Arginase-Producing Myeloid Suppressor Cells in Renal Cell Carcinoma Patients: A Mechanism of Tumor Evasion

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Abstract

Myeloid suppressor cells with high arginase activity are found in tumors and spleen of mice with colon and lung cancer. These cells, described as macrophages or immature dendritic cells, deplete arginine and impair T cell proliferation and cytokine production. Although arginase activity has been described in cancer patients, it is thought to originate from tumor cells metabolizing arginine to ornithine needed to sustain rapid cell proliferation. The goal of this study was to determine whether myeloid suppressor cells producing high arginase existed in renal cell carcinoma patients. Peripheral blood mononuclear cells from 123 patients with metastatic renal cell carcinoma, prior to treatment, were found to have a significantly increased arginase activity. These patients had a markedly decreased cytokine production and expressed low levels of T cell receptor CD3ζ chain. Cell separation studies showed that the increased arginase activity was limited to a specific subset of CD11b+, CD14−, CD15+ cells with a polymorphonuclear granulocyte morphology and markers, instead of macrophages or dendritic cells described in mouse models. Furthermore, these patients had low levels of arginine and high levels of ornithine in plasma. Depletion of the CD11b+, CD14− myeloid suppressor cells reestablished T cell proliferation and CD3ζ chain expression. These results showed, for the first time, the existence of suppressor myeloid cells producing arginase in human cancer patients. In addition, it supports the concept that blocking arginase may be an important step in the success of immunotherapy. (Cancer Res 2005; 65(8): 3044-8)

Introduction

Immunotherapy with cytokines is a standard of treatment in renal cell carcinoma (RCC) patients. Unfortunately, only 5% to 20% respond to treatment. Tumor-induced anergy may in part explain the low response rates. However, the mechanisms of immune dysfunction in cancer patients remain unclear. Suppressor myeloid cells with high arginase activity, recently described in mice with cancer, deplete arginine from the microenvironment profoundly inhibiting T cell proliferation, cytokine production, and the expression of T cell receptor CD3ζ chain (1, 2). We asked whether a similar mechanism was present in patients. In collaboration with the Cytokine Working Group, we studied peripheral blood mononuclear cells (PBMC) from 123 patients with metastatic RCC, collected prior to treatment. Results showed high arginase activity in PBMC compared with normal controls. Additional studies in 15 new patients determined the types of cells producing arginase and their effect on arginine and ornithine levels and T cell function.

Materials and Methods (details in Supplemental Information)

Samples. Frozen pretreatment PBMC from 123 metastatic RCC patients participating in a Cytokine Working Group trial (3) and 33 normal controls with similar age and gender distributions were initially studied. Additional samples from 15 new RCC patients and 10 normal controls were used for cell separation studies at a later time.

Flow cytometry. Surface markers for T cells, natural killer cells, monocytes, dendritic cells, and polymorphonuclear granulocytes were tested. Results were expressed as the percentage of positive cells and mean fluorescence intensity.

Arginase activity. Cytoplasmic extracts of PBMC and purified subpopulations from 118 patients were tested for arginase expression by Western blot and arginase activity by conversion of L-arginine to L-ornithine (nanomoles of ornithine per million cells per hour), described elsewhere (2).

RT-PCR. RNA from 2×10^6 PBMC or purified cell subsets were extracted, treated with DNase I (Invitrogen, Carlsbad, CA), and reverse-transcribed using Superscript II. PCR amplification was done using primers for arginase I, indoleamine 2,3-dioxygenase (IDO), and β-actin (see Supplemental data for sequences). Fragment sizes: arginase I, 466 bp; IDO, 413 bp; and β-actin, 661 bp. PCR products were visualized in ethidium bromide agarose gel.

Western blots. Thirty micrograms of cell lysates were electrophoresed in 10% Tris-glycine gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with anti-human-arginase I, arginase II (Santa Cruz Biotechnology, Santa Cruz, CA) and glyceraldehyde-3-phosphate dehydrogenase (RDI, Flanders, NJ).

