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Arginase I Production in the Tumor Microenvironment by Mature Myeloid Cells Inhibits T-Cell Receptor Expression and Antigen-Specific T-Cell Responses

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ABSTRACT

T cells infiltrating tumors have a decreased expression of signal transduction proteins, a diminished ability to proliferate, and a decreased production of cytokines. The mechanisms causing these changes have remained unclear. We demonstrated recently that peritoneal macrophages stimulated with interleukin 4 + interleukin 13 produce arginase I, which decreases the expression of the T-cell receptor CD3ζ chain and impairs T-cell responses. Using a 3LL murine lung carcinoma model we tested whether arginase I was produced in the tumor microenvironment and could decrease CD3ζ expression and impair T-cell function. The results show that a subpopulation of mature tumor-associated myeloid cells express high levels of arginase I, whereas tumor cells and infiltrating lymphocytes do not. Arginase I expression in the tumor was seen on day 7 after tumor injection. Tumor-associated myeloid cells also expressed high levels of cationic amino acid transporter 2B, which allowed them to rapidly incorporate L-Arginine (L-Arg) and deplete extracellular L-Arg *in vitro*. L-Arg depletion by tumor-associated myeloid cells blocked the re-expression of CD3ζ in stimulated T cells and inhibited antigen-specific proliferation of OT-1 and OT-2 cells. The injection of the arginase inhibitor *N*-hydroxy-nor-L-Arg blocked growth of s.c. 3LL lung carcinoma in mice. High levels of arginase I were also found in tumor samples of patients with non-small cell carcinoma. Therefore, arginase I production by mature myeloid cells in the tumor microenvironment may be a central mechanism for tumor evasion and may represent a target for new therapies.

INTRODUCTION

T cells infiltrating tumors have alterations in signal transduction and function (1, 2), which may impair the therapeutic efficacy of various forms of immunotherapy. Several potential mechanisms for tumor-induced immuno-suppression have been described, but their presence *in vivo* has been difficult to demonstrate (3, 4). Tumor cells and immature myeloid cells have been linked to the induction of T-cell dysfunction in cancer (5, 6) through the production of reactive oxygen species and the depletion of tryptophan (3, 7). Other reports have shown that immature myeloid cells from the spleen of tumor bearing mice can impair alloreactive T-cell function through a nitric oxide-dependent mechanism (8, 9). More recently, *in vitro* models suggest that stimulation of T cells in an L-Arginine (L-Arg) poor microenvironment results in the induction of signal transduction and functional alterations (10) similar to those seen in tumor-infiltrating lymphocytes and peripheral blood T cells of patients with cancer.

L-Arg is metabolized by arginase I, arginase II, and the inducible nitric oxide synthase (11). Arginase I and arginase II are encoded by two distinct genes and are located in the cytoplasm and mitochondria, respectively. Both enzymes hydrolyze L-Arg 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-Arg can also be metabolized by inducible nitric oxide synthase to produce citrulline and nitric oxide, important in vascular homeostasis and cytotoxic mechanisms of macrophages (12). High arginase activity has been described in patients with various malignancies including gastric, colon, breast, and lung cancers (13, 14). Most reports have associated the increased arginase activity with the need for malignant cells to produce polyamines to sustain their rapid proliferation (15). Recent *in vitro* models suggest that macrophages stimulated by interleukin (IL)4 and IL13 produce arginase I and can cause T-cell anergy by decreasing the expression of the T-cell receptor CD3ζ chain (16). Using mice bearing the Lewis lung carcinoma (3LL), we asked whether one or more of the enzymatic pathways that metabolize L-Arg were present in the tumor microenvironment and could explain the molecular alterations seen in T cells from patients with cancer. The results show that a unique subpopulation of mature tumor-associated myeloid cells but not tumor cells or immature myeloid cells express arginase I and cationic amino acid transporter 2B. Tumor-associated myeloid cells inhibit CD3ζ expression and antigen-specific T-cell responses. Inhibition of arginase *in vivo* decreased tumor growth in mice and, therefore, may represent a target for new therapies. High levels of arginase I and a decreased CD3ζ levels in the infiltrating T cells were also found in human non-small cell lung cancer samples.

MATERIALS AND METHODS

Murine Tumor Model and Antigen-Specific T Cells. Lewis lung carcinoma (3LL) cells, a murine lung carcinoma cell line (American Type Culture Collection, Manassas, VA), was maintained in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 25 mM HEPES (Life Technologies Inc.-Invitrogen, Carlsbad, CA), 4 mM L-glutamine (BioWhittaker), and 100 units/ml of penicillin-streptomycin (Life Technologies Inc.-Invitrogen). Six-week-old female C57BL/6 mice (Harlan, Indianapolis, IN) and C57BL/6 Prkdc^{scid} (The Jackson Laboratories, Bar Harbor, ME) mice were s.c. injected with 1×10^6 3LL cells and sacrificed at different periods of time, and tumors and spleens were harvested. Splenocytes from transgenic mice OT-1 and OT-2 (H-2K^b) and OVA-derived peptide 257–264 (SIINFEKL) and 323–339 (ISQAVHAAHAEINEAGR) were kindly provided by Dr. Jason Brayer and Dr. Eduardo Sotomayor (University of South Florida, Tampa, FL). The endotoxin levels of these peptides were below the limit of detection of the LAL QCL-1000 kit (BioWhittaker). All of the experiments using animals were performed in compliance with the relevant laws and Louisiana State University animal care facility guidelines.

Antibodies and Reagents. CD3ζ and CD3ϵ expression in murine T lymphocytes were measured by flow cytometry using anti-mouse-CD3ϵ-fluorescein isothiocyanate (FITC; PharMingen-Becton Dickinson, San Diego, CA) and anti-mouse-CD3ζ-phycoerythrin (PE; Santa Cruz Biotechnology, Santa Cruz, CA). Human CD3ζ and CD3ϵ expression was detected using anti-human CD3ζ-PE and anti-human CD3ϵ-FITC (Beckman-Coulter, Miami, FL). Mouse IgG1-FITC-IgG2b-PE, rat IgG1-FITC, and rat-IgG2-PE (PharMingen-Becton

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Dickinson) were used as isotype controls. Antibodies against inducible nitric oxide synthase (Santa Cruz Biotechnology), murine arginase I (Transduction-Becton Dickinson, San Jose, CA), human arginase I (Santa Cruz Biotechnology), and arginase II (a kind gift of Dr. Sidney Morris Jr., University of Pittsburgh, Pittsburgh, PA) were used for Western blots. Other antibodies included anti-CD11b, CD16/CD32, I-A/I-E, H-2D^b, CD80, CD86, CD45RB, CD40, CD11c, CD4, CD8a, CD14, B220, and CD49f labeled with FITC or PE (PharMingen-Becton Dickinson), anti-DEC-205 FITC, anti-CD68 FITC (Serotech, Raleigh, NC), and anti-F4/80 (Caltag, Burlingame, CA).

The specific arginase inhibitor *N*-hydroxy-nor-L-Arg (Calbiochem, San Diego, CA) was used to confirm the role of arginase in the loss of CD3ζ and CD3ε *in vitro*. In addition, *N*-hydroxy-nor-L-Arg was injected s.c. in mice at 20 mg/kg/day, 40 mg/kg/day, and 80 mg/kg/day. Animals were also injected s.c. with L-Arg (500 mg/kg/day). Analogs of L-Arg symmetrical N^G-N^G-dimethyl-L-Arginine and *N*-nitro-L-Arg were purchased from Calbiochem and used at 1 mM. The amino acid L-Lysine (Sigma) was used at 1 mM.

Dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) and dihydroethidium (Molecular Probes) were used to measure reactive oxygen species. Catalase (200 units/ml; Roche Diagnostics, Indianapolis, IN) was used to test the role of hydrogen peroxide in the down-regulation of CD3ζ.

Cell Subset Isolation from 3LL Tumors. Tumors were removed from mice under sterile conditions after 7, 14, and 21 days of tumor injection. Tumors were digested with trypsin-EDTA (Invitrogen) for 3 h, and the single cell suspension was passed through a 40-μm cell strainer (Becton Dickinson-Falcon, Franklin Lakes, NJ). Cells were then stained for CD11b, CD16, CD32, I-A/I-E, CD49f, CD4, CD8, or Gr-1 and separated into different subsets, as discussed in the "Results," using a Sorter Epics Altra (Beckman-Coulter) or anti-FITC and anti-PE immunomagnetic beads (Miltenyi Biotec, Auburn, CA). Purity of the subsets ranged between 93% and 99%. T cells were isolated from spleens of mice by T-cell enrichment columns (R&D Systems, Minneapolis, MN) according to the manufacturer's specifications. T-cell purity (CD3$^+$) ranged between 89% and 95%.

Cocultures in Transwells (Boyden Chambers). Cell subpopulations isolated from 3LL tumors were cultured in the bottom chamber of a transwell (six-well plates) for 24 h using RPMI containing 150 μM L-Arg (physiological levels). In parallel, 1×10^6 normal splenic T cells were stimulated with 1 μg/ml anti-CD3 plus 500 ng/ml anti-CD28 (PharMingen-Becton Dickinson, San Diego, CA), in the absence of L-Arg for 24 h. The stimulated T cells were then cultured in the upper chamber of a transwell system, which has 0.4-μm pores (Falcon-Becton Dickinson). The expression of CD3ζ in the T cells was tested after 24, 48, and 72 h. Results were expressed as mean fluorescence intensity. In addition, T-cell proliferation was tested by [^{3}H]thymidine incorporation.

