L-Arginine Depletion Blunts Antitumor T-cell Responses by Inducing Myeloid-Derived Suppressor Cells

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Abstract

Enzymatic depletion of the nonessential amino acid L-Arginine (L-Arg) in patients with cancer by the administration of a pegylated form of the catabolic enzyme arginase I (peg-Arg I) has shown some promise as a therapeutic approach. However, L-Arg deprivation also suppresses T-cell responses in tumors. In this study, we sought to reconcile these observations by conducting a detailed analysis of the effects of peg-Arg I on normal T cells. Strikingly, we found that peg-Arg I blocked proliferation and cell-cycle progression in normal activated T cells without triggering apoptosis or blunting T-cell activation. These effects were associated with an inhibition of aerobic glycolysis in activated T cells, but not with significant alterations in mitochondrial oxidative respiration, which thereby regulated survival of T cells exposed to peg-Arg I. Further mechanistic investigations showed that the addition of citrulline, a metabolic precursor for L-Arg, rescued the antiproliferative effects of peg-Arg I on T cells in vitro. Moreover, serum levels of citrulline increased after in vivo administration of peg-Arg I. In support of the hypothesis that peg-Arg I acted indirectly to block T-cell responses in vivo, peg-Arg I inhibited T-cell proliferation in mice by inducing accumulation of myeloid-derived suppressor cells (MDSC). MDSC induction by peg-Arg I occurred through the general control nonrepressed-2 eIF2α kinase. Furthermore, we found that peg-Arg I enhanced the growth of tumors in mice in a manner that correlated with higher MDSC numbers. Taken together, our results highlight the risks of the L-Arg-depleting therapy for cancer treatment and suggest a need for cotargeting MDSC in such therapeutic settings. Cancer Res; 1–9. © 2014 AACR.

Introduction

Therapeutic modulation of specific cellular metabolic pathways in solid and hematologic malignancies represents a major strategy for the development of new treatments (1–4). Metabolism of the nonessential amino acid L-Arginine (L-Arg) plays a central role in several biologic systems including the modulation of immune responses and tumor growth (5). We and others described the therapeutic benefit of depleting L-Arg in hematologic and solid tumors through a pegylated form of the human L-Arg metabolizing enzyme arginase I (peg-Arg I; refs. 6–10). Pegylation of arginase I increased its stability in vivo, without altering its activity (7). Moreover, peg-Arg I was effective against malignant cells that depended upon exogenous L-Arg for growth (auxotrophic) and did not induce significant toxic side effects (3). The antitumor effect induced by peg-Arg I was mediated by the induction of malignant cell apoptosis (6) and controlled through the phosphorylation of eukaryotic translation initiation factor 2a (eIF2α) and the expression of general control nonrepressed-2 eIF2α kinase (GCN2; ref. 11). Although peg-Arg I was highly effective in controlling the growth of leukemic T cells (6, 11), its effect on normal T cells remains unclear. Initial in vitro studies showed that L-Arg starvation blocked proliferation of activated normal T cells (12–14). In addition, we found that peg-Arg I delayed development of GVHD and increased burden of Listeria monocytogenes (15, 16), both conditions linked to impaired T-cell function. However, the mechanisms by which peg-Arg I could impair T-cell responses in vivo and how normal activated T cells maintain survival under L-Arg starvation remain unknown.

Specific energy metabolic pathways regulate the activation and proliferation of normal T cells. Production of ATP and reactive oxygen species (ROS) from the mitochondria control the initial T-cell–activation phase, whereas aerobic glycolysis modulates proliferation and effector T-cell functions (17–21). Although specific energy metabolic programming regulates global function of T cells, it remains unknown the effect of L-Arg in the modulation of energy metabolism.

Accumulation of myeloid-derived suppressor cells (MDSC), a heterogeneous population of immature myeloid cells expressing CD11b+ Gr1+, is a hallmark of chronic inflammation and a major mediator for the induction of T-cell suppression in various tumors (22, 23). MDSCs block T-cell responses through the metabolism...
of L-Arg by the enzymes arginase I and inducible nitric oxide synthase (iNOS), which promote L-Arg depletion and production of peroxynitrite, respectively (24, 25). Although the role of L-Arg metabolism on the T-cell suppression induced by MDSC is well understood, the effect of the deprivation of L-Arg in the accumulation and function of MDSC remains unknown.

