechanisms, and by arginase I (ASE I) and arginase II (ASE II) to synthesize L-ornithine and urea, the first being the precursor for the production of polyamines needed for cell proliferation. L-Arginine availability can modulate T cell function. Human T cells stimulated and cultured in the absence of L-arginine lose the expression of the TCR ζ-chain (CD3ζ) and have an impaired proliferation and a decreased cytokine production. The aim of this work was to test whether activated macrophages could modulate extracellular levels of L-arginine and alter T cell function, and to determine which metabolic pathway was responsible for this event. The results show that macrophages stimulated with IL-4 + IL-13 up-regulate ASE I and cationic amino acid transporter 2B, causing a rapid reduction of extracellular levels of L-arginine and inducing decreased expression of CD3ζ and diminished proliferation in normal T lymphocytes. Competitive inhibitors of ASE I or the addition of excess L-arginine lead to the re-expression of CD3ζ and recovery of T cell proliferation. In contrast, inducible nitric oxide synthase or ASE II failed to significantly reduce the extracellular levels of L-arginine and modulate CD3ζ expression. These results may provide new insights into the mechanisms leading to T cell dysfunction and the down-regulation of CD3ζ in cancer and chronic infectious diseases.** ***The Journal of Immunology,*** **2003, 17: 1232–1239.**

L-Arginine plays a central role in several functions of the immune system (1–3). It is metabolized in macrophages, endothelial cells, hepatocytes, kidney cells, and certain tumors by three enzymatic pathways, the inducible nitric oxide synthase (iNOS),[3] arginase I (ASE I), and arginase II (ASE II) (3). In macrophages, L-arginine is metabolized by iNOS to produce citrulline and nitric oxide, which is one of the principal cytotoxic mechanisms in these cells (4, 5). Alternatively, ASE I and ASE II metabolize L-arginine to L-ornithine and urea, the first being the precursor for the production of polyamines that are essential for cell proliferation and the second being an important mechanism for detoxification (3). The expression of ASE I and iNOS in murine macrophages is differentially regulated by Th1 and Th2 cytokines (6, 7). Stimulation of murine macrophages with IFN-γ up-regulates iNOS exclusively, whereas IL-4, IL-10 and IL-13 induce ASE I (8, 9). The mitochondrial isoform ASE II is not significantly modulated by Th1 or Th2 cytokines (7, 10).

An increase in circulating ASE and a decrease in L-arginine levels has been described in patients and rodents after liver transplantation or trauma (11–13) and is accompanied by a marked T cell dysfunction characterized by diminished proliferation and decreased production of cytokines (14). The infusion of high doses of L-arginine results in a recovery of T cell proliferation and an increase in the number of $CD4^+$ cells (15–17). However, the mechanisms by which low levels of L-arginine impair T cell function have remained unclear. We recently reported that Jurkat cells cultured in the absence of L-arginine have diminished proliferation and decreased expression of the ζ-chain (CD3ζ) (18, 19), the principal signal-transduction element of the TCR (20). CD3ζ down-regulation induced by L-arginine starvation was not caused by apoptosis, but rather through posttranscriptional mechanisms leading to decreased CD3ζ mRNA stability (19). The depletion of other nonessential amino acids such as L-glutamine does not alter CD3ζ expression (18, 19). However, the effect of L-arginine depletion on normal T cells and the mechanisms that may limit the availability of L-arginine during an immune response are unclear. Using a co-culture system, we studied whether one or more of the enzymatic pathways that metabolize L-arginine in macrophages, namely iNOS, ASE I, or ASE II, could regulate the availability of L-arginine and possibly modulate CD3ζ expression and T cell function. The results demonstrate that macrophages producing ASE I and expressing the cationic amino acid (CAT) transporter 2B rapidly reduce the extracellular concentration of L-arginine and cause the down-regulation of CD3ζ in activated T lymphocytes, whereas iNOS and ASE II do not induce these changes.

## Materials and Methods

### *Tissue culture media and reagents*

RPMI 1640 without L-arginine or with 150 μM L-arginine (Invitrogen, Carlsbad, CA) were used to culture Jurkat cells, T cells, and peritoneal macrophages (PM). The normal physiological concentration of L-arginine in serum ranges between 50 and 150 μM. Media were supplemented with 4% FCS (Hyclone, Logan, UT), 25 mM HEPES (Gibco-Invitrogen), 4 mM

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[3] Abbreviations used in this paper: iNOS, inducible nitric oxide; ASE I, arginase I; ASE II, arginase II; CAT, cationic amino acid transporters; PM, peritoneal macrophages; NOHA, *N*-hydroxy-L-arginine; nor-NOHA, *N*-hydroxy-nor-L-arginine; L-NIL, L-*N*-iminoethyllysine; L-NMMA, L-monomethyl-L-arginine; L-SDMA, *sym*-$N^G,N^G$-dimethyl-L-arginine; L-NNA, *N*-nitro-L-arginine; L-NAME, *N*-nitro-L-arginine methyl ester.



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L-glutamine (Biowhittaker, Walkersville, MD), and 100 U/ml penicillin/streptomycin (Invitrogen). In some experiments, 2 mM exogenous L-arginine (Sigma-Aldrich, St Louis, MO) was added to cocultures containing IL-4 + IL-13 or IFN-$\gamma$-stimulated PM and T cells. Murine rIL-4, rIL-13, and rIFN-$\gamma$ (R&D Systems, Minneapolis, MN) were titrated for their ability to induce the expression of iNOS or ASE I (data not shown). The optimal concentrations needed to induce ASE I or iNOS were 50 U/ml IL-4 plus 50 ng/ml IL-13 and 100 U/ml IFN-$\gamma$, respectively. Inhibitors of ASE and/or iNOS included *N*-hydroxy-L-arginine (NOHA; 100 $\mu$M), an inhibitor of iNOS and ASE (Calbiochem, San Diego, CA), the specific ASE inhibitor *N*-hydroxy-nor-L-arginine (Nor-NOHA, 50 $\mu$M), and the NOS inhibitor L-*N*-iminoethyllysine (L-NIL, 5 $\mu$g/ml). Hydrogen peroxide scavenger catalase (Roche Diagnostics, Indianapolis, IN) was used at a final concentration of 200 U/ml. Analogs of L-arginine purchased from Calbiochem were used at 1 mM and included L-monomethyl-L-arginine (L-NMMA), *sym-*$N^G$,$N^G$-dimethyl-L-arginine (L-SDMA), *N*-nitro-L-arginine (L-NNA), and *N*-nitro-L-arginine methyl ester (L-NAME). L-Lysine (Sigma-Aldrich) was used at 1 mM.