Immunohistochemistry. 3LL tumors were fixed in buffered 10% formalin and embedded in paraffin. Serial 4 μm-thick sections were cut and mounted on poly-L-lysine coated Probe-On slides (Fisher Biotech). Tissue sections were heated overnight at 37°C, deparaffinized using xylene, and rehydrated in graded alcohol dilutions. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide solution. After washing, nonspecific binding was reduced by incubation in 1% normal goat serum diluted with PBS. Tissue sections were then incubated with anti-arginase I (Transduction, Becton Dickinson) or IgG mouse isotype control (Becton Dickinson) for 2 h at room temperature. After washing, slides were incubated with biotinylated goat anti-mouse antibody (1:200; Dako, Carpinteria, CA) for 30 min at room temperature. Thereafter, sections were rinsed and incubated with streptavidin-biotin-peroxidase complex (Strept-ABComplex; Dako) for 30 min at room temperature. Diaminobenzidine (Sigma-Aldrich, St. Louis, MO) was used as a chromogen and counterstained with hematoxylin.

Detection of Endocytotic Capacity and Nonspecific Esterases. To further study the endocytotic pathway, 1×10^5 tumor-associated myeloid cells, non-tumor-associated myeloid cells and peritoneal macrophages were pulsed with FITC-labeled K-12 *Escheria coli* bioparticles (relation 1:200) using the Vybrant Phagocytosis Assay kit (Molecular Probes) following the vendor's recommendations. Trypan blue was used as extracellular quenching probe. Non-specific esterases were measured in cytospins prepared from 3×10^5 tumor-associated myeloid cells, non-tumor-associated myeloid cells, and peritoneal macrophages using α-naphthyl acetate esterase (Sigma-Aldrich).

Reactive Oxygen Species Detection. Dyes sensitive to oxidation by reactive oxygen species were used to determine oxidative stress in cells isolated from 3LL tumors. Dichlorodihydrofluorescein diacetate (Molecular Probes) was used to test the production of hydrogen peroxide, peroxinitrites, and hydroxyl radical. Dihydroethidium (Molecular Probes) was used to test the production of superoxide anion. Cells were labeled for 5 min 37°C in RPMI 1640 using 2 μM of the respective dye, washed twice with RPMI 1640 and fluorescence determined by flow cytometry.

L-Arg Measurement and L-Arg Incorporation. L-Arg concentration in tissue culture medium was measured by high-performance liquid chromatography with electron capture detection using an ESA-CoulArray Model 540 (ESA Inc., Chelmsford, MA) with an 80 × 3.2 column with 120A pore size. Briefly, supernatants were deproteinized in methanol. After centrifugation at 6000 × *g* for 10 min at 4°C, the supernatant was derivatized with 0.2 M *O*-phtaldialdehyde containing 7 mM β-mercaptoethanol. Fifty microliters of the sample were injected into the column. The retention time for L-Arg was 10.2 min. Standards of L-Arg were prepared in methanol. [^{3}H]L-Arg incorporation was measured at 3, 6, 12, and 24 h. One million cells isolated from 3LL tumors were cultured in RPMI 1640 containing 150 μM L-Arg and 5 μCi of [^{3}H]L-Arg.

Northern Blot. Two million cells were used for RNA extraction using lysis with TRIzol (Invitrogen) following the manufacturer's specifications. Five 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 prehy-

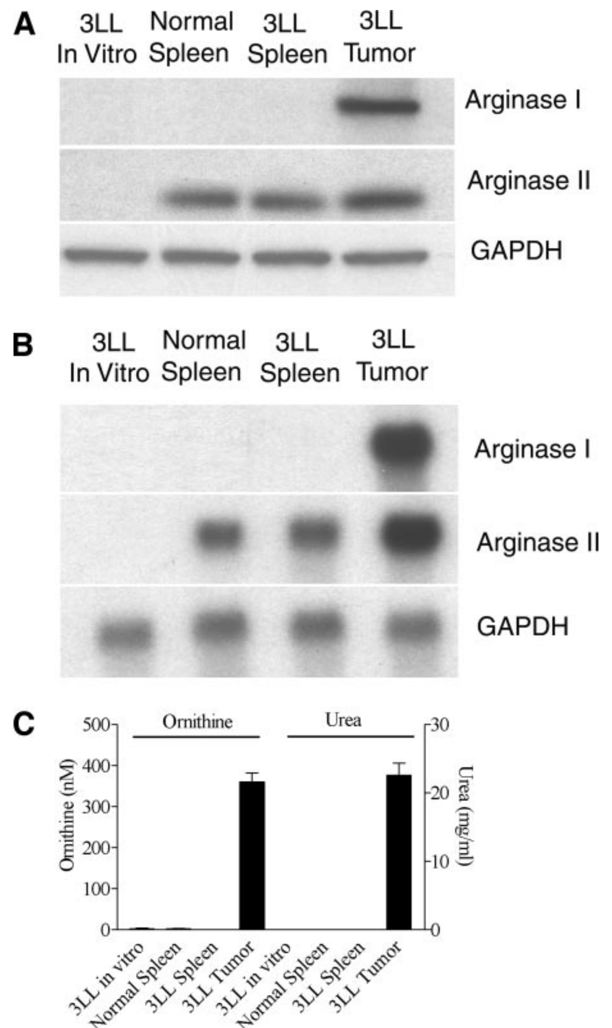

Fig. 1. Single cell suspension from 3LL tumors express high levels of arginase I and arginase II . *A*, cytoplasmic extracts were isolated from 2×10^6 cells and immunoblotted with anti-arginase I, II, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *B*, 5 μg of total RNA were transferred and hybridized using specific arginase I, II, and GAPDH probes. *C*, cytoplasmic extracts were tested for arginase activity by conversion of L-Arg onto urea or ornithine. Experiments were done using 8 mice; *bars*, ±SD.

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bridized at 42°C in ULTRAhyb buffer (Ambion, Austin, TX) and hybridized overnight with 1×10^6 cpm/ml of ^{32}P-labeled probe. Probes for detection of arginase I, arginase II, cationic amino acid transporter 1, cationic amino acid transporter 2B, and glyceraldehyde-3-phosphate dehydrogenase (Clontech, Palo Alto, CA) mRNA were labeled by random priming using a RediPrime Kit (Amersham, Buckinghamshire, United Kingdom) and [α-^{32}P]dCTP (3,000 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.

Reverse Transcription-PCR. Two micrograms of total RNA were treated with DNase I (Invitrogen) and converted to cDNA using SuperScript kit (Invitrogen), following the manufacturer's specifications. PCR reactions using recombinant Taq polymerase (Invitrogen) were done to determine the expression of indoleamine 2, 3-dioxygenase (740 bp), cationic amino acid transporter 2A (115 bp), cationic amino acid transporter 2B (121 bp), and β-actin (697 bp). The linearity of the PCRs was determined based on the volume of cDNA (2 μl) and the number of cycles: 30 for indoleamine 2, 3-dioxygenase and β-actin, 31 for cationic amino acid transporter 2A, and 32 for cationic amino acid transporter 2B. The primers used were described previously (17, 18): β-actin, forward 5′-AGCAAGAGAGGTATCCTG-3′, reverse 5′-CCTTACGGATGTCAACGTC-3′; indoleamine 2, 3-dioxygenase, forward 5′-GTACATCACCATGGCGTATG-3′, reverse 5-GCTTTCGTCAAGTCTTCATTG-3′; and cationic amino acid transporter 2B, forward 5′-CCCAATGCCTCGTGTAATCTA-3′ and reverse 5′-TGCCACTGCACCCGATGACAA-3′. Amplification products were run in 1.5% agarose gels, visualized by ethidium bromide (Sigma) and analyzed in a Gel-Doc instrument (Bio-Rad Laboratories).

Western Blots. Cell extracts were obtained as described previously (16). The expression of arginase I, arginase II, and glyceraldehyde-3-phosphate dehydrogenase was detected by immunoblot using 30 μg of cell extracts. 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 antibodies. The reactions were detected using the enhanced chemiluminescence kit (Amersham).

Arginase Activity Assay. Cell lysates (5 μg) from 3LL cells cultured *in vitro*, tumor-associated myeloid cells, and non-tumor-associated myeloid cells selections were tested for arginase activity by measuring the production of L-ornithine and urea. Briefly, cell lysates were added to 25 μl 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 arginase. Then, a solution containing 150 μl carbonate buffer (100 mM; Sigma) and 50 μl L-Arg (100 mM) was added and incubated at 37°C for 20 min. The hydrolysis reaction from L-Arg to L-ornithine was identified by a colorimetric assay after the addition of ninhydrin solution and incubation at 95°C for 1 h. In addition, the hydrolysis reaction from L-Arg to urea was detected with diacetyl monoxime (Sigma) and incubation at 95°C for 10 min.

Statistical Analysis. Comparisons of CD3ζ expression, L-Arg incorporation, L-Arg concentrations, arginase activity, and tumor volume were done with one way ANOVA test using the Graph Pad statistical program (Graph Pad, San Diego, CA).