L-Arginine and L-ornithine serum levels. High-performance liquid chromatography with electrochemical detection was done using an ESA-CoulArray Model 540, with an 80 × 3.2 column with 120 Å pore size (4). Plasma was deproteinized and derivatized with 0.2 mol/L orthopthaldialdehyde/β-mercaptoethanol. Fifty microliters were injected per sample. Standards of L-arginine and ornithine in methanol were run with each experiment.

Cytokine production and cell proliferation. PBMC were stimulated with anti-CD3 (30 ng/mL) plus anti-CD28 (100 ng/mL), supernatants were collected at 48 and 72 hours and cytokines measured using the Th1/Th2 panel Bio-Plex assay (Bio-Rad, Hercules, CA) following the manufacturer's instructions. T cell proliferation was measured by changes in fluorescence using carboxy-fluoresceindiacetate succinimidyl ester.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Statistics. Differences between the groups were determined by Wilcoxon's rank sum test. Relationships between two variables were quantified by Spearman's rank correlation coefficient.

Results

We initially tested PBMC from 123 metastatic RCC patients collected before the start of treatment. A highly significant ($P < 0.01$) increase in arginase activity was seen in patient's PBMC (mean = 128.5 ± 130.0, range = 0-654.8) compared with controls (mean = 32.6 ± 48.6, range = 0-182; Fig. 1*A*). This was accompanied by a decreased CD3ζ chain expression (mean fluorescence intensity = 21.39 versus control mean fluorescence intensity = 35.4, $P < 0.01$; Fig. 1*B*) and a diminished production of IFN-γ and IL-2 (Fig. 1*C*) and IL-4 and IL-10 (Supplemental Information, Fig. 1) after stimulation with anti-CD3+ anti-D28. These results could not be readily explained by major differences in the distribution of cell subpopulations or an increased apoptosis of T cells (data not shown). Unfortunately, additional samples from these initial patients were not available. Therefore, 15 newly diagnosed RCC patients were added for further research.