Because the potential contradictory effect of L-Arg depletion as an antitumor therapy and as a mechanism for inhibition of immune responses, we aimed to understand the effects of peg-Arg I on normal T cells. Our results show the regulatory effect of peg-Arg I on T-cell proliferation and the ability of T cells to resist peg-Arg I through de novo L-Arg synthesis. Moreover, L-Arg deprivation induced the accumulation of MDSC, which inhibited T-cell proliferation in mice. These results support the novel role of MDSC in the regulation of T-cell responses by L-Arg starvation and suggest the need to therapeutically target MDSC in peg-Arg I–based therapies.

Materials and Methods

Mice and cells

C57BL/6 mice were purchased from Harlan Laboratories. CD45.1+, GCN2−/−, and anti-OVA357–364 (siinfekl) OT-1 mice were from The Jackson Laboratories. Lewis lung carcinoma cells (3L) were obtained in 2012 from the ATCC and injected s.c. into the mice (26). 3L cells were periodically tested (last test May 2014) and validated to be mycoplasma-free, using an ATCC kit. All mice studies were achieved using an approved Institutional Animal Care and Use Committee protocol from Louisiana State University-Health Sciences Center, New Orleans, LA. T cells were isolated from spleens and lymph nodes of mice using T-cell-negative isolation kits (Dynal, Life Technologies). Then, T cells were activated using 0.5 μg/mL plate bound anti-CD3 plus anti-CD28 (26). MDSCs were isolated from spleens of mice using Gr-1 antibody (RB6–8C5) or IgG control twice a week, starting the day before the adoptive transfer. For MDSC proliferation, mice were treated with 200 mg anti–Gr-1 antibody (RB6–8C5) or IgG control twice a week, starting the day before the adoptive transfer. For MDSC proliferation, mice were treated with peg-Arg I every other day for 7 days, after which BrdUrd uptake into CD11b+ Gr-1+ cells was tested. Analysis of nuclear DNA content was achieved using the CycleTEST-DNA Kit (BD Biosciences). T-cell apoptosis was tested using Annexin V–FITC Apoptosis Detection Kit (BD Biosciences). Staurosporine (1 μmol/L) was added 24 hours prior the apoptosis analysis as positive control.

[3H]-glucose uptake

Glucose uptake was tested after pulsing activated T cells cultured with peg-BSA or peg-Arg I (48 hours) with 1 μCi/mL glucose-[3H] (Perkin Elmer Life Sciences). Eight hours later, T cells were washed in PBS, collected, and tested for radioactivity (counts per minute).

Extracellular flux analysis

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using the XF-24-extracellular flux analyzer (Seahorse Bioscience). In brief, 5 × 105 activated T cells treated for 24 to 72 hours with peg-Arg I or peg-BSA were plated in XF-24 cell culture plates coated with 15 μg CellTak (BD Biosciences) in XF media (DMEM containing 11 mmol/L glucose and 100 mmol/L sodium pyruvate). For OCR, T cells were analyzed under basal conditions and in response to 10 μmol/L oligomycin, 1 μmol/L fluorocarbonyl-cyanide-phenylhydrazone (FCCP), and 1 μmol/L rotenone (Sigma-Aldrich). For ECAR, T cells were plated in XF media lacking glucose and monitored under basal conditions and in response to 10 μmol/L glucose, 10 μmol/L oligomycin, and 100 mmol/L 2-Deoxy-D-glucose (2-DG; Sigma-Aldrich).

siRNA transfection of primary T cells

Argininosuccinate synthetase (ASS-1) siRNA, nontargeting control siRNA, and FAM-labeled nontargeting control siRNA were obtained from Thermo Scientific (Accell-SMARTpool). Activated T cells (2 × 106) were transfected (2 μmol/L siRNA) using Accell siRNA delivery media plus 2% FBS, following the vendor’s protocol. Transfection efficiency ranged from 90% to 95% and viability from 95% to 99%. Peg-Arg I (1 μg/mL) or peg-BSA (1 μg/mL) were added 12 hours after transfection.