RESULTS

Arginase I Expression and Activity in 3LL Lung Carcinoma *in Vivo*. C57BL/6 mice were injected s.c. with 1×10^6 3LL cells. After 14 days the tumors and spleens were excised, made into a single cell suspension and tested for arginase I, arginase II, and inducible nitric oxide synthase expression (mRNA and protein) and activity. Splenocytes from non-tumor-bearing mice and 3LL cells kept in culture were used as controls. All of the cell preparations, except 3LL cultured *in vitro*, expressed arginase II, whereas only the tumor single cell suspension (3LL tumor) expressed arginase I (Fig. 1, *A* and *B*). Furthermore, high arginase activity, as measured by the conversion of L-Arg to ornithine and urea, was only present in the 3LL tumor single cell suspension (Fig. 1*C*; $P < 0.0001$). Inducible nitric oxide synthase, the third enzymatic pathway metabolizing L-Arg, was not detectable in any of the cell preparations (data not shown). The cells producing arginase I in the tumor microenvironment were unknown, although prior reports have shown that some tumor cell lines and peritoneal macrophages can produce arginase *in vitro* (13–15). The 3LL cells expanded *in vitro* and used to generate the tumors in mice did not express arginase I, suggesting that either tumor cells developed the ability to produce arginase I after being injected into mice or that other cells infiltrating the tumor were producing arginase I. Immunohisto-

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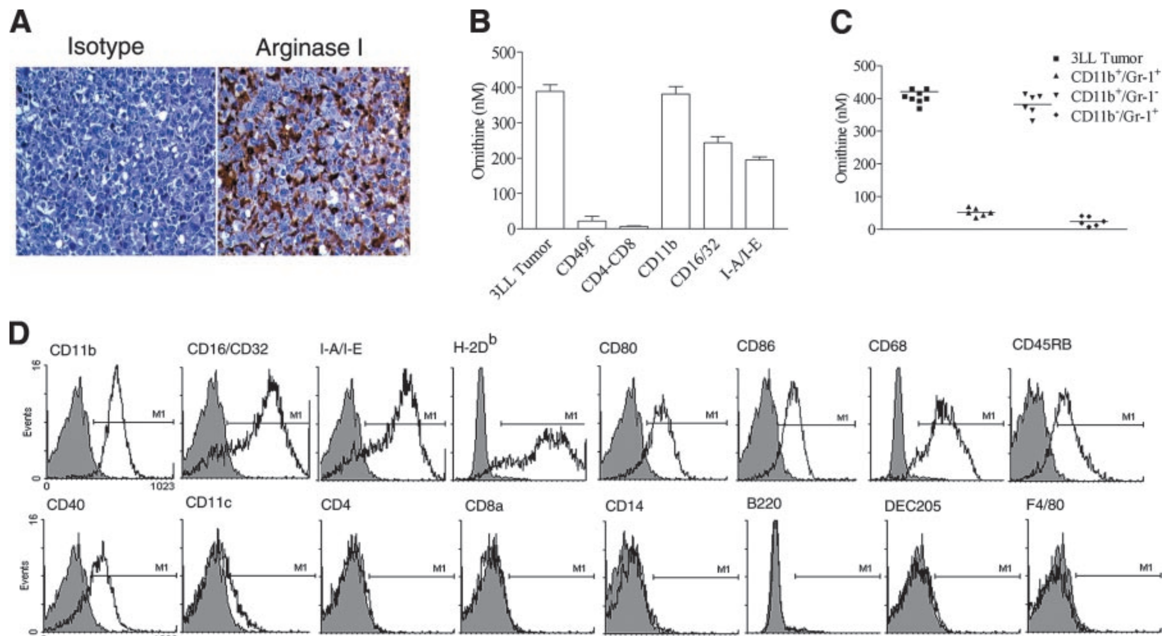

Fig. 2. Arginase I is produced in the tumor by mature myeloid cells. *A,* tumor sections were tested for arginase I by immunohistochemistry. IgG1 isotype was used as control. *B,* single cell suspensions of the 3LL tumor were stained with anti-CD49f (tumor cells), anti-CD4 plus anti-CD8 (T cells), and the myeloid markers CD11b, CD16/CD32, or I-A/I-E, after which the cells were selected using immunomagnetic beads. Arginase activity was tested in the subpopulations as described in "Materials and Methods." *C,* single cell suspensions of 3LL tumors were stained with anti-CD11b FITC and GR-1 PE and sorted using flow cytometry. Cytoplasmic extracts (5 μg) from $CD11b^+/GR-1^+$, $CD11b^+/GR-1^-$, $CD11b^-/GR-1^+$ were then used to determine arginase activity. *D,* $CD11b^+$, $CD16/CD32^+$, $I\text{-}A/I\text{-}E^+$ cells were sorted from a single cell suspension, after which they were stained against different markers and their expression detected by flow cytometry.

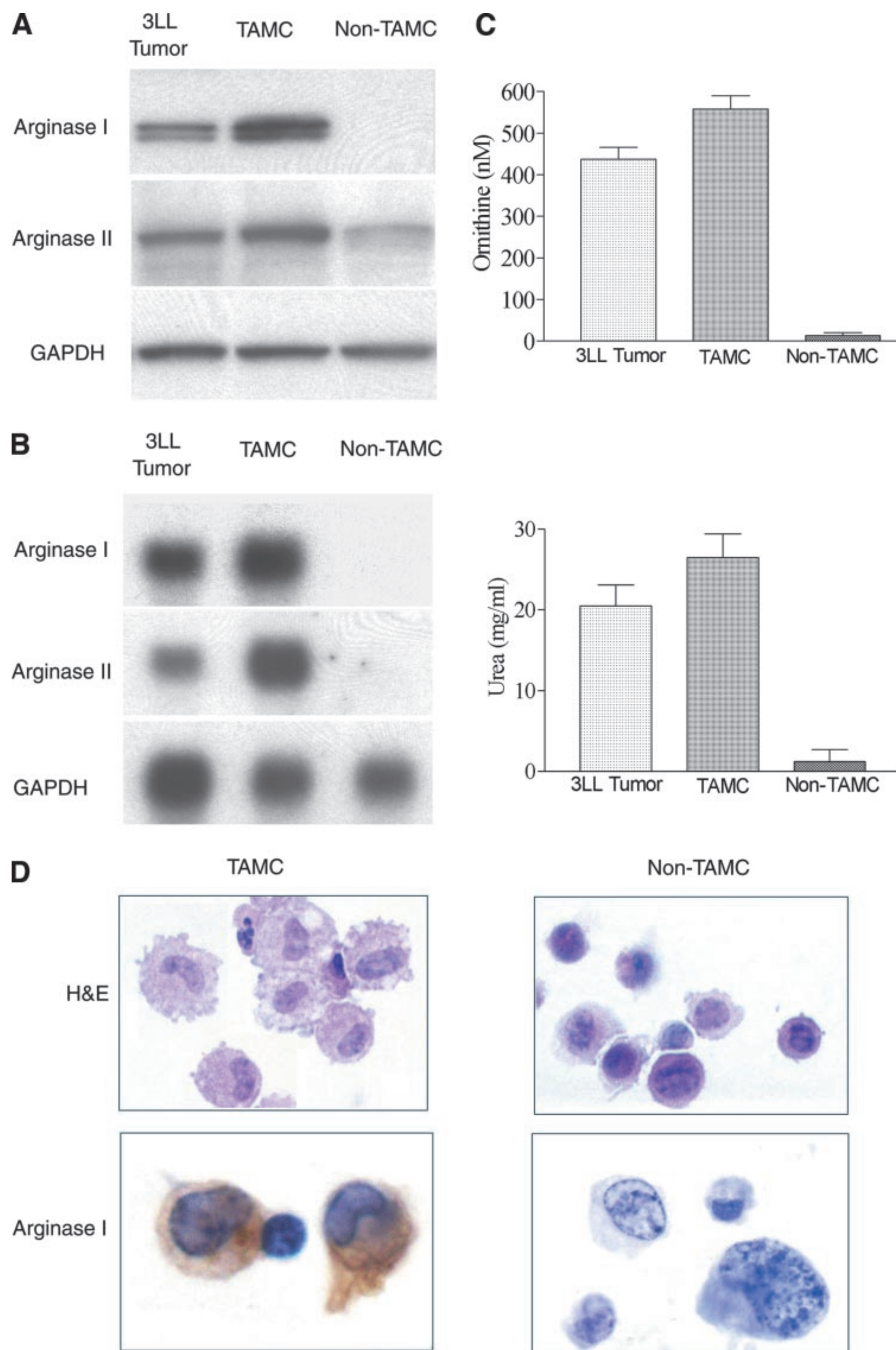

Fig. 3. Tumor-associated myeloid cells (*TAMC*) produce arginase I. TAMC were stained with anti-CD11b, CD16/CD32, I-A/I-E, and sorted using magnetic beads. The remainder cells, composed mostly of tumor cells and T cells, were labeled non-TAMC. *A,* cells were lysed and tested for arginase I and arginase II expression by Western blot. *B,* total RNA from TAMC and non-TAMC were obtained by TRIzol and tested for arginase I and arginase II expression by Northern blot. *C,* 5 μg of cytoplasmic extracts were used to determine arginase activity by the conversion of L-Arg to ornithine and urea. *D,* hematoxylin and eosin staining (*top*) were used to determine the morphology of TAMC and non-TAMC populations. Immunohistochemistry was used to determine arginase I expression in the TAMC and non-TAMC cytospins (*bottom*); *bars,* ±SD.

chemistry done on day 14 tumor sections showed a significant number of arginase I-positive cells, which had a macrophage morphology, whereas tumor cells and infiltrating lymphocytes appeared to be negative (Fig. 2*A*). Analysis of cell markers in the single cell suspensions from the 3LL tumors showed the majority of the cells to be CD49f$^+$ tumor cells (43.07% ± 1.56), infiltrating CD4$^+$ or CD8$^+$ T cells (11.91% ± 3.94), and CD11b$^+$ myeloid cells (39.34% ± 4.23), with ~1% of the cells being CD56$^+$ natural killer cells or CD19$^+$ B cells. A very low expression of CD3 (CD3ε) was seen in the CD4$^+$ and CD8$^+$ T cells (data nor shown). In addition, a significant proportion of the CD11b$^+$ myeloid cells were also positive for CD16$^+$/CD32$^+$ and I-A/I-E$^+$ (data not shown). To further characterize the population producing arginase I, the single cell suspensions of 3LL tumors were stained and sorted into three distinct populations, tumor cells (CD49f$^+$), myeloid cells (CD11b$^+$, CD16/CD32$^+$, and I-A/I-E$^+$), and T cells expressing CD4$^+$ or CD8$^+$, which together accounted for >95% of the cells in the tumor preparations. As shown in Fig. 2*B,* cells expressing CD11b, CD16/CD32, and I-A/I-E had the highest arginase activity as compared with tumor cells (CD49f$^+$) or infiltrating T lymphocytes.