### *Cell lines and mice*

Jurkat T cells, a $CD4^+$ cell line (clone E6-1) (American Type Culture Collection, Manassas, VA) were used as an indicator cell line because they undergo a rapid loss of CD3$\zeta$ in the absence of L-arginine. Female C57BL/6 mice (6 wk old; Harlan Sprague-Dawley, Indianapolis, IN) were used to isolate PM and T cells from spleen. PM were isolated by injection of 2 ml of thioglycolate (BD Biosciences, San Jose, CA) into the peritoneal cavity of mice for 3 days. Mice were then sacrificed, and PM were isolated by washing the peritoneum with HBSS. After 2 days of culture in RPMI with 4% FBC, unattached cells were washed off, and attached cells were used in cocultures with Jurkat or anti-CD3- plus anti-CD28-stimulated mouse T cells.

### *T cell isolation and Ag stimulation*

T cells were isolated from spleen of mice by T cell enrichment columns (R&D Systems) according to the manufacturer's specifications. T cell purity ($CD3^+$) ranged between 89 and 95%. The cycle of internalization and re-expression of the TCR-CD3 complex was induced by T cell stimulation with 1 $\mu$g/ml anti-CD3 plus 500 ng/ml anti-CD28 (BD PharMingen, San Diego, CA). Cells were stimulated in the absence of L-arginine and added to the cocultures 24 h after stimulation. T cell proliferation was tested by CFSE (Molecular Probes, Eugene, OR). Briefly, T cells were stained with 1 $\mu$M CFSE and then stimulated with anti-CD3 plus anti-CD28 and cocultured with PM. Fluorescence was tested by flow cytometry after 96 h.

### *Cocultures in Transwells (Boyden chambers)*

PM were cultured in six-well plates in RPMI containing 150 $\mu$M L-arginine and stimulated with murine rIL-4 (50 U/ml) and murine rIL-13 (50 ng/ml), or murine rIFN-$\gamma$ (100 U/ml) for 24 h. One million normal T cells stimulated with anti-CD3 plus anti-CD28 in the absence of L-arginine for 24 h were cultured in the top chamber of a Transwell system containing 0.4-$\mu$m pores (BD Biosciences, Franklin Lakes, NJ). PM were detached using trypsin-EDTA (Invitrogen), and the expression of protein and RNA for CAT-2B, ASE I, ASE II, and iNOS was tested by Western blot and Northern blot. Cytoplasmic extracts from PM were used to test ASE activity. Supernatants from stimulated PM were used to measure NO production using the Griess reagent method (Molecular Probes). In addition, the extracellular concentration of L-arginine was tested in supernatants by HPLC (Dr. G. Wu, Texas A&M University, College Station, TX) as described previously (21).

### *Abs and probes*

CD3$\varepsilon$ and CD3$\zeta$ expression was detected by flow cytometry in murine T lymphocytes and Jurkat cells using anti-mouse CD3-FITC (BD PharMingen), anti-mouse CD3$\zeta$-PE (Santa Cruz Biotechnologies, Santa Cruz, CA), and anti-human CD3$\varepsilon$-FITC, and anti-human CD3$\zeta$-PE (Beckman-Coulter, Miami, FL), respectively. Rat IgG1-FITC, rat IgG2-PE (BD PharMingen) and mouse IgG1-FITC, mouse IgG1-PE (Beckman-Coulter) were used as isotype controls for mouse and human T cell detection, respectively. mAbs against iNOS (Santa Cruz Biotechnologies), ASE I (BD Transduction Laboratories, Lexington, KY), and ASE II (a gift of Dr. S. Morris, Jr.) were used for Western blots. To test the expression of CAT transporters mRNA, specific amplification products from RT-PCR of CAT-1, CAT-2A, CAT-2B, and CAT-3 were purified from agarose gels and used as probes to detect CAT expression by Northern blot. Murine full-length cDNA for GAPDH (1.6 kb; Clontech, Palo Alto, CA) was used as housekeeping control.

### *Flow cytometry*

Flow cytometry analysis was performed as previously described (18). Briefly, $5 \times 10^5$ Jurkat cells or T lymphocytes were washed once with Dulbecco's phosphate-buffered saline 1$\times$ (D-PBS) and resuspended in 200 $\mu$l of D-PBS containing 1 $\mu$g of anti-CD3$\varepsilon$-FITC or isotype control. Cells were incubated for 15 min at 4°C, washed with D-PBS, and resuspended in 200 $\mu$l of D-PBS containing 500 $\mu$g/ml digitonin plus 1 $\mu$g of anti-CD3$\zeta$ or 1 $\mu$g of isotypic control. Cells were incubated for 8 min at 4°C after which they were washed and resuspended in 400 $\mu$l of D-PBS. Fluorescence acquisition and analysis were done using a Coulter-EPICS XL flow cytometer (Beckman-Coulter) with a 488 nm argon laser. Data are expressed as mean channel fluorescence intensity.