Results

Effects of peg-Arg I on T-cell proliferation and activation

Our previous results showed that peg-Arg I blocked malignant T-cell proliferation through the induction of cellular apoptosis; however, the effect of peg-Arg I on normal T cells remains unclear (6, 11). To test this, we first investigated the effect of peg-Arg I on the proliferation of CFSE-labeled activated T cells. A dose-dependent inhibition of T-cell proliferation was found after the addition of peg-Arg I, but not with control peg-BSA (Fig. 1A). The antiproliferative effect triggered by peg-Arg I was similarly noted in both CD4+ and CD8+ T cells and was associated with a lower IFNγ production (Supplementary Fig. S1A and S1B). To validate our results in vivo, we tested the effect of peg-Arg I in the proliferation of anti-OVA357–364 (siinfekl) transgenic CD8+ OT-1 T cells. Thus, CD45.2+ mice were adaptively transferred with CD45.1+ CD8+ OT-1 cells, followed by immunization with siinfekl peptide. Mice were injected i.p. with peg-Arg I or peg-BSA the day before T-cell transfer and then every two days. A significant decrease in CD45.1+ OT-1 cell proliferation, as tested by BrdUrd uptake, was found in immunized mice treated with peg-Arg I, compared with those injected with peg-BSA (Fig. 1B). To evaluate a potential T-cell toxicity side effect, we measured T-cell subsets in spleens of naïve mice treated for a week with...
increasing concentrations of peg-Arg I or peg-BSA. Peg-Arg I treatment was well tolerated and did not significantly alter the percentages of CD3⁺, CD4⁺, and CD8⁺ T cells in spleen (Supplementary Fig. S2A–S2C). Therefore, peg-Arg I blocked proliferation of activated T cells, but did not affect homeostatic naive T-cell numbers.

To better understand the mechanisms by which peg-Arg I blocked T-cell proliferation, we tested the effect of peg-Arg I in the induction of T-cell apoptosis and cell-cycle progression. A similar low expression of the apoptosis marker Annexin V was found in activated T cells treated with peg-Arg I or peg-BSA (Fig. 1C). Conversely, upregulation of Annexin V was noted in T cells treated with the apoptosis-inducer staurosporine. Furthermore, an arrest in the G₀–G₁ phase of the cell cycle, which correlated with a decreased expression of cyclin D3 and cdk4, was found in activated T cells cultured with peg-Arg I, compared with peg-BSA–treated T cells (Fig. 1D and E). We next evaluated the effect of peg-Arg I on T-cell activation by testing the expression of the early activation T-cell markers CD25 and CD69, and the production of IL2. Similar upregulation of CD25 and CD69 and comparable percentages of IL2⁺ cells were found in activated T cells treated with increasing doses of peg-Arg I or peg-BSA (Fig. 1F and G). Also, in agreement with the results showing the activation-independent antiproliferative effect induced by peg-Arg I, we found that addition of peg-Arg I...
after 24 hours of activation still inhibited T-cell proliferation (Fig. 1H). Therefore, peg-Arg I blocks proliferation and cell-cycle progression of T cells without leading to apoptosis or impairing the expression of early activation mediators.

Effects of peg-Arg I on T-cell energy metabolic pathways

Specific energy metabolic pathways control the proliferation and activation of normal T cells. Proliferating T cells are highly dependent on the production of energy through aerobic glycolysis, whereas mitochondrial oxidative respiration fuels T-cell activation (20). Thus, we tested the effect of peg-Arg I on T-cell energy metabolism using extracellular flux analysis. A significant decrease in glycolysis, as measured by ECAR, was noted in activated T cells treated with peg-Arg I, compared with T cells cultured with peg-BSA (Fig. 2A and Supplementary Fig. S3A and S3B). Moreover, a lower glucose-[3-3H] uptake and decreased expression of the glucose transporter Glut-1 were found in T cells cultured in the presence of peg-Arg I (Fig. 2B and C), compared with peg-BSA-treated T cells, suggesting that peg-Arg I blocked glycolysis in normal activated T cells.