Some subsets of myeloid cells have been shown to have a suppressive activity. Gabrilovich *et al.* (19) and Bronte *et al.* (20) reported

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that immature myeloid cells expressing CD11b$^+$ GR-1$^+$ and isolated from the spleen of tumor-bearing mice could suppress T-cell functions *in vitro*. Therefore, the CD11b$^+$ myeloid cells isolated were additionally sorted into three subpopulations, namely, CD11b$^+$/GR-1$^+$, CD11b$^+$/GR-1$^-$, and CD11b$^-$/GR-1$^+$. High arginase activity was only found in the mature CD11b$^+$/GR-1$^-$ subpopulation but not in the immature CD11b$^+$/GR-1$^+$ myeloid cells or the CD11b$^-$/GR-1$^+$ cells (Fig. 2*C*). The mature CD11b$^+$, GR1$^-$ cells also expressed CD16/CD32, I-A/I-E, H-2D^b, CD80, CD86, CD68, CD40, and CD45RB, and were negative for dendritic cell markers CD11c, B220, DEC-205, CD4, and CD8a, and for other myeloid cells markers such as CD14 and F4/80 (Fig. 2*D*). These cells will be referred from here on as tumor-associated myeloid cells. Tumor-associated myeloid cells stimulated with lipopolysaccharide or CD40L failed to further differentiate into CD11c$^+$ dendritic cells (data not shown).

Arginase activity and expression was then tested in the unseparated single cell suspension, tumor-associated myeloid cells, and the remaining cells that mainly included tumor cells and infiltrating T cells and were labeled non-tumor-associated myeloid cells. Tumor-associated myeloid cells expressed high levels of protein and mRNA for arginase I and arginase II (Fig. 3, *A* and *B*) and had a high arginase activity (Fig. 3*C*). In contrast, non-tumor-associated myeloid cells did not express arginase I and arginase II nor had arginase activity. Again, inducible nitric oxide synthase expression was not detected in the tumor-associated myeloid cell or non-tumor-associated myeloid cell populations (data not shown). Tumor-associated myeloid cells had a distinct morphology with an irregular cytoplasm and a high content of intracytoplasmic vacuoles (Fig. 3*D, top left*), whereas the non-tumor-associated myeloid cells were negative for arginase I and included a mixture of tumor cells and infiltrating lymphocytes (Fig. 3*D, top right*). Arginase I expression was additionally confirmed by immunohistochemistry, showing positive staining for arginase I in the tumor-associated myeloid cells (Fig. 3*D, bottom left,* note that a contaminant lymphocyte is negative for arginase I) but not the non-tumor-associated myeloid cells (Fig. 3*D, bottom right*). Cytokine profiles by RNase protection assay of tumor-associated myeloid cells and non-tumor-associated myeloid cells demonstrated an increased expression of IL-10, IL-1α, IL-1β, IL-1RN, and IL-6 in the tumor-associated myeloid cells but not in the non-tumor-associated myeloid cells (data not shown). Enriched cell subpopulations were also tested for phagocytic ability and the production of esterases. Tumor-associated myeloid cells had a lower capacity to phagocytose FITC-labeled *E. coli* K-12 bioparticles than did control peritoneal macrophages (Fig. 4, *top*), whereas non-tumor-associated myeloid cells had no phagocytic activity. In addition, 89% of tumor-associated myeloid cells were positive for non-specific esterases (Fig 4, *bottom*), as compared with only 33% of the non-tumor-associated myeloid cells and 99% of the peritoneal macrophages.

The kinetics of arginase I production in the tumor showed an increased arginase I expression (Fig. 5*A*) and activity (Fig. 5*B*) as early as day 7 after s.c. injection of the tumor cells, which continued increasing throughout the 21 days of tumor growth. The increasing activity was not caused by a higher percentage of tumor-associated myeloid cells infiltrating the tumor (Fig. 5*C*), suggesting instead an increase in arginase I activity on a per cell basis. Simultaneously, we tested the expression of CD3ζ and CD3ε chains in T cells infiltrating the tumors, finding a major decrease by day 7 (Fig. 5, *D* and *E*). In contrast, splenic T cells from tumor-bearing mice only showed a detectable decrease in CD3ζ by day 14 and CD3ε by day 21, which is in accordance with the original description by Mizoguchi *et al.* (21). Interestingly, little Arginase activity or cells with a tumor-associated myeloid cells phenotype were detected in the spleens of tumor bearing mice even by day 21 (data not shown).

Increased L-Arg Incorporation by Tumor-Associated Myeloid Cells. L-Arg depletion can occur by the secretion of arginase into the microenvironment or an increased uptake of L-Arg into the cells. Culture of tumor-associated myeloid cells failed to demonstrate arginase activity in the tissue culture medium (data not shown). However, analysis of the expression of the cationic amino acid transporters showed that the unseparated tumor cell suspension expressed both cationic amino acid transporter 2B and cationic amino acid transporter 1 (Fig. 6*A*), with cationic amino acid transporter 2B being preferentially expressed in the tumor-associated myeloid cells and cationic amino acid transporter 1 in the non-tumor-associated myeloid cells.

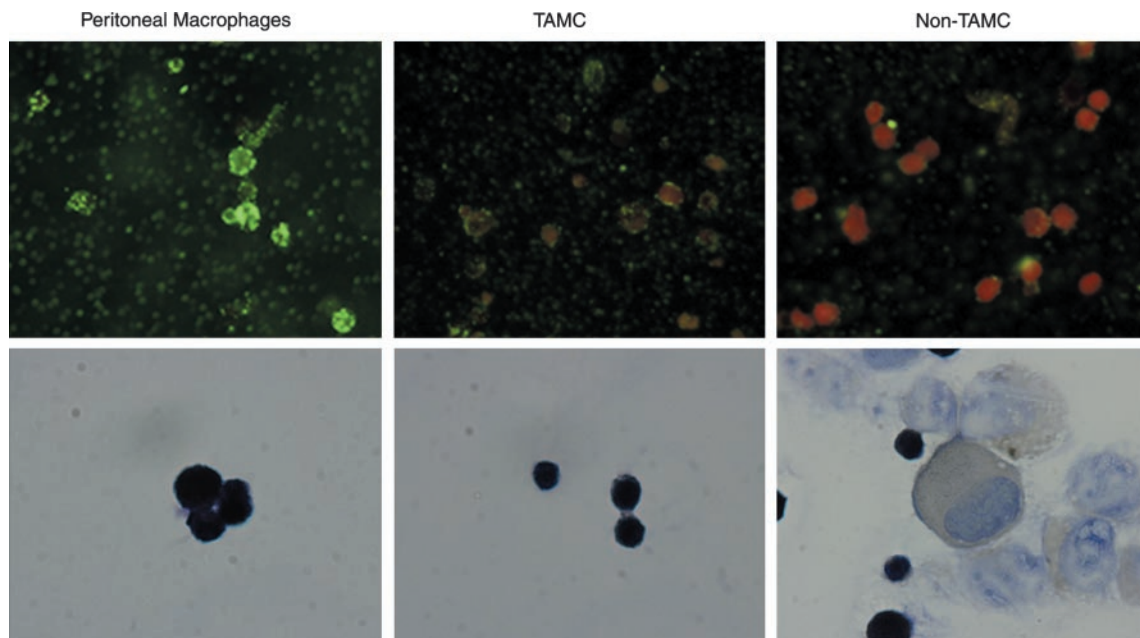

Fig. 4. Tumor-associated myeloid cells (*TAMC*) have a low phagocytic capacity but express esterases. *Top,* peritoneal macrophages, TAMC and non-TAMC, were tested for their ability of phagocyte FITC-labeled *E. coli* (strain K-12). *Bottom,* cytospins from 3 × 10^4 cells were tested for non-specific esterase activity. Experiments were repeated using cells isolated from 5 mice.