### *Northern blot*

Two million PM stimulated with IL-4 + IL-13 or IFN-$\gamma$ were used for RNA extraction using lysis with TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's specifications. Four micrograms of total RNA from each sample were 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 $1 \times 10^6$ cpm/ml $^{32}$P-labeled probe. Probes for detection of CAT transporters and GAPDH mRNA were labeled by random priming using a RediPrime Kit (Amersham, Little Chalfont, UK) and [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol; NEN Life Science Products, Boston, MA). Membranes were washed and subjected to autoradiography at −70°C using Kodak Biomax-MR films (Eastman Kodak, New Haven, CT) and intensifying screens.

### *Extracts and Western blot analysis*

Cells were resuspended in lysis buffer (50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1 mM $NaVO_4$, and 0.5% Triton) containing 50 $\mu$g/ml aprotinin, 50 $\mu$g/ml leupeptin, 100 $\mu$g/ml trypsin-chymotrypsin inhibitor, and 2 mM PMSF. Lysates were centrifuged at 3000 $\times$ *g* for 10 min at 4°C. The expression of ASE I, ASE II, and iNOS was detected by immunoblot using PM extracts. GAPDH was used as housekeeping protein. Cytoplasmic extracts were electrophoresed in 12 or 8% Tris-glycine gels (Novex, San Diego, CA), transferred to polyvinylidene difluoride membranes, and immunoblotted with the appropriate Abs. The reactions were detected using the ECL kit (Amersham).

### *ASE activity assay*

Cell lysates (2 $\mu$g) from PM stimulated with IL-4 + IL-13 or IFN-$\gamma$ were tested for ASE activity by measuring the production of L-ornithine and urea. In brief, cell lysates from PM stimulated with IL-4 + IL-13 or IFN-$\gamma$ in the presence or absence of NOHA or Nor-NOHA were added to 50 $\mu$l of Tris-HCl (50 mM; pH 7.5) containing 10 mM $MnCl_2$. This mixture was heated at 55–60°C for 10 min to activate ASE. The hydrolysis reaction from L-arginine to L-ornithine was identified by a colorimetric assay after the addition of ninhydrin solution and incubation at 37°C for 1 h. In addition, the hydrolysis reaction from L-arginine to urea was detected with diacetyl monoxime (Sigma-Aldrich).

## Results

### *Peritoneal macrophages stimulated with IL-4 + IL-13 deplete extracellular L-arginine*

Previous reports have shown that the expression of iNOS and ASE I in murine macrophages is differentially regulated by Th1 and Th2 cytokines (6–8). PM stimulated with IL-4 + IL-13 up-regulate ASE I but not ASE II or iNOS, whereas those stimulated with IFN-$\gamma$ increase iNOS expression and NO production, as measured by the production of nitrites, and decrease ASE I expression (Fig. 1, *A* and *B*). Kinetic studies showed that IL-4 + IL-13 stimulation increases ASE I mRNA expression in the first 4 h (data not shown), with an increase in ASE I protein starting at 12 h, whereas the mitochondrial isoform ASE II remains unchanged (Fig. 1*C*). Activation of PM by Th1 or Th2 cytokines also had different effects on the extracellular levels of L-arginine. PM stimulated with IL-4 + IL-13 rapidly reduced L-arginine in the tissue culture medium to levels below 15 $\mu$M in the first 12 h of culture (Fig. 1*D*)

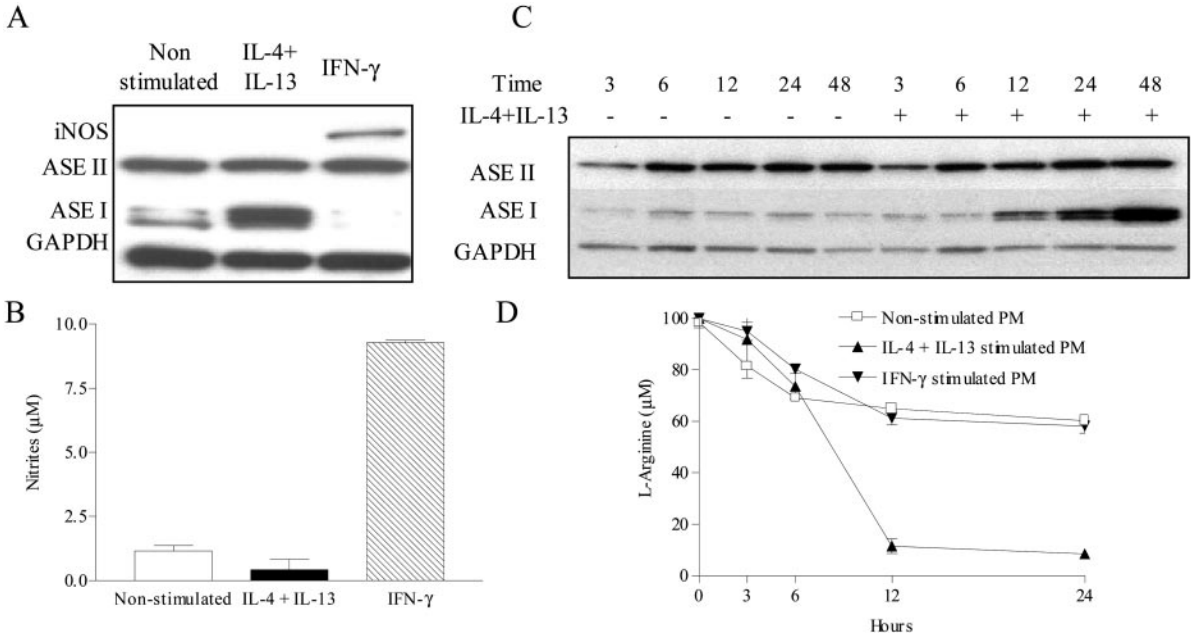


**FIGURE 1.** PM stimulated with IL-4 + IL-13 increase ASE I expression and decrease the extracellular levels of L-Arg. *A*, Cytoplasmic extracts were isolated from $2 \times 10^6$ PM stimulated with IL-4 + IL-13 or IFN-γ for 24 h and immunobloted for iNOS, ASE I, and ASE II. *B*, Nitrite levels were measured in the supernatants using Griess reagent as an indirect measure of NO production. Bars represent the mean levels of nitrites in three different experiments ± SD. *C*, Cytoplasmic extracts from PM stimulated with IL-4 + IL-13 were harvested at 3, 6, 12, 24, and 48 h. *D*, Supernatants from cultures of PM stimulated with IL-4 + IL-13 or IFN-γ were tested for L-arginine concentration by HPLC at 3, 6, 12, and 24 h. Results show the mean ± SD of three different experiments.