Next, we evaluated the effect of peg-Arg I on mitochondrial oxidative respiration by measuring OCR. Activated T cells were cultured in the presence of peg-Arg I or peg-BSA for 24 hours, after which basal OCR was tested. A similar upregulation of OCR levels was noted in activated T cells cultured with peg-Arg I or peg-BSA, compared with nonactivated T cells (Fig. 2D). We then tested mitochondrial function by evaluating ATP-linked respiration using the mitochondrial ATP synthase inhibitor oligomycin, maximal respiration using the uncoupling ionophore FCCP, and nonmitochondrial OCR using the electron transport chain inhibitor rotenone. ATP-linked and nonmitochondrial OCR were similar in the peg-Arg I and peg-BSA–treated T cells, though the peg-Arg I–treated cells did have a slightly lower OCR reserved capacity (Fig. 2E). Furthermore, in agreement with the lack of effect of peg-Arg I on mitochondrial respiration, we noticed a similar production of mitochondrial ROS (MitoSOX) and mitochondrial biogenesis (MitoTracker) in activated T cells cultured with peg-Arg I or peg-BSA (Fig. 2F and G). Next, we elucidated whether energy production by mitochondrial respiration is needed for the survival of T cells to peg-Arg I. Addition of oligomycin resulted in a

Figure 2.
Effects of peg-Arg I on T-cell energy metabolic pathways. A, T cells were treated with 1 μg/mL peg-Arg I or peg-BSA for 24 to 72 hours. Then, ECAR was measured via an extracellular flux analyser. B, glucose-[3-3H] uptake in activated T cells treated with peg-Arg I or peg-BSA for 48 hours. C, expression of Glut-1 was tested in T cells cultured in the presence of 1 μg/mL peg-Arg I or peg-BSA. D and E, OCR in activated T cells cultured with peg-Arg I or peg-BSA for 24 hours was analyzed under basal conditions (D) and in response to oligomycin, FCCP, and rotenone (E). F, activated T cells treated with peg-Arg I or peg-BSA for 48 hours were stained with MitoSOX and analyzed by flow cytometry. G, activated T cells treated with peg-Arg I or peg-BSA for 72 hours were stained with Mitotracker Green-FM and DAPI and images were captured at equal exposure. Voxel quantification was achieved using Mask analysis. H, Annexin V staining in activated T cells treated with peg-Arg I ± oligomycin (100 nmol/L) for 48 hours. Results are expressed as mean ± SEM from three experiments. Nonstatistical significant differences (ns), *P > 0.05.

***, P < 0.001.
significant increase of apoptosis in peg-Arg I–treated T cells, but not in controls (Fig. 2H), suggesting the importance of the mitochondrial ATP production in the survival of normal T cells to peg-Arg I.

L-Arg synthesis from citrulline and T-cell resistance to peg-Arg I

De novo synthesis of L-Arg occurs from citrulline and is reliant upon enzymes ASS-1 and argininosuccinate lyase (ASL; ref. 23). Because complete culture medium lacks citrulline, we asked whether the addition of exogenous citrulline or L-Arg rescued the proliferation arrest induced by peg-Arg I. Addition of citrulline, but not L-Arg, restored proliferation of T cells cultured in the presence of peg-Arg I (Fig. 3A). The lack of effect of the supplemented L-Arg can be explained by its immediate metabolism by peg-Arg I (6). Then, we compared the expression of ASS-1 and ASL in activated T cells cultured with peg-Arg I or peg-BSA. A similar expression of both enzymes was observed in T cells independent of the activation or the presence of peg-Arg I (Fig. 3B). To understand the role of L-Arg synthesis in the proliferation of T cells cultured with peg-Arg I and citrulline, we silenced the expression of ASS-1 using siRNA (Supplementary Fig. S4). Silencing of ASS-1 significantly impaired the ability of peg-Arg I–treated T cells to proliferate in citrulline-supplemented media (Fig. 3C) without resulting in T-cell apoptosis (Fig. 3D). Because normal T cells were resistant to peg-Arg I in the presence of citrulline, we evaluated the availability of citrulline in the serum of peg-Arg I–treated mice. A dose-dependent increase in citrulline levels was noted in the serum of mice treated with peg-Arg I, but not in peg-BSA controls (Fig. 3E), indicating the elevation of citrulline as a systemic response to acute L-Arg deprivation in vivo.