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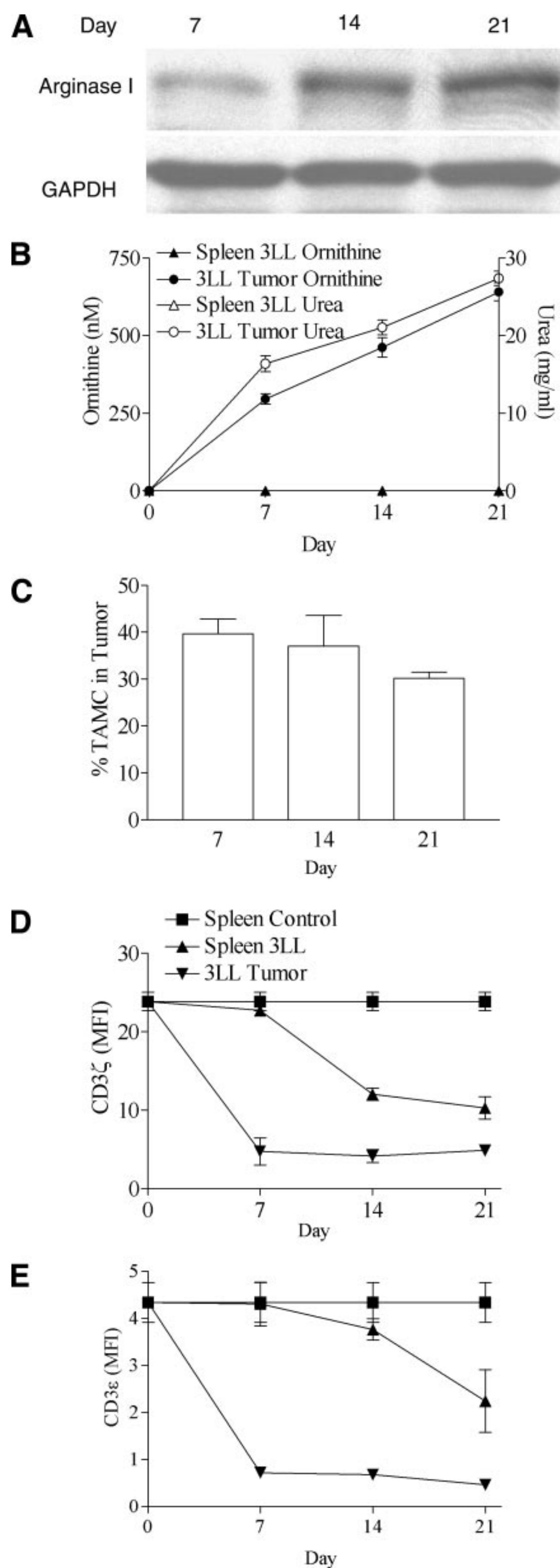

Fig. 5. Kinetics of arginase I and CD3ζ expression in mice injected with 3LL tumors. Cytoplasmic extracts from single cell suspensions of 3LL tumors after 7, 14, and 21 days of injection were used to determine arginase I expression (*A*) and arginase activity (*B*). *C*, the percentage of $CD11b^+$, $CD16^+/32^+$, I-A/I-E^+ cells was determined in tumor suspensions at day 7, 14, and 21 after tumor injection. CD3ζ (*D*) and CD3ϵ (*E*) chain expression was determined in tumor and splenic T cells from tumor-bearing mice and control mice at days 7, 14, and 21; *MFI,* mean fluorescence intensity; *bars,* ±SD.

The increased cationic amino acid transporter 2B expression was confirmed by reverse transcription-PCR using specific primers (Fig. 6*B*). Tumor-associated myeloid cells incorporated [^{3}H]L-Arginine five times higher than the non-tumor-associated myeloid cells and approximately three times higher than the unseparated tumor single cell suspension, in the first 12–24 h of culture (Fig. 6*C*), resulting in a rapid depletion of L-Arg in the tissue culture medium (Fig. 6*D*). The addition of cationic amino acid transporter 2B inhibitors, symmetric dimethyl arginine and L-Lysine, decreased the L-Arg incorporation in the tumor-associated myeloid cell population to levels similar to the non-tumor-associated myeloid cells population (Fig. 6*E*). In contrast, the L-Arg analog, *N*-nitro-L-Arg, which is not transported by cationic amino acid transporter 2, did not affect L-Arg incorporation suggesting that cationic amino acid transporter 2B was mediating L-Arg transport into tumor-associated myeloid cells.

Previous studies have suggested that reactive oxygen species produced by macrophages and granulocytes from tumor-bearing mice or cancer patients can cause the loss of CD3ζ chain and induce T-cell dysfunction (22). To test this possibility purified tumor-associated myeloid cells and non-tumor-associated myeloid cells were labeled with dihydroethidium and dichlorodihydrofluorescein diacetate. Dihydroethidium is a marker of superoxide anion, whereas dichlorodihydrofluorescein diacetate is oxidized by hydrogen peroxide, peroxinitrites, and hydroxyl radicals. Freshly isolated enriched tumor-associated myeloid cells did not have detectable levels of reactive oxygen species (Fig. 7*A*) as evidenced by the absence of fluorescence with dihydroethidium and dichlorodihydrofluorescein diacetate. However, stimulation with phorbol 12-myristate 13-acetate demonstrated their ability to produce reactive oxygen species. In contrast, non-tumor-associated myeloid cells displayed high accumulation of reactive oxygen species, even without additional stimulation by phorbol 12-myristate 13-acetate. Similarly, the production of indoleamine 2, 3-dioxygenase by dendritic cells has been reported as a mechanism that can inhibit T-cell proliferation (7). A low level of indoleamine 2, 3-dioxygenase expression was detected in the non-tumor-associated myeloid cell fraction (Fig. 7*B*), but not in the tumor-associated myeloid cell subpopulation. Thus, the data suggest that tumor-associated myeloid cells are a unique subset of mature myeloid cells that produce high levels of arginase I in the tumor microenvironment but not reactive oxygen species or indoleamine 2, 3-dioxygenase.

Tumor-Associated Myeloid Cells Inhibit CD3ζ Expression and Antigen-Specific Proliferation of T Cells. T cells stimulated with anti-CD3 + anti-CD28 in conventional RPMI 1640 containing L-Arg undergo a cycle of internalization and re-expression of the T-cell receptor proteins including CD3ζ. However, in the absence of L-Arg the re-expression of CD3ζ is blocked, preventing the assembly of new T-cell receptors on the cell membrane (16). Coculture of freshly isolated tumor-associated myeloid cells with T cells stimulated with anti-CD3 + anti-CD28 in transwells (Boyden Chambers) resulted in a similar effect with an inability of T cells to re-express CD3ζ and CD3ϵ chains (Fig. 8, *A* and *B*). In contrast, nontumor-associated myeloid cells did not alter the normal cycling of CD3ζ and the CD3ϵ. This effect was prevented by the addition of the Arginase inhibitor *N*-hydroxy-nor-L-Arg or excess exogenous L-Arg (2 mM) into the culture but not by the addition of catalase, a hydrogen peroxide scavenger (Fig. 8, *C* and *D*), demonstrating that the depletion of L-Arg by tumor-associated myeloid cells caused the impaired T-cell receptor expression. Histograms of CD3ζ and CD3ϵ expression in activated T cells cocultured with tumor-associated myeloid cells for 72 h confirmed the decreased expression of the T-cell receptor chains and their normal re-expression with the addition of the Arginase inhibitor *N*-hydroxy-nor-L-Arg (Fig. 8, *E* and *F*)

We also tested the effect of tumor-associated myeloid cells on

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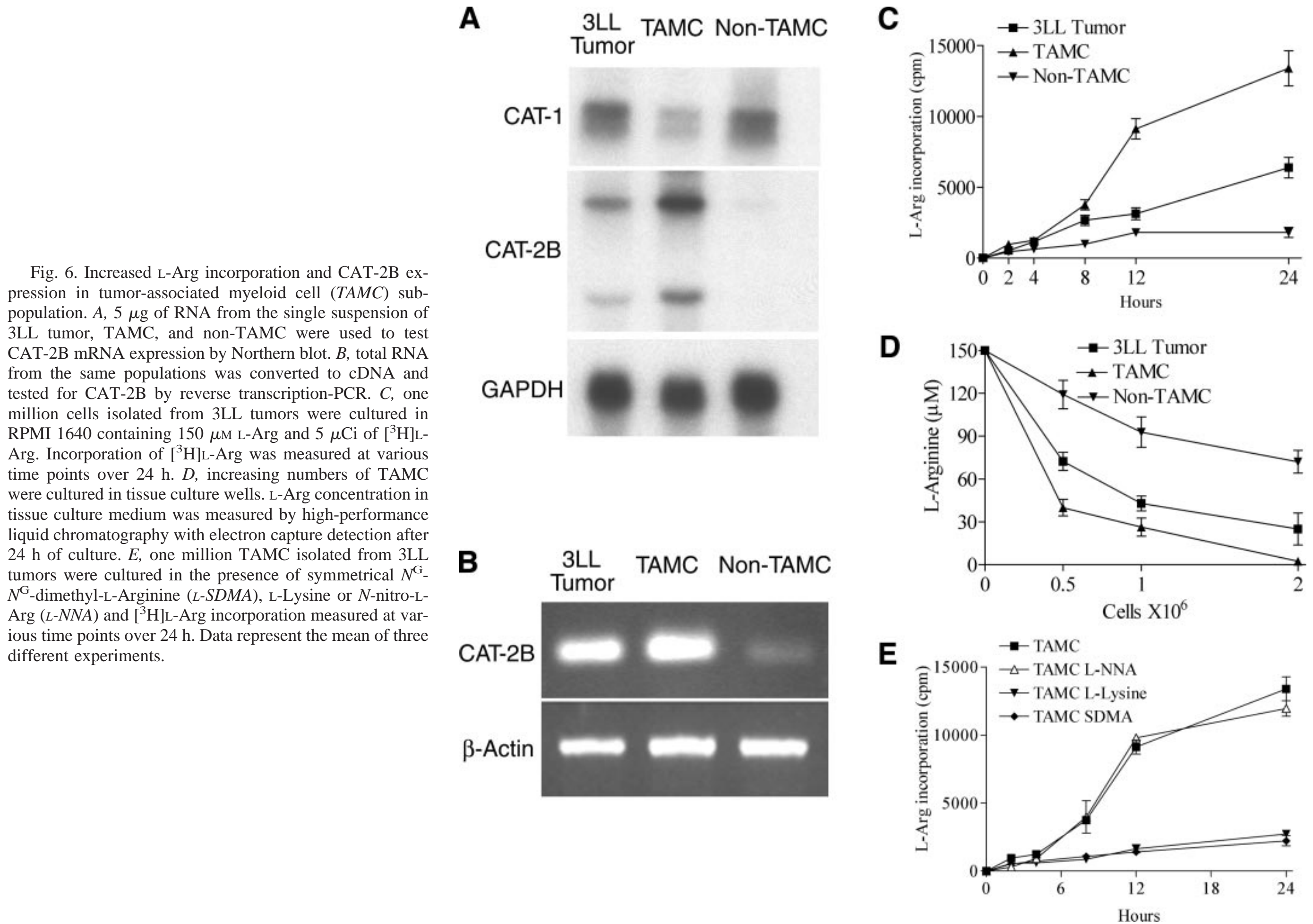