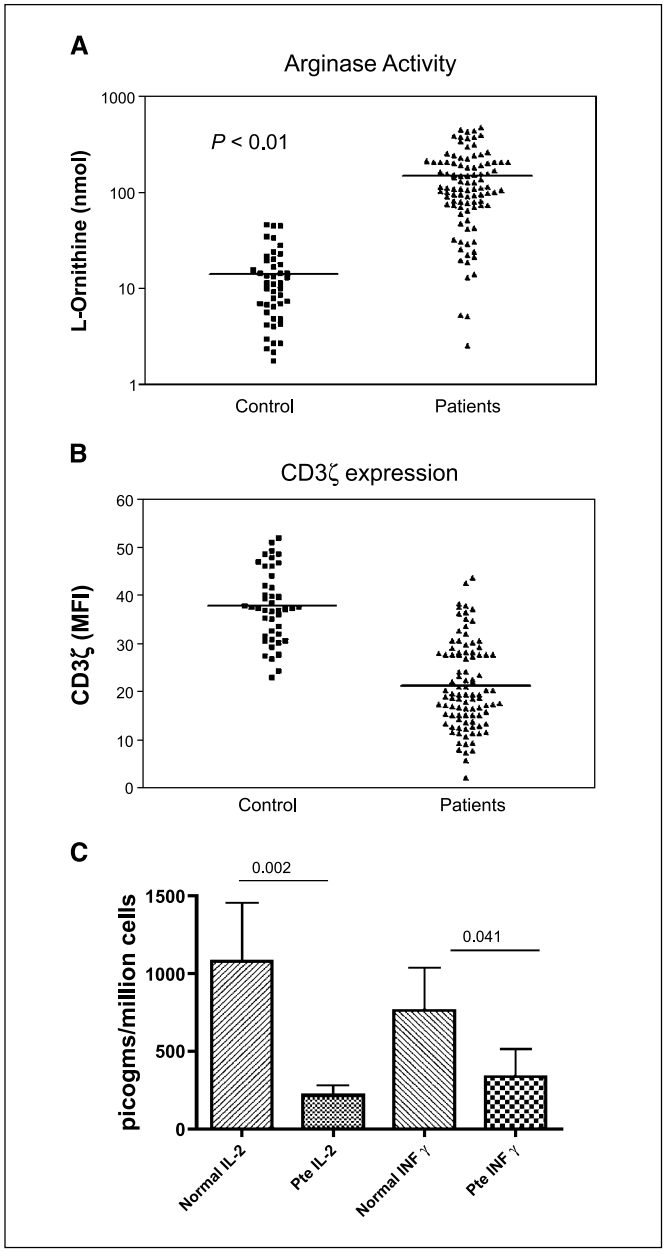

Figure 1. PBMC from 123 patients and 33 normal controls were tested for arginase activity (*A*) and CD3ζ expression (*B*). Cytokine production by PBMC from 49 RCC patients and 15 normal controls were tested at 48 hours after stimulation with anti-CD3 + anti-CD28 (*C*).

Previous reports suggested that increased arginase in cancer patients came from tumor cells metabolizing arginine to polyamines needed to sustain rapid cell proliferation (5). Murine models suggested instead that it came from macrophages infiltrating tumors or immature myeloid cells in spleen (2, 6). The role of these cells in patients was unknown. Patient's PBMC showed a significant increase in a CD11b+,CD14− subset of cells ($P = 0.037$, Fig. 2*A*), which accounted for the majority of cells in the monocyte flow cytometry gate, compared with normal controls (Fig. 2*B*). Phenotyping revealed that CD11b+,CD14− cells also expressed CD15, but were negative for CD80, CD83, CD86, MHC II, and CD11a (Fig. 2*C*, *bottom*), in contrast to mature monocytes from patients or controls (Fig. 2*C*, *top*). Interestingly, Giemsa staining of the CD11b+,CD14− arginase-producing cells revealed a polymorphonuclear granulocyte morphology distinct from the mononuclear CD11b+,CD14+ monocytes (Fig. 2*D*).

Consequently, an increased arginase I expression was found in freshly isolated patient's PBMC (Fig. 3*A*), specifically in CD11b+,CD14− cells, but not in CD11b+,CD14+ mature monocytes (Fig. 3*B*). Statistical analysis, of 15 new patients tested showed a positive correlation between the percentage of CD11b+,CD14− cells in peripheral blood and arginase activity ($r = 0.9833$, Fig. 3*C*), and a negative correlation between arginase activity and CD3ζ chain expression ($r = -0.8167$, Fig. 3*D*). Arginase metabolizes arginine to ornithine. Plasma arginine levels were decreased in the 15 additional patients studied (mean = 28.48 ± 32.51 μmol/L) compared with controls (mean = 90.0 ± 15.56 μmol/L, $P < 0.025$). Conversely, ornithine levels were increased in patients (172.1 ± 61.01) compared with controls (81.5 ± 26.16, $P < 0.001$; Fig. 3*E*).

Other mechanisms of immunosuppression including IDO were not detected in CD11b+,CD14− cells by RT-PCR (Supplemental Information, Fig. 2). Both CD11b+,CD14− suppressor myeloid cells and CD11b+,CD14+ monocytes produced similar amounts of H_2O_2 at rest and upon stimulation with phorbol 12-myristate 13-acetate (data not shown), suggesting that this was not the principal suppressor mechanism of these cells.

Depletion of CD11b+ cells, but not CD14+ cells reestablished proliferation (Fig. 4*A*), cytokine production (Fig. 4*B*), and CD3ζ chain expression (Fig. 4*C*). Representative data from one of three patient samples depleted of CD11b+ or CD14+ cells (Fig. 4) show that 48 hours after depletion and stimulation with anti-CD3 + anti-CD28, only cells depleted of CD11b+ myeloid suppressor cells recovered the capacity to proliferate and produce IFN-γ, while those depleted of CD14+ cells did not (Fig. 4*A* and *B*). Furthermore, CD3ζ chain expression was reexpressed 48 to 72 hours after depletion of the CD11b+ myeloid suppressor cells (but not the CD14+ cells) and culture in medium with physiologic concentrations of arginine (150 μmol/L, Fig. 4*C*). Similar results were obtained when depleting with anti-CD15, also expressed in the myeloid suppressor subpopulation (data not shown).