($p < 0.005$). In contrast, PM stimulated with IFN-γ produced only a moderate decrease in L-arginine, similar to unstimulated PM.

### *ASE I depletes L-arginine and induces CD3ζ down-regulation*

Jurkat cells have been shown to rapidly lose CD3ζ in the absence of L-arginine (19). Therefore, we tested whether L-arginine reduction by macrophages had any effect on CD3ζ expression in Jurkat cells and syngeneic normal T cells. PM stimulated with IL-4 + IL-13 induced a rapid decrease in the expression of CD3ζ in cocultured Jurkat cells (separated by 0.4-μm pore size Transwells), which was dependent on the number of PM and the time in culture (Fig. 2). This effect coincided with the increase in ASE I expression (Fig. 1*C*) and the reduction in the extracellular levels of L-arginine (Fig. 1*D*). In contrast, PM stimulated with IFN-γ or unstimulated PM did not alter CD3ζ expression (Fig. 2*B*). Control cultures of Jurkat cells (without PM) with IL-4 + IL-13 or IFN-γ did not show changes in CD3ζ expression (data not shown).

The effect of arginine depletion by IL-4 + IL-13-stimulated PM was then tested on normal murine T cells. Control cultures show that T cells stimulated and cultured in RPMI containing 150 μM L-arginine undergo a normal cycle of internalization and re-expression of CD3ζ. However, the absence of this amino acid prevents the normal re-expression of CD3ζ after the stimulation with anti-CD3 plus anti-CD28 (Fig. 3*A*). Similarly, the coculture of stimulated T cells with PM activated with IL-4 + IL-13 in Transwells (0.4-μm pores), also resulted in a decreased expression of CD3ζ chain (Fig. 3*B*). In contrast, T cells cocultured with resting PM or PM stimulated with IFN-γ displayed the normal cycle of internalization and re-expression of CD3ζ within 48 h, as did T cells cultured in RPMI containing 150 μM L-arginine. Resting T cells

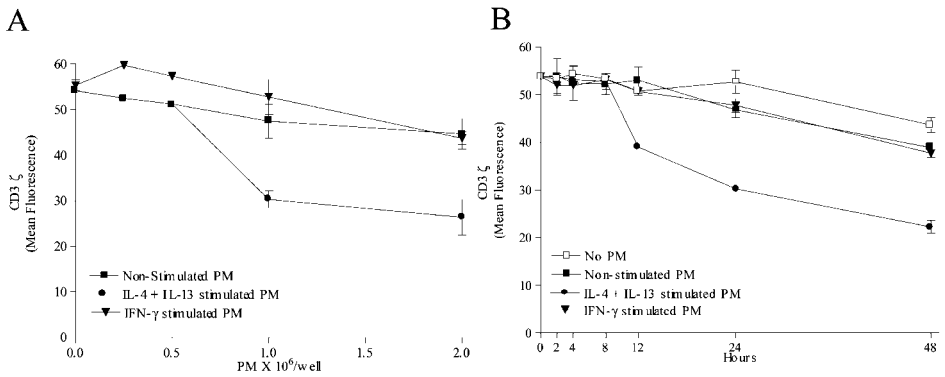


**FIGURE 2.** Coculture of Jurkat cells with IL-4 + IL-13-stimulated PM decreases CD3ζ expression. *A*, Coculture of PM stimulated with IL-4 + IL-13 or IFN-γ and Jurkat cells ($1 \times 10^6$) were done in Transwells using $0.25–2.0 \times 10^6$ PM/well. All cells were cultured in RPMI containing 150 μM L-arginine. CD3ζ expression was measured by flow cytometry at 24 h of coculture. *B*, Cocultures of PM ($2 \times 10^6$) and Jurkat cells ($1 \times 10^6$) were done as described previously. Jurkat cells were harvested and tested for CD3ζ expression by flow cytometry at different times in culture.

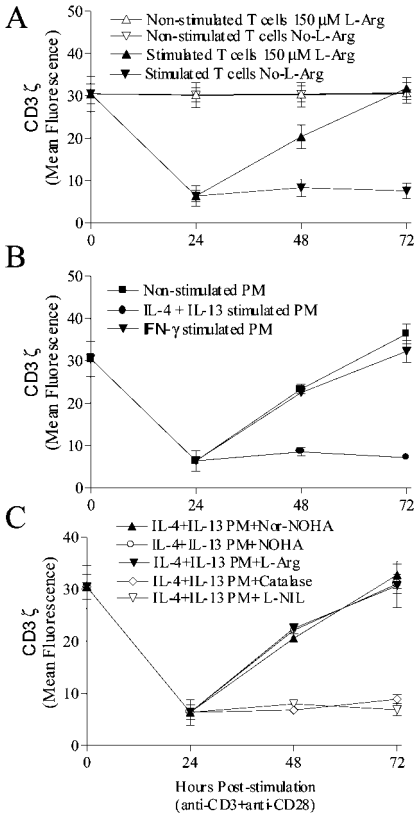


**FIGURE 3.** ASE I but not iNOS, ASE II, or hydrogen peroxide prevents the re-expression of CD3ζ in T lymphocytes after stimulation. *A*, Murine T cells were stimulated with anti-CD3 plus anti-CD28 in L-arginine-free RPMI; after 24 h cells were cultured in RPMI (150 μM L-arginine) or in L-arginine-free RPMI. Control unstimulated T cells were cultured in the absence and the presence of L-arginine from time 0. *B*, PM stimulated for 24 h with IL-4 + IL-13 or IFN-γ in RPMI containing 150 μM L-arginine were cocultured with $1 \times 10^6$ stimulated T lymphocytes. *C*, PM were stimulated with IL-4 + IL-13 for 24 h in RPMI containing 150 μM L-arginine in the presence of NOHA (NO), Nor-NOHA, L-NIL, excess L-arginine (2 mM), or catalase. Stimulated T cells were then added onto the upper chamber of Transwells, and after an additional 48 h of culture, CD3ζ was tested by flow cytometry. Data represent the mean fluorescence intensity of CD3ζ ± SD of three different experiments.