Fig. 6. Increased L-Arg incorporation and CAT-2B expression in tumor-associated myeloid cell (*TAMC*) subpopulation. *A,* 5 μg of RNA from the single suspension of 3LL tumor, TAMC, and non-TAMC were used to test CAT-2B mRNA expression by Northern blot. *B,* total RNA from the same populations was converted to cDNA and tested for CAT-2B by reverse transcription-PCR. *C,* one million cells isolated from 3LL tumors were cultured in RPMI 1640 containing 150 μM L-Arg and 5 μCi of [^{3}H]L-Arg. Incorporation of [^{3}H]L-Arg was measured at various time points over 24 h. *D,* increasing numbers of TAMC were cultured in tissue culture wells. L-Arg concentration in tissue culture medium was measured by high-performance liquid chromatography with electron capture detection after 24 h of culture. *E,* one million TAMC isolated from 3LL tumors were cultured in the presence of symmetrical N^G-N^G-dimethyl-L-Arginine (*L-SDMA*), L-Lysine or *N*-nitro-L-Arg (*L-NNA*) and [^{3}H]L-Arg incorporation measured at various time points over 24 h. Data represent the mean of three different experiments.

antigen-specific T cells from OT-1 and OT-2 transgenic mice that express antigen receptors specific for the OVA peptides 257–264 (SIINFEKL) and 323–339 (ISQAVHAAHAEINEAGR), respectively. Splenocytes from OT-1 and OT-2 transgenic mice were stimulated with their respective peptide for 24 h in the absence of L-Arg. Simultaneously, tumor-associated myeloid cells or non-tumor-associated myeloid cells isolated from tumor-bearing C57BL/6 mice were cultured in the lower chamber of transwells for 24 h in RPMI 1640 containing 150 μM L-Arg. The arginase inhibitor *N*-hydroxy-nor-L-Arg was added to some of the tumor-associated myeloid cell cultures. The stimulated OT-1 and OT-2 splenocytes were then transferred into the upper chamber of the transwells containing the tumor-associated myeloid cells or non-tumor-associated myeloid cells for an additional 72 h, after which proliferation was tested by [^{3}H]thymidine incorporation. Coculture of activated splenocytes with tumor-associated myeloid cells completely blocked OT-1 (Table 1) and OT-2 (Table 2) proliferation to OVA peptides ($P < 0.001$). This suppressive effect of tumor-associated myeloid cells was prevented by the addition of arginase inhibitor *N*-hydroxy-nor-L-Arg or exogenous L-Arg. In contrast, OT-1 and OT-2 cells stimulated with OVA and cocultured with non-tumor-associated myeloid cells proliferated similarly to control cells.

Arginase Activity in Human Non-Small Cell Lung Carcinoma. Individual tumor samples from 4 non-small cell lung carcinoma patients were made into single cell suspensions and tested for arginase I expression and activity. Peripheral blood mononuclear cells from normal individuals were used as controls. Samples from tumors tested showed a high arginase activity and increased arginase I protein expression (Fig. 9, *A* and *B*) as compared with the normal peripheral blood mononuclear cells. Furthermore, the CD3ζ chain expression in the infiltrating T cells was markedly decreased in patients as compared with T cells from normal controls (Fig. 9*C*). Unfortunately normal surrounding lung tissue was not available at the time.

Arginase I Inhibition Reduces Tumor Growth in Vivo. If arginase is indeed a mechanism for tumor evasion, then its inhibition should result in a reduction in tumor growth. C57BL/6 mice were injected in the right flank with 1×10^6 3LL tumor cells. Simultaneously, they were injected s.c. in the left flank once a day during 18 days with varying doses of the arginase inhibitor *N*-hydroxy-nor-L-Arg or *N*-hydroxy-nor-L-Arg plus L-Arg. The L-Arg dose tested (500 mg/kg) is the same used in some trauma patients as an "immune reconstituting" dose. As shown in Fig. 10*A*, tumor growth on day 14 was significantly inhibited by *N*-hydroxy-nor-L-Arg in a dose-dependent manner. This anti-tumor effect was similar with the combination of *N*-hydroxy-nor-L-Arg plus L-Arg. In both treatments the anti-tumor effect was observed during the whole period of the experiment (Fig. 10*B*). In addition, the expression of arginase I was markedly decreased in the tumor-associated myeloid cells infiltrating the tumors of mice receiving *N*-hydroxy-nor-L-Arg (Fig. 10*C*).

To determine whether the anti-tumor effect of *N*-hydroxy-nor-L-Arg was in part mediated by the immune response, C57BL/6-Prkdc^{scid} mice bearing 3LL tumors were injected with 80 mg/kg/day of *N*-hydroxy-nor-L-Arg. C57BL/6-Prkdc^{scid} mice have a mutation in the gene encoding the catalytic subunit of DNA activated protein kinase, which does not allow the rearrangement of genes that code for antigen-specific receptors and, therefore, lack functional T and B

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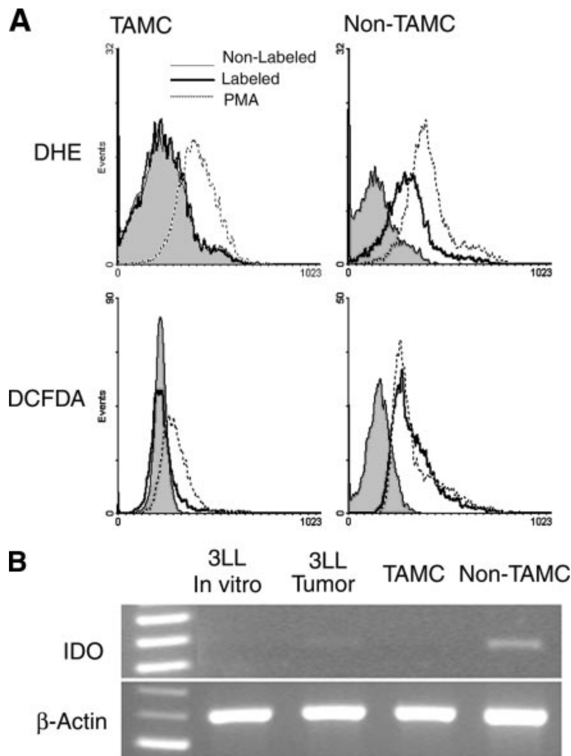

Fig. 7. Tumor-associated myeloid cells (*TAMC*) do not produce reactive oxygen species nor indoleamine 2, 3-dioxygenase (*IDO*). *A,* 1×10^6 TAMC or non-TAMC were labeled with 2 μM of dihydroethidium (*DHE*) or dichlorodihydrofluorescein diacetate (*DCFDA*), washed twice with RPMI 1640 and fluorescence determined by flow cytometry. Negative control included non-labeled cells, whereas positive control consisted of labeled cells stimulated 10 min 37°C with 50 ng/ml phorbol 12-myristate 13-acetate. *B,* indoleamine 2, 3-dioxygenase expression was tested in the same subpopulations by reverse transcription-PCR.

cells. The inhibition in tumor growth seen in wild-type C57BL/6 mice treated with *N*-hydroxy-nor-L-Arg was not observed in the 3LL-bearing C57BL/6-Prkdc^{scid} mice (Fig. 10*D*), suggesting that the anti-tumor effect caused by the inhibition of arginase is in part dependent on lymphocyte function.

DISCUSSION

L-Arg is a nonessential amino acid that plays a central role in several biological systems including the immune response. L-Arg is metabolized by arginase I, arginase II, and inducible nitric oxide synthase (11). Arginase I and arginase II are encoded by two distinct genes and hydrolyze L-Arg into urea and L-ornithine, the latter being the main substrate for the production of polyamines that are required for cell cycle progression. L-Arg is also metabolized by inducible nitric oxide synthase to citrulline and nitric oxide, a highly reactive compound important in vascular homeostasis, and as part of the cytotoxic mechanism of macrophages (12). The importance of L-Arg on the immune response was initially suggested by the association between an impaired T-cell function and the reduction in serum L-Arg levels found in patients and rodents after liver transplantation or trauma, a process that was rapidly reversed by the enteral or parenteral supplementation of L-Arg (23). However, the mechanisms by which L-Arg starvation impaired T-cell function were unknown. We initially reported that T cells cultured in the absence of L-Arg loose CD3ζ expression and are unable to proliferate (10). More recently, Rodriguez *et al.* (16) showed that murine peritoneal macrophages stimulated with T-helper 2 cytokines (IL-4 + IL-13) produce arginase I, rapidly reducing L-Arg concentration in the microenvironment and inducing T-cell dysfunction. In contrast, macrophages producing arginase II do not deplete L-Arg from the microenvironment and do not impair T-cell function. However, it was unclear whether these *in vitro* mechanisms were present in disease.