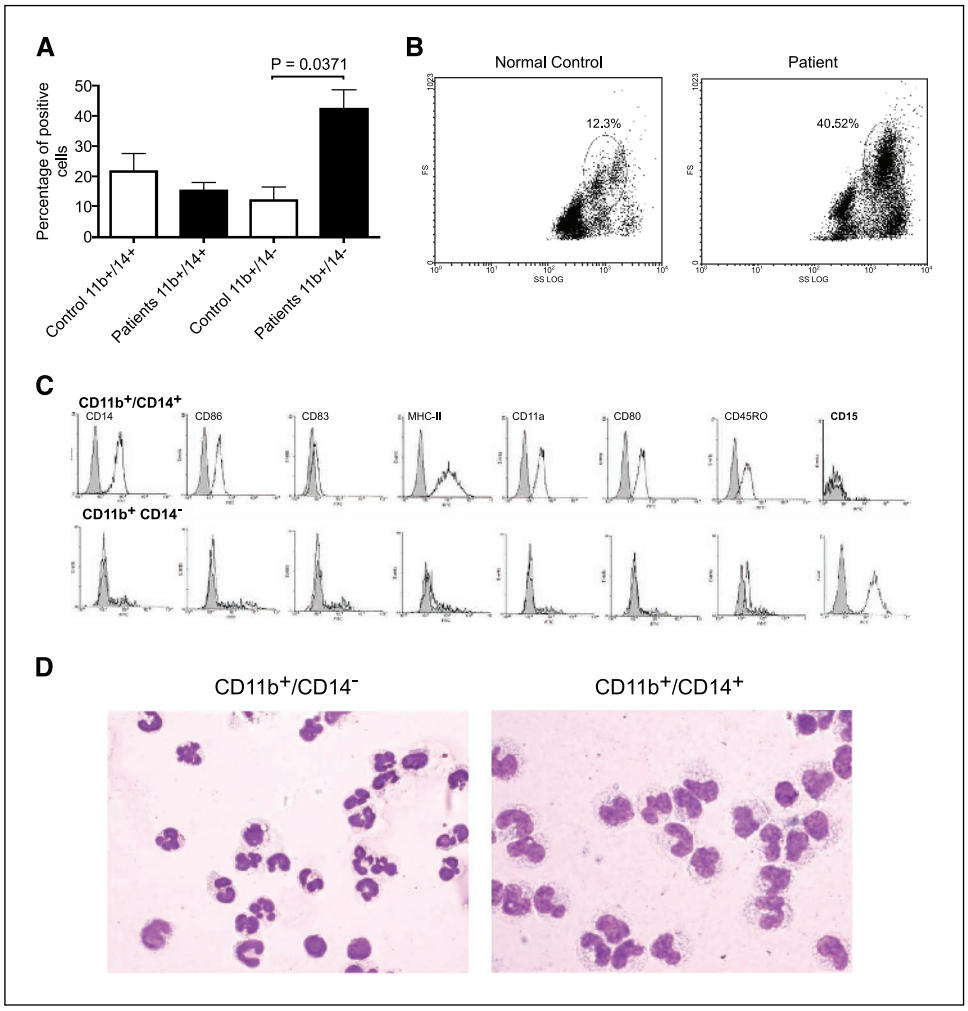

Figure 2. Additional flow cytometry studies done in seven RCC patients and seven normal controls showed an increased CD11b+,CD14− subpopulation in PBMC (*A*) and the monocytes gate (*B*) of RCC patients compared with normal controls. Giemsa staining of CD11b+,CD14− cells showed polymorphonuclear morphology (*C*) compared with the mononuclear morphology of CD11b+CD14+ monocytes both cells from RCC patients. *D,* separation and phenotyping of the CD11b+,CD14− and CD11b+,CD14+ subpopulations from patients.

Discussion

The mechanisms of tumor-induced T cell anergy in patients remains incompletely understood. Potential mechanisms include T cell apoptosis (7), regulatory T cells (8), and H_2O_2 (9) and IDO (10) production by immature dendritic cells. Myeloid suppressor cells found in the spleen of mice with colon cancer block T cell function through nitric oxide and arginase production, requiring cell-cell contact (11, 12). Recently, Rodríguez et al. (2) characterized highly suppressive myeloid cells infiltrating mouse lung carcinomas, which had high arginase activity and rapidly depleted arginine, blocking T cell proliferation, cytokine production, and CD3ζ chain expression. They did not produce H_2O_2, NO, or IDO. Blocking arginase eliminated the suppressor function *in vitro* and induced an antitumor effect *in vivo*.