did not lose CD3ζ in the absence of L-arginine. A similar effect has been seen in normal human T lymphocytes where the absence of arginine impairs the re-expression of ζ chain by blocking its synthesis.[4]

Inhibitors of ASE and iNOS and a hydrogen peroxide scavenger were then used to further determine the role of these enzymatic pathways in the regulation of extracellular L-arginine and the expression of CD3ζ. The addition of ASE inhibitors NOHA or Nor-NOHA, or the excess of L-arginine in the cultures, partially prevented the reduction of L-arginine in the tissue culture medium of IL-4 + IL-13-stimulated PM (Table I). When these inhibitors or exogenous L-arginine were added into cocultures of stimulated T cells and IL-4 + IL-13-activated PM, there was a normal cycle of internalization and re-expression of CD3ζ in T cells (Fig. 3*C*). In contrast, the iNOS inhibitor L-NIL did not prevent the prolonged CD3ζ down-regulation. Catalase was included in some control cultures because previously published data had shown that hydrogen peroxide released by activated macrophages and neutrophils could induce a decrease in CD3ζ expression (22, 23). Catalase, however, did not prevent the decrease of CD3ζ in T cells induced by IL-4 + IL-13-stimulated PM.

[4] A. Zea. L-Arginine modulates CD3ζ expression and function in activated T cells. *Submitted for publication.*

Table I. *Arginase inhibitors prevent the depletion of extracellular L-arginine levels induced by PM stimulated with IL-4 plus IL-13*

| PM Cultures | Nonstimulated | IL-4 + IL-13 |
|---|---|---|
| PM[a] | 64.9 (6.2)[b] | 7.8 (4.9) |
| PM + NOHA | 101.6 (9.6) | 75.6 (13.1) |
| PM + Nor-NOHA | 109.8 (8.3) | 73.8 (18.4) |
| PM + Exogenous L-Arg | 1267 (123.6) | 59.8 (11.4) |

[a] Unstimulated PM and PM stimulated with IL-4 (50 U/ml) plus IL-13 (50 ng/ml) were cultured in RPMI containing 150 μM L-arginine. L-Arginine concentrations were tested after 24 h. NOHA (100 μM), Nor-NOHA (50 μM), or L-arginine (2 mM) were added at time 0.

[b] Mean L-arginine concentration in micromolar (± SD) of three different experiments.

### *ASE I inhibits* in vitro *T cell proliferation*

We also tested whether the loss of CD3ζ induced by ASE I had any effect on T cell proliferation. As shown in Fig. 4, stimulated T cells cultured in RPMI with 150 μM L-arginine or cocultured with resting PM showed a normal proliferation as seen by the reduction (halving) of CFSE fluorescence (23.63 and 22.04%, respectively) and the appearance of additional fluorescent peaks of proliferating T cells. In contrast, stimulated T cells cultured in the absence of L-arginine or cocultured with IL-4 + IL-13-activated PM failed to proliferate. However, if the ASE inhibitor Nor-NOHA was added to the cocultures of T cells and IL-4 + IL-13-activated PM, T cells showed active proliferation (20.123%), suggesting that ASE I not only depleted L-arginine and induced a decreased CD3ζ expression but also impaired T cell proliferation. NOHA and Nor-NOHA did not alter ASE I up-regulation after IL-4 + IL-13 stimulation (Fig. 5*A*) but instead blocked intracellular ASE activity (Fig. 5, *B* and *C*).

### *ASE I/CAT-2B increase L-arginine uptake in macrophages*

A significant reduction in L-arginine levels is found in the serum of patients and rodents in the first 24 h after liver transplantation and trauma and appears to be caused by a rapid increase in circulating ASE (11, 12). Therefore, the decrease in L-arginine levels induced by IL-4 + IL-13-stimulated PM could be the result of ASE I released into the tissue culture media. Interestingly, there was no

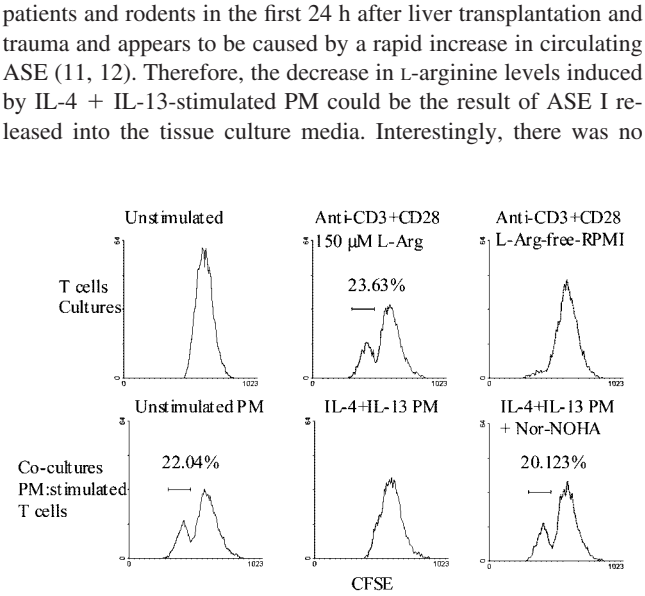


**FIGURE 4.** ASE I inhibits T cells proliferation in vitro. *Top*, Normal T cells ($1 \times 10^6$) were stained with 1 μM CFSE, stimulated with anti-CD3 plus anti-CD28, and cultured in RPMI containing 150 μM L-arginine or L-arginine-free RPMI. *Bottom*, Stimulated T cells were cocultured with unstimulated PM or PM stimulated with IL-4 + IL-13, with or without the addition of Nor-NOHA.