An increased Arginase activity has been described in patients with different types of tumors (13, 14) and has been associated with the need of malignant cells to produce polyamines to sustain their rapid proliferation (15). However, the source of Arginase and its potential role as a mechanism for tumor evasion were unclear. The results shown here demonstrate that in the 3LL model a distinct subpopulation of tumor-infiltrating myeloid cells, and not tumor cells, produces high levels of arginase I and are potent inhibitors of T-cell receptor expression and antigen-specific T-cell responses. These cells have also been shown to be potent inducers of regulatory T cells (24) and, therefore, may represent a unique subpopulation with the ability to suppress the protective immune response through various mechanisms. Other cells within the tumor microenvironment including the malignant cells, T lymphocytes, and even other myeloid subpopulations did not produce arginase I and did not impair T-cell function in this tumor model. Furthermore, the almost complete inhibition of the suppressive function of tumor-associated myeloid cells by an Arginase inhibitor suggested that arginase I may represent one of the principal mechanisms used by these cells to impair T-cell function. Therefore, the increase in arginase I expression may not only facilitate tumor growth, but may also have as a secondary effect, the local reduction of L-Arg levels allowing tumors to escape the immune response.

Previous reports have suggested that Arginase activity in cancer patients might be coming from the tumor cells to sustain their rapid proliferation (15). However, our data evaluating multiple human lung carcinoma cell lines failed to demonstrate any Arginase activity or arginase I expression.[6] Which signal(s) initiate the production of arginase I by the tumor-associated myeloid cells is still unclear. Murine peritoneal macrophages stimulated with IL-4 and IL-13 produce arginase I but not inducible nitric oxide synthase, whereas macrophages stimulated with interferon γ produce inducible nitric oxide synthase and not arginase I. However, initial studies of the 3LL cell line fail to show the production of either cytokine.[6] Therefore, it is possible that T cells, which preferentially produce T-helper 2 cytokines in mice with progressively growing tumors (25), may initiate the production of arginase I by tumor-associated myeloid cells. The sequence of signals from tumor derived factors and cytokines from infiltrating T cells that initiate and sustain the production of arginase in tumor-associated myeloid cells remains to be fully understood.

How low do L-Arg levels need to be to alter CD3ζ chain expression and T-cell function, and can these levels occur *in vivo*? L-Arg levels in serum of normal individuals ranges from 50 μM to 150 μM. *In vitro* data show that concentrations below 40 μM cause the rapid decrease of CD3ζ chain in Jurkat cells and impair its re-expression in stimulated T cells.[7] Consistent with this, rodents and patients with trauma or patients undergoing liver transplantation have a rapid decrease in circulating L-Arg levels to concentrations below 40 μM (26, 27). This phenomenon is paralleled by poor T-cell function (27) and, as shown recently by Ichihara *et al.* (28), with a decreased expression of CD3ζ chain. Additional experiments with transgenic mice that overexpress arginase I in enterocytes show a selective depletion of L-Arg in serum, concomitant with a poor thymic development (29). Preliminary experiments have shown large variations in the levels of L-Arg in the serum of tumor-bearing mice in the first 14 days after tumor implan-

[6] P. Rodriguez, unpublished observations.
[7] P. Rodriguez and A. Zea, unpublished observations.

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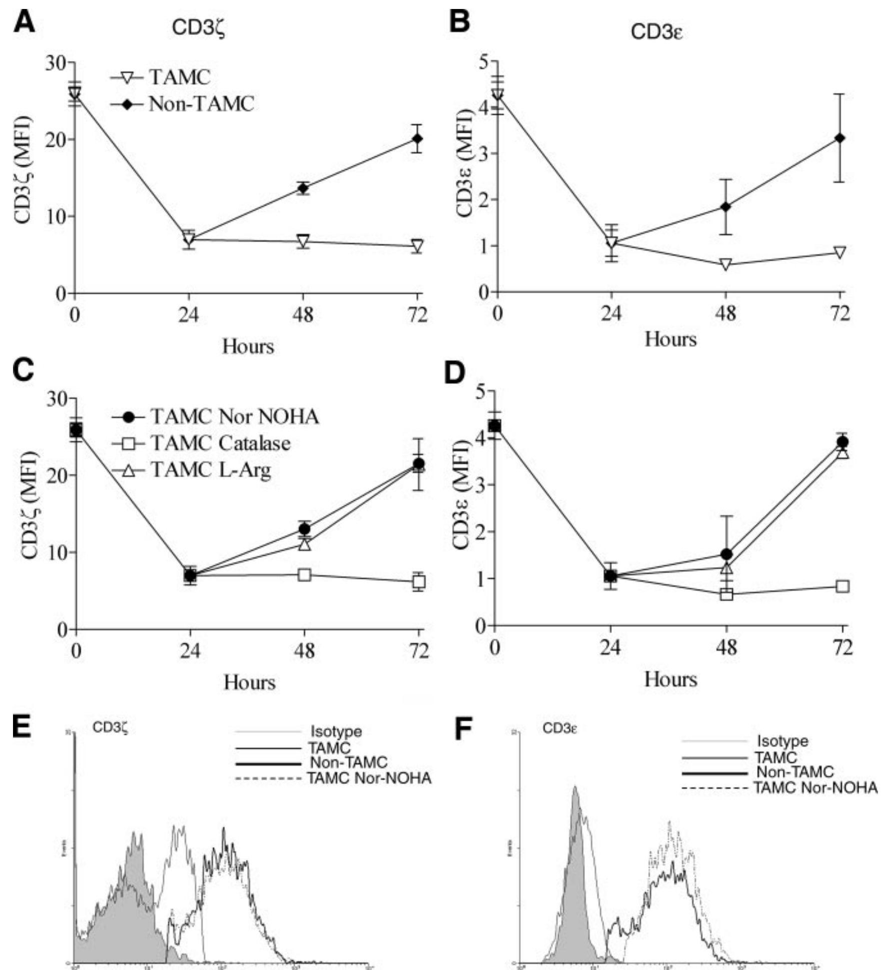

Fig. 8. Arginase I produced by tumor-associated myeloid cells (*TAMC*) prevents the re-expression of CD3ζ and the T-cell receptor in T lymphocytes stimulated with anti-CD3 plus anti-CD28. Murine T cells were stimulated in L-Arg-free RPMI 1640. Simultaneously, TAMC or non-TAMC populations were cultured for 24 h in RPMI 1640 containing 150 μM L-Arg. Stimulated T cells were then added onto the upper chamber of transwells and after an additional 24 and 48 h of culture, CD3ζ (*A*) and CD3ε (*B*) were tested by flow cytometry. Some wells included *N*-hydroxy-nor-L-Arg (50 μM), excess L-Arg (2 mM), or catalase (200 units/ml; *C* and *D*). Data represent the mean fluorescence intensity (*MFI*) of CD3ζ and CD3ε of three different experiments; *bars,* ±SD. *E* and *F* represent an example of histograms of CD3ζ and CD3ε of activated T cells cocultured for 72 h with TAMC, non-TAMC, or TAMC and *N*-Hydroxy-nor-L-Arg.

Table 1 *Arginase I produced by TAMC inhibits OVA-specific OT-1 responses in vitro*

	No cocultures	TAMC	Non-TAMC	TAMC + Nor-NOHA	TAMC + L-Arg
Nonstimulated	**33***	**48.5**	**33**	**88.5**	**42**
	(41)	(23)	(36)	(40)	(32)
OVA$^{257–264}$	**20713**	**217**	**23842**	**20517**	**24562**
	(1050)	(47.3)	(5476)	(2849)	(3023)
CD3 + CD28	**26062**	**507**	**28996**	**27116**	**28562**
	(3535)	(78)	(4462)	(1254)	(4082)

NOTE. OT-1 were stimulated with 1 μg/ml OVA$^{257–264}$ (SIINFEKL) peptide and cocultured with TAMC, non-TAMC, or TAMC plus Nor-NOHA or exogenous L-Arg. Proliferation was tested after 72 h of culture.

* **Mean of three experiments** (±SD).

tation (data not shown). It is possible that serum levels of L-Arg fail to reflect the changes occurring within the tumor microenvironment.

Arginase activity, however, is not detected to a great extent in the tissue culture medium of the tumor-associated myeloid cells, suggesting that L-Arg incorporation could be the mechanism of L-Arg consumption. The cationic amino acid transporter 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 (30). In accordance with these observations, tumor-associated myeloid cells, which express high levels of cationic amino acid transporter 2B and arginase I, have an increased incorporation of L-Arg and rapidly reduce extracellular concentration of L-Arg. In contrast, tumor cells and other lymphoid-infiltrating cells (non-tumor-associated myeloid cells), which express cationic amino acid transporter -1, do not.