In this article, we report for the first time the existence of myeloid suppressor cells with high arginase activity in RCC patients. Interestingly, the arginase-producing cells in RCC are polymorphonuclear cells and not macrophages or immature dendritic cells as suggested in murine tumor models. It is likely that these represent a subpopulation of myeloid cells induced by tumor-derived factors as suggested by Gabrilovich (13). Although their morphology and cell markers are those of polymorphonuclear cells, they could represent an immature stage of myeloid differentiation such as promonocytes, myelocytes, metamyelocytes, and even immature dendritic cells. Suppressor polymorphonuclear cells were described by Schmielau and Finn (9) in pancreatic cancer patients. These cells produced H_2O_2, but were not tested for arginase. Recently Munder et al. (14) reported that granulocytes can produce arginase I as a potent antifungal mechanism.

How low does arginine have to be to impair T cell function? Arginine concentrations <60 μmol/L decrease T cell proliferation, cytokine production, and CD3ζ expression *in vitro* (15). Trauma patients, who rapidly increase arginase activity in PBMC, profoundly deplete arginine to 0 to 50 μmol/L (normal levels range 50-150 μmol/L), resulting in T cell anergy and loss of CD3ζ (16, 17). Therefore, arginase producing cells may decrease arginine levels in circulation, a phenomenon that may be more profound in the tumor or lymphoid organ microenvironment where these cells are also found. Unfortunately, replenishment of arginine alone does not seem to be a simple solution. Although arginine replenishment does reestablish CD3ζ chain expression, it may also stimulate tumor growth (2, 18). Therefore, depletion of CD11b+,CD14− suppressor cells, or inhibition of the signals that induce arginase 1 in these cells may be an alternative approach. Depletion of CD11b+ cells does indeed reestablish T cell proliferation (Fig. 4*A*) and reexpression of CD3ζ chain. The latter experiments were done in physiologic levels of arginine (150 μmol/L), because standard RPMI has very high concentrations of this amino acid (1 mmol/L) which will cause