MDSCs mediate antiproliferative effects of peg-Arg I in vivo

Because the elevation of citrulline in peg-Arg I–treated mice and the ability of T cells to produce L-Arg from citrulline, we sought to determine whether an indirect mechanism was mediating the impaired T-cell proliferation after peg-Arg I treatment. To test the
potential role of MDSC, CD8+ T cells from CD45.1+ OT-1 mice were adoptively transferred into cohorts of CD45.2+ mice treated with peg-Arg I, peg-BSA, or PBS, which received repeated injections with anti-Gr-1 or control IgG antibodies and were immunized with siinfekl. A significant restoration in OT-1 cell proliferation was detected in peg-Arg I–treated mice receiving anti-Gr-1, but not in those receiving IgG (Fig. 4A), suggesting that MDSC regulated T-cell suppression induced by peg-Arg I in vivo. In addition, a dose-dependent accumulation of CD11b+ Gr1+ cells occurred in spleens of mice treated with peg-Arg I, but not in those treated with peg-BSA or PBS (Fig. 4B), which correlated with a higher CD11b+ Gr1+ cell proliferation, as tested by BrdUrd (Fig. 4C). Moreover, the increase in MDSC by peg-Arg I treatment was the result of the expansion of granulocytic-MDSC (G-MDSC, CD11b+ Ly6G+ Ly6Clow), but not monocytic-MDSC (M-MDSC, CD11b+ Ly6Gneg Ly6Chigh; Fig. 4D).

Next, we tested the ability of peg-Arg I–induced CD11b+ Gr1+ cells to block T-cell responses in vitro. CD11b+ Gr1+ cells isolated from peg-Arg I–treated mice, but not from those treated with peg-Arg I–induced MDSC (1:1/2 ratio) after addition of NN (200 μmol/L), L-NMMA (500 μmol/L), or L-Arg (2 mmol/L). H, 1 x 10^6 3LL cells were injected s.c. into mice (n = 5), followed by peg-Arg I, peg-BSA, or PBS injections i.p. (1 mg) every 3 days. I, CD11b+ Gr1+ within spleens of peg-Arg I, peg-BSA, or PBS-treated 3LL-bearing mice (17 days). Results are expressed as mean ± SEM from three experiments. Nonstatistical significant differences (ns), P > 0.05; **, P < 0.01; *** P < 0.001.
discussion

Peg-Arg I–induced MDSC accumulation is mediated by GCN2

We aimed to determine the molecular mediators by which peg-Arg I induced the accumulation of MDSC. Kinase GCN2 is a key mediator of the effects induced by amino acid starvation (11, 29, 30–33). Therefore, we sought to examine the effect of GCN2 in the MDSC induction by peg-Arg I. An impaired accumulation of MDSC was detected in peg-Arg I–treated GCN2-deficient mice compared with controls (Fig. 5A), suggesting the key role of GCN2 in the accumulation of MDSC by peg-Arg I. To determine whether GCN2 also mediated the inhibitory function of peg-Arg I–induced MDSC, we compared on a per cell basis the ability of CD11b+ Gr1+ cells from wild-type and GCN2-deficient mice to impair T-cell proliferation and to express arginase I. An identical ability to suppress T-cell proliferation and a similar expression of arginase I were found in sorted MDSC from wild-type and GCN2-deficient mice treated with peg-Arg I (Fig. 5B and C), suggesting that GCN2 is involved in the accumulation of MDSC, but not in the per-cell function of peg-Arg I–induced MDSC.

Discussion

Previous studies established the therapeutic benefit of peg-Arg I in various preclinical malignancies (6–10). Moreover, peg-Arg I is undergoing evaluation in phase I/II trials for adults with liver carcinoma, with favorable safety profiles (34). Because l-Arg depletion is also an important mechanism for suppression of T-cell responses in tumors (22), we sought to reconcile these observations by conducting a detailed analysis of the effects of peg-Arg I on normal T cells. Our findings show the regulatory effect of peg-Arg I on T-cell proliferation and the ability of T cells to resist peg-Arg I through l-Arg synthesis. In addition, we show the new role of MDSC in the inhibition of T-cell responses by l-Arg starvation.

Our findings showed the ability of T cells to synthesize l-Arg de novo and indicated the elevation of serum citrulline as a systemic response to acute depletion of l-Arg. The increased citrulline synthesis occurring under systemic low arginine levels has been shown to be mediated by enterocytes using diet-based glutamine (35–39). Also, in agreement with the role of citrulline after l