Recent publications have suggested a close correlation between the availability of certain amino acids and the immune response. Munn *et al.* (7) described that tryptophan metabolism by macrophages producing indoleamine 2, 3-dioxygenase inhibits T-cell proliferation and sensitizes activated T cells to apoptosis (18). Tumor-associated myeloid cells, however, did not express indoleamine 2, 3-dioxygenase, and there was only a low-level expression in the non-tumor-associated myeloid cell population. Several reports have also shown the presence of immature myeloid cells expressing CD11b$^+$, GR-1$^+$, F4/80$^+$ in the spleen of tumor-bearing mice. These cells produce reactive oxygen species and low levels of arginase I, which can increase after stimulation with IL-4. These immature myeloid suppressor cell lines can impair alloreactivity *in vitro* through a nitric oxide-dependent mechanism (8, 9, 31) and induce T-cell apoptosis; however, they require cell-cell contact to impair T-cell function (8, 31, 32). In contrast, the tumor-associated myeloid cell subpopulation reported here expresses mature myeloid markers, does not require cell to cell contact to inhibit

Table 2 *Arginase I produced by TAMC inhibits OVA-specific OT-2 responses in vitro*

	No cocultures	TAMC	Non-TAMC	TAMC + Nor-NOHA	TAMC + L-Arg
Nonstimulated	**19.5***	**22**	**61**	**33**	**23**
	(37)	(39)	(41)	(29)	(45)
OVA$^{323–339}$	**17073**	**164**	**15159**	**15562**	**19562**
	(2050)	(67.8)	(1023)	(652)	(2156)
CD3 + CD28	**19209**	**356**	**18572**	**23267**	**22341**
	(1858)	(64)	(1429)	(2467)	(3602)

NOTE. OT-2 splenocytes were stimulated with 3 μg/ml OVA$^{323–339}$ (ISQAVHAA-HAEINEAGR) and co-cultured with TAMC, non-TAMC or TAMC plus Nor-NOHA or exogenous L-Arg. Proliferation was tested after 72 h of culture.

* **Mean of three experiments** (±SD).

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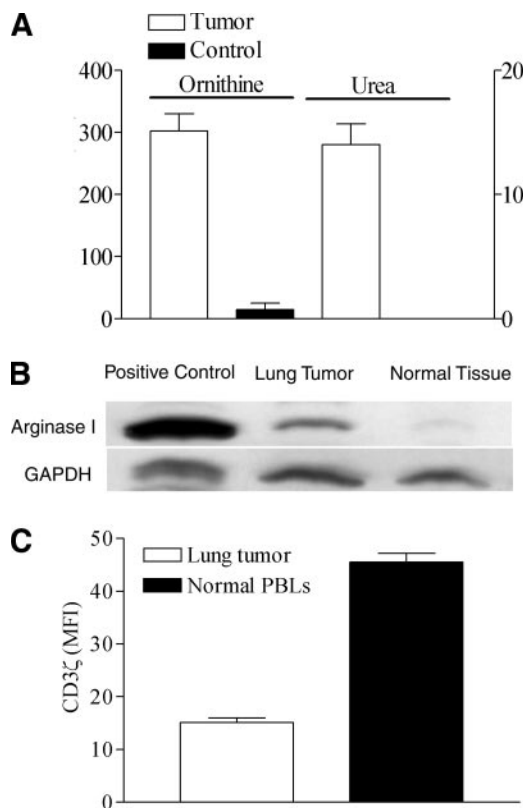

Fig. 9. Single cell suspensions from human non-small cell lung tumors have increased expression of Arginase I and decreased expression of CD3ζ. *A,* cytoplasmic extracts (100 μg) from single cell suspensions of 4 non-small cell lung tumors and peripheral blood leukocytes of 3 normal individuals were tested for arginase activity. *B,* a representative example of the increased expression of arginase I in tumors of non-small cell lung carcinoma patients. Human liver was used as positive control, whereas peripheral blood leukocytes from a normal individual were used as normal tissue. *C,* the expression of CD3ζ was tested by flow cytometry in tumor infiltrating T lymphocytes of 4 patients with non-small cell lung carcinoma and peripheral blood leukocytes of 4 normal individuals; *bars,* ±SD.

T-cell activity (as shown in the transwell experiments), and does not cause T-cell apoptosis in cocultures (data not shown). Therefore, the CD11b$^+$ tumor-associated myeloid cells producing arginase I may represent a unique subpopulation of potent suppressor cells within the tumor microenvironment.

How amino acid starvation impairs T-cell function is unclear. The absence of the essential amino acid leucine has been associated with an increase in the synthesis and stability of mRNA for *CHOP* (33). This gene encodes a transcription factor that interacts with CCAAT/enhancer-binding proteins family, which, in turn, may inhibit the normal proliferation of cells (34). Our data show that metabolism of the non-essential amino acid L-Arg may also control T-cell function through the modulation of CD3ζ expression. In Jurkat cells the decrease of CD3ζ appears to be caused by a decreased CD3ζ mRNA stability (10), associated with *de novo* synthesis of a protein that releases a ribonucleoprotein complex bound to the 3′ untranslated region of CD3ζ mRNA.[6] In normal human T cells, however, L-Arg starvation appears to alter CD3ζ re-expression by a specific decrease in CD3ζ translation.[8] L-Arg starvation and accumulation of empty tRNA for L-Arg could induce the activation of GCN2, which phosphorylates eIF2α and inhibits the access of methionyl tRNA to the ribosome and, therefore, impairs the initiation of translation (35).

The injection of *N*-hydroxy-nor-L-Arg and *N*-hydroxy-nor-L-Arg plus L-Arg in tumor-bearing mice significantly inhibited tumor growth in a dose-dependent manner. This was associated with a lower expression of arginase I in the tumor-associated myeloid cells population but no changes in the percentage of these cells infiltrating the tumor. The effect of *N*-hydroxy-nor-L-Arg appears to be in part mediated by a lymphocyte response. Ongoing studies are aimed at additionally elucidating the anti-tumor mechanism of arginase inhibition.

Arginase I production alone is unlikely to be the only mechanism

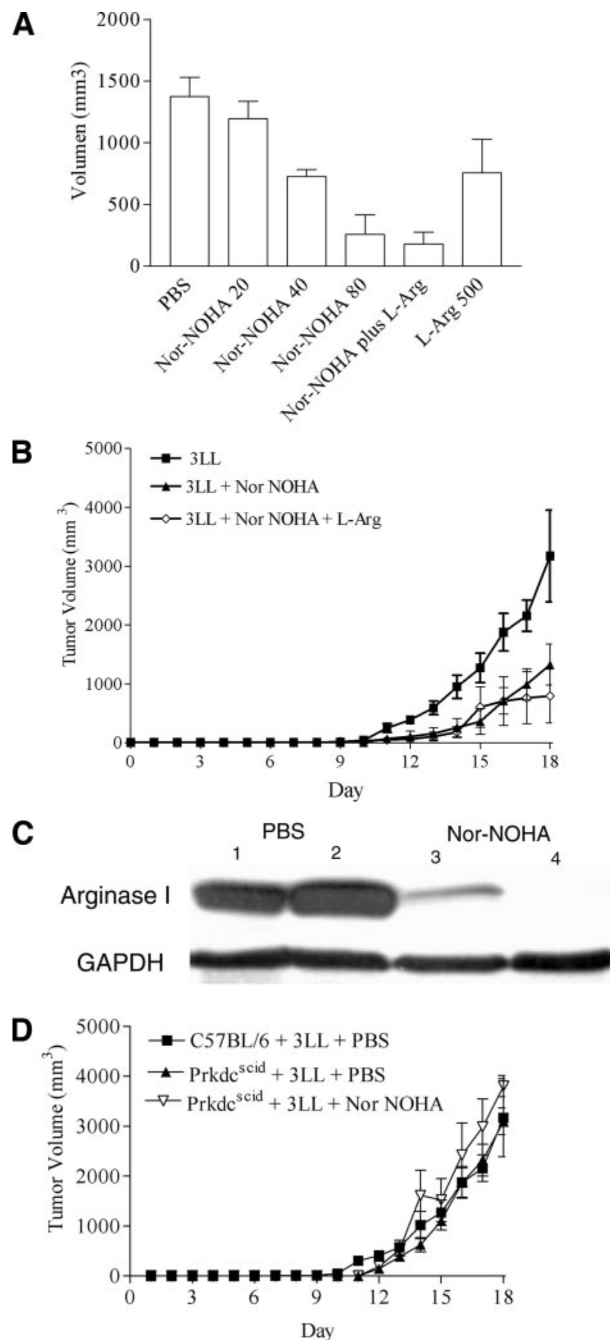

Fig. 10. Arginase inhibitor *N*-hydroxy-nor-L-Arg (*Nor-NOHA*) inhibits tumor growth. *A,* mice injected s.c. with 1×10^6 3LL cells were injected daily in the opposite flank with PBS, Nor-NOHA (20, 40 or 80 mg/kg), L-Arg (500 mg/kg), or Nor-NOHA plus L-Arg (80 mg/kg and 500 mg/kg, respectively). Tumor volume was determined after 15 days of tumor injection by using the formula: smaller diameter^2 × larger diameter × 0.5. Data include groups of 6 mice. *B,* tumor volume during the first 18 days of following. *C,* Arginase I expression was tested in the TAMC of 2 Nor-NOHA treated (80 mg/kg) and 2 untreated mice. *D,* Prkdc^{scid} mice injected s.c. with 1×10^6 3LL cells were injected daily in the opposite flank with PBS or Nor-NOHA (80 mg/kg). Data represents mean of tumor volume of 8 mice per condition.

[8] A. Zea, unpublished observations.

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by which tumors impair T-cell function. Fas-Fas ligand interactions, the production of hydrogen peroxide, or indoleamine 2, 3-dioxygenase by macrophages or neutrophils may also play an important role in this process (3, 36). Additional studies will determine the relative contribution of each of these mechanisms in impairing T-cell function and possibly blocking the therapeutic potential of immunotherapy.

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