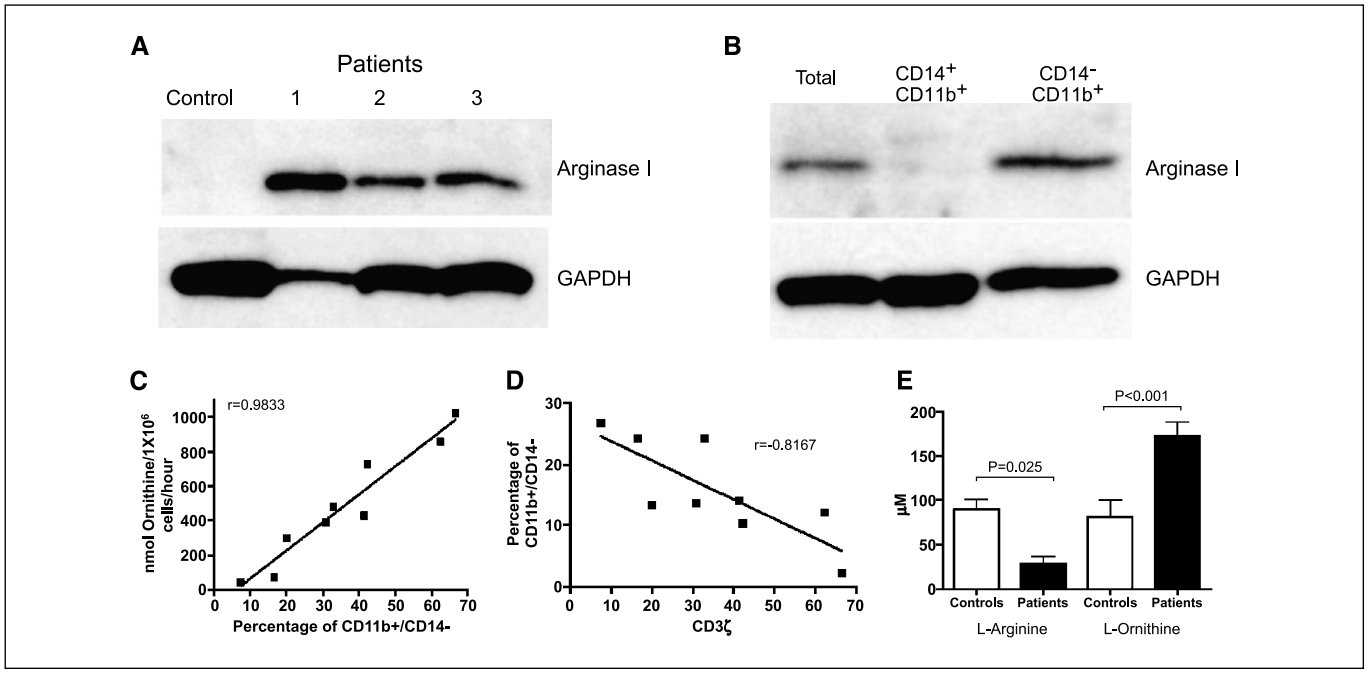

Figure 3. *A,* arginase expression by Western blotting was present in the PBMC of RCC patients. Representative data from three of seven patients tested. *B,* arginase activity was limited to CD11b+,CD14− cells. *C,* analysis by Spearman's rank correlation shows a positive correlation between percent CD11b+,CD14− cells and arginase activity, and a negative correlation between arginase activity and CD3ζ chain expression in peripheral blood T cells in 15 RCC patients tested (*D*). Arginine and ornithine levels measured in plasma from 15 RCC patients and 7 normal controls (*E*).

CD3ζ reexpression even in the presence of arginase-producing suppressor myeloid cells.

In summary, regulation of the immune response by depletion of specific amino acids including tryptophan by IDO (10) and arginine by arginase seems to be an important mechanism for tumor evasion. These mechanisms may provide new approaches to block suppressor mechanisms and enhance the therapeutic effect of immunotherapy.