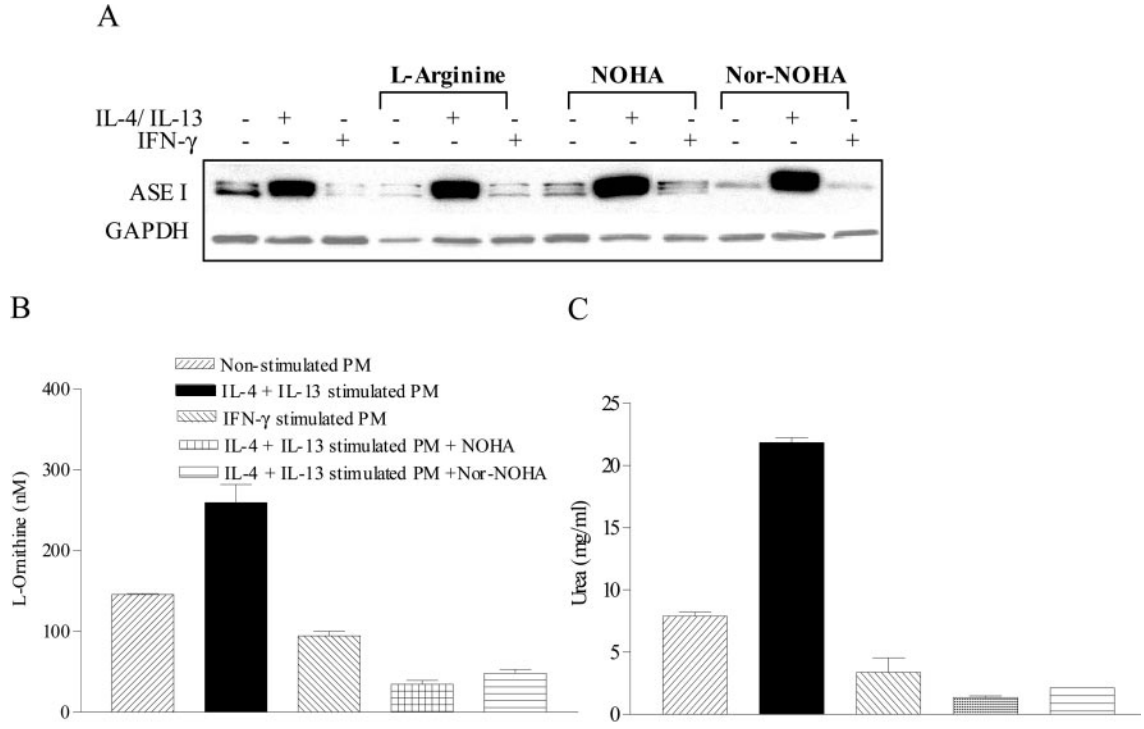


**FIGURE 5.** ASE inhibitors do not affect the expression of ASE I. *A*, Western blot for ASE I was done using cytoplasmic extracts from macrophages stimulated with IL-4 + IL-13 or IFN-γ in the presence or absence of NOHA or Nor-NOHA. These cytoplasmic extracts were also tested for ASE activity by measuring L-ornithine *(C)* and urea *(D)* production in the presence or absence of NOHA or Nor-NOHA. All experiments were repeated at least three times.

significant ASE activity in the supernatants from the cultures of IL-4 + IL-13-stimulated PM (data not shown). Therefore, we tested whether L-arginine uptake by PM could in part explain the depletion of extracellular L-arginine. PM stimulated with IL-4 + IL-13 showed a rapid increase in L-[$^3$H]arginine uptake at 12 and 24 h, which was significantly higher than PM stimulated with IFN-γ or nonstimulated PM ($p < 0.005$) (Fig. 6*A*). L-Arginine is transported across the cell membrane by the cationic amino acid transporter $Y^+$ family of receptors (CAT). The increase in the L-[$^3$H]arginine uptake and the decreased extracellular levels of L-arginine coincided with an increased CAT-2B mRNA expression in IL-4 + IL-13-stimulated PM (Fig. 6*B*). In contrast, there was no increase in CAT-2B mRNA in PM stimulated with IFN-γ. Furthermore, L-arginine analogs L-NMMA and L-SDMA and the amino acid L-lysine, which competitively inhibit CAT-2B (24), allowed a partial recovery of CD3ζ expression in stimulated T lymphocytes cocultured with IL-4 + IL-13-activated PM (Fig. 6*C*). Instead, other L-arginine analogs, not transported by CAT-2B such as L-NNA and L-NAME (24), failed to recover CD3ζ expression. Stimulation with IL-4 + IL-13 or IFN-γ did not induce significant changes in the expression of other CAT transporters including CAT-1 and CAT-2A. In addition, CAT-3, which is mostly expressed in brain (25), was not detected in resting and activated PM (data not shown).

## Discussion

L-Arginine is a nonessential amino acid that plays a central role in several biological systems including the immune response. In macrophages, L-arginine is metabolized by ASE I, ASE II, and the nitric oxide synthase (NOS) family of enzymes (1). ASE I and ASE II are encoded by two distinct genes and hydrolyze L-arginine into urea and L-ornithine, the latter being the main substrate for the production of polyamines (putrescine, spermidine, and spermine) that are required for cell cycle progression. L-Arginine is also metabolized in macrophages by iNOS to citrulline and nitric oxide, a highly reactive compound important in the cytotoxic mechanism of these cells (4, 5). The importance of L-arginine on the immune response was suggested by the marked association of impaired T cell function in patients and rodents after liver transplantation or trauma (11, 12, 26, 27), which rapidly recovered with the enteral or parenteral supplementation of L-arginine (14–16, 28). L-Arginine levels in serum range from 50 to 150 μM. The mechanism by which L-arginine depletion causes T cell dysfunction is incompletely understood. We recently showed that T cells cultured in low concentrations of L-arginine lose CD3ζ expression, are unable to proliferate, and have a decreased cytokine production (18, 19).[4] However, it was unclear how L-arginine availability might be regulated during an immune response. We therefore asked which of the metabolic pathways that use L-arginine in macrophages could modulate the availability of L-arginine and whether this would alter the expression of CD3ζ chain and proliferation in normal T cells.

Our initial data confirmed previous observations demonstrating the reciprocal regulation of iNOS and ASE I in macrophages by Th1 and Th2 cytokines (6–9). Stimulation of PM with IFN-γ induced the expression of iNOS, whereas IL-4 plus IL-13 stimulation increased ASE I. In contrast, the isoform ASE II was not regulated by these cytokines. The coculture experiments demonstrated that PM expressing ASE I but not iNOS and ASE II rapidly decrease extracellular L-arginine concentrations to levels below 15 μM. A similar decrease in L-arginine levels had been previously reported by Chang et al. (29) and Que et al. (30) using an in vitro model with ASE I-transfected cell lines. In addition, the coordinated expression of CAT proteins that transport L-arginine from the extracellular microenvironment into the cell appears to play an

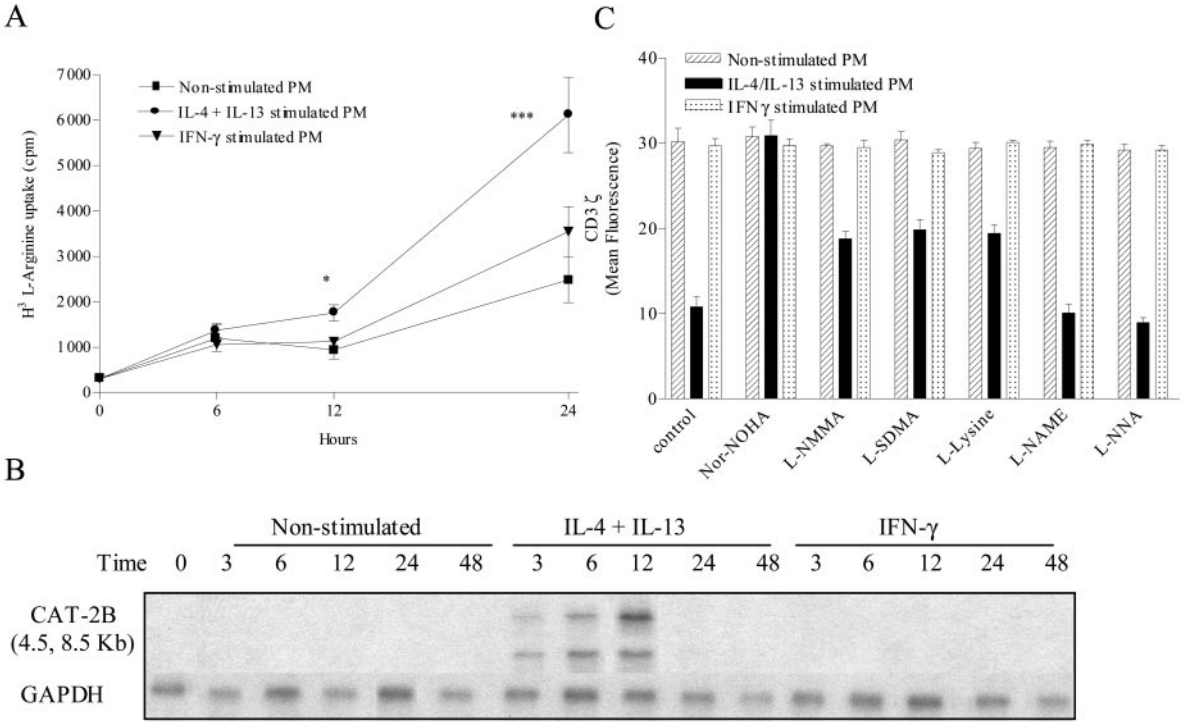


**FIGURE 6.** Increased L-arginine uptake and CAT-2B expression in PM stimulated with IL-4 + IL-13. *A*, PM ($1 \times 10^6$) were stimulated with IL-4 + IL-13 or IFN-γ and cultured in RPMI containing 150 μM L-arginine and 5 μCi of L-[$^3$H]arginine. L-[$^3$H]arginine uptake was measured at 6, 12, and 24 h. ***, $p < 0.005$. *B*, PM ($2 \times 10^6$) were stimulated with IL-4 + IL-13 or IFN-γ and RNA isolated at 3, 6, 12, 24, and 48 h. CAT-2B mRNA expression was measured by Northern blot. *C*, One million T cells were stimulated with anti-CD3 + anti-CD28 and cultured for 24 h in the absence of L-arginine. PM ($2 \times 10^6$) were stimulated for 24 h with IL-4 + IL-13 or IFN-γ in the presence of 1 mM concentrations of the L-arginine analogs L-NMMA, L-SDMA, L-NNA, L-NAME, or 1 mM concentrations of L-lysine. All cultures were done in RPMI containing 150 μM L-arginine. Cocultures of stimulated T cells and PM were then done in 0.4-μm pore size Transwells for an additional 48 h, after which CD3ζ was tested by flow cytometry. All experiments were repeated at least three times.