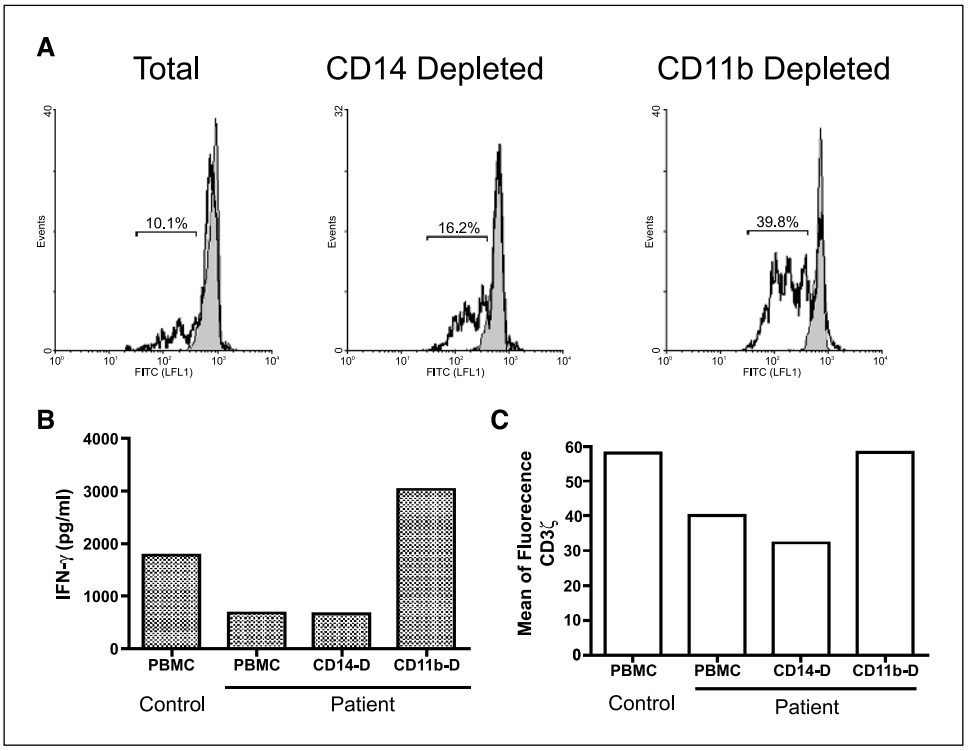

Figure 4. PBMC from RCC patients were depleted of CD11b cells (CD11b-D) or CD14 cells (CD14-D) and stimulated with anti-CD3 + anti-CD28. Representative data from one of three patient samples depleted of suppressor myeloid cells. Proliferation was measured by carboxyfluorescein diacetate succinimidyl ester fluorescence (*A*) and cytokine production (*B*) were measured at 24, 48, and 72 hours. Results at 48 hours from one of three patient samples tested. *C,* CD3ζ chain was tested after depletion of CD11b+ or CD14+ cells and culture in RPMI with physiologic levels of arginine (50 μmol/L) without stimulation.

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References

1. Rodriguez PC, Zea AH, DeSalvo J, et al. L-Arginine consumption by macrophages modulates the expression of CD3ζ chain in T lymphocytes. J Immunol 2003; 171:1232–9.
2. Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res 2004;64: 5839–49.
3. McDermott DF, Regan MM, Clark JI, et al. Randomized phase III trial of high-dose interleukin-2 versus subcutaneous interleukin-2 and interferon in patients with metastatic renal cell carcinoma. J Clin Oncol 2005; 23:133–41.
4. Tcherkas YV, Kartsova LA, Krasnova IN. Analysis of amino acids in human serum by isocratic reversed-phase high-performance liquid chromatography with electrochemical detection. J Chromatogr 2001; 913:303–8.
5. Singh R, Pervin S, Karimi A, Cederbaum S, Chaudhuri G. Arginase activity in human breast cancer cell lines: *N*(omega)-hydroxy-L-arginine selectively inhibits cell proliferation and induces apoptosis in MDA-MB-468 cells. Cancer Res 2000;60:3305–12.
6. Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P. L-Arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol 2003;24:302–6.
7. Rabinowich H, Reichert TE, Kashii Y, Gastman BR, Bell MC, Whiteside TL. Lymphocyte apoptosis induced by Fas ligand expressing ovarian carcinoma cells: Implications for altered expression of T cell receptor in tumor associated lympocytes. J Clin Invest 1998;101: 2579–88.
8. Terabe M, Berzofsky JA. Immunoregulatory T cells in tumor immunity. Curr Opin Immunol 2004;16:157–62.
9. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. Cancer Res 2001;61: 4756–60.
10. Lee GK, Park HJ, Macleod M, Chandler P, Munn DH, Mellor AL. Tryptophan deprivation sensitizes activated T cells to apoptosis prior to cell division. Immunology 2002;107:452–60.
11. Apolloni E, Bronte V, Mazzoni A, et al. Immortalized myeloid suppressor cells trigger apoptosis in antigen-activated T lymphocytes. J Immunol 2000;165: 6723–30.
12. Bronte V, Serafini P, De Santo C, et al. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. J Immunol 2003;170:270–8.
13. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. Nat Rev Immunol 2004;4:941–52.
14. Munder M, Mollinedo F, Calafat J, et al. Arginase I is constitutively expressed in human granulocytes and participates in fungicidal activity. Blood 2005;105:2549–56.
15. Taheri F, Ochoa JB, Faghiri Z, et al. L-Arginine regulates the expression of the T-cell receptor ζ chain (CD3ζ) in Jurkat cells. Clin Cancer Res 2001;7:958–65s.
16. Bernard AC, Mistry SK, Morris SM Jr, et al. Alterations in arginine metabolic enzymes in trauma. Shock 2001;15:215–9.
17. Ichihara F, Kono K, Sekikawa T, Matsumoto Y. Surgical stress induces decreased expression of signal-transducing ζ molecules in T cells. Eur Surg Res 1999; 31:138–46.
18. Park KG, Heys SD, Blessing K, et al. Stimulation of human breast cancers by dietary L-arginine. Clin Sci (Lond) 1992;82:413–7.