important role in the regulation of extracellular levels of L-arginine. This particular carrier system is characterized by its high affinity for basic amino acids, its independence from $Na^+$, and the ability of substrate on the opposite (*trans*) side of the membrane to increase transport activity (31). CAT genes have been recently cloned and designated CAT-1, CAT-2A, CAT-2B, and CAT-3. Whereas CAT-1, CAT-2B, and CAT-3 are high affinity ($K_M$ 100 μmol/L) transporters for L-arginine, CAT-2A is an alternatively spliced variant of CAT-2B that possesses low affinity for L-arginine ($K_M$ 1 to 2 mmol/L) (32). In accordance with Louis et al. (10), our data show that PM stimulated with IL-4 + IL-13 up-regulate the expression of CAT-2B, displaying a similar kinetics to ASE I (Figs. 1*C* and 6*B*). Furthermore, inhibitors of CAT-2B, L-NMMA, L-lysine, and L-SDMA partially allowed the re-expression of CD3ζ in T cells cocultured with IL-4 + IL-13-activated PM (Fig. 6*C*), suggesting a possible role of CAT-2B in the regulation of extracellular L-arginine and CD3ζ expression. Although L-NMMA can also inhibit iNOS activity, we have used L-SDMA and L-lysine, which do not inhibit iNOS at the concentration used (1 mM) (24). In addition, iNOS inhibitors L-NIL, L-NNA, and L-NAME, which are not transported by CAT-2B failed to recover CD3ζ expression after T cell stimulation. L-NAME has also been reported to be an ASE inhibitor in vitro and in vivo (33); however, we did not observe an inhibitory effect on ASE activity assay at the concentrations used (data not shown). Stimulation of PM with IL-4 + IL-13 did not induce major changes in the expression of CAT-1 and CAT-2A in PM (data not shown). In addition, CAT-3, which is mostly expressed in the brain (25), was not detected in PM-unstimulated or activated with IL-4 + IL-13 or IFN-γ.

The molecular mechanisms involved in the control of gene expression by amino acid deprivation have been extensively studied in yeast. However, the effect of starvation of different amino acids in mammalian cells is less clear (34). Recent publications have suggested a close correlation between the availability of certain amino acids and the immune response. Munn et al. (35) described that tryptophan metabolism by macrophages producing indoleamine 2,3-dioxygenase inhibits T cell proliferation (35). This group also suggested that tryptophan starvation induced cell cycle arrest in normal T lymphocytes and sensitized activated T cells to apoptosis before cell division (36). The absence of other amino acid such as leucine have been associated with an increase in the synthesis and stability of mRNA for *CHOP* (37). This gene encodes a transcription factor that interacts with CCAAT/enhancer-binding proteins family, which in turn may inhibit the normal proliferation of cells (38). Therefore, essential amino acids appear to induce changes in T cells that ultimately inhibit their normal proliferation. Our data show that metabolism of the nonessential amino acid L-arginine can also control T cell function through the modulation of CD3ζ expression. In Jurkat cells, the decrease of CD3ζ appears to be caused by decreased CD3ζ mRNA stability (19), associated with de novo synthesis of a protein that releases a ribonucleoprotein complex bound to the 3′-untranslated region of CD3ζ mRNA (P. Rodriguez, unpublished data). In normal human T cells, L-arginine starvation appears to alter CD3ζ re-expression by specific decrease in CD3ζ translation.[4]

How low must L-arginine levels be to alter CD3ζ chain expression and T cell function, and can these levels occur in vivo? In vitro data show that concentrations below 40 μM cause the rapid

decrease of CD3ζ chain in Jurkat cells and impair the re-expression of this chain in stimulated T cells (P. Rodriguez and A. Zea, unpublished results). Consistent with this, rodents and patients with trauma or undergoing liver transplantation have a rapid decrease in circulating L-arginine levels to concentrations below 40 μM (11, 14), paralleled by poor T cell function (14). In addition, Ichihara et al. (39) recently demonstrated the trauma patients may lose CD3ζ, although they did not associate this change with L-arginine reduction. Furthermore, transgenic mice that overexpress ASE I in their enterocytes have a selective depletion of L-arginine in serum, concomitant with a poor thymic development (40). However, de Jonge et al. (41) showed that the major dysfunction in these transgenic mice occurs in B cell maturation rather than T cell development. This observation is not contrary to our data because, as shown in Fig. 3, L-arginine depletion affects only activated T cells and not resting T lymphocytes. It is likely that most T cells in these mice are in a resting state and therefore are not susceptible to the effects of L-arginine depletion in vivo.

ASE I is also produced by several tumors including gastric, colon, breast, and lung cancers (42–46). Most reports have associated the increased ASE expression by tumor cells with the need to produce polyamines to sustain the rapid malignant cell proliferation (29). Our data also suggest that the increase in ASE I expression may have as a secondary effect the local (or systemic) reduction of L-arginine levels resulting in the decreased expression of CD3ζ and inhibition of proliferation in activated T cells, allowing tumors to escape the immune response. A recent report by Bronte et al. (47) suggested that ASE I produced by myeloid suppressor cells isolated from mice bearing the C26-GM tumor impaired T cell function. Our preliminary data in murine tumor models suggest that ASE I production in tumor-bearing mice can modulate CD3ζ expression in vivo (P. Rodriguez, manuscript in preparation). It is possible that L-arginine levels may be decreased locally in certain tumors, at sites where ASE I is being produced by tumor cells or infiltrating APC. This might explain the observation that CD3ζ is more profoundly decreased in tumor-infiltrating T cells than in circulating T lymphocytes (48).

ASE I production in cancer may not be the only mechanism by which CD3ζ is decreased and T cell function impaired. Previous studies have also demonstrated that Fas-Fas ligand interactions and the production of hydrogen peroxide by macrophages or neutrophils may cause these changes (22, 23). Therefore, additional studies in tumor models in vivo and in cancer patients will help elucidate the contribution of these different mechanisms in the induction of T cell anergy and CD3ζ down-regulation in cancer and determine their possible impact on immunotherapy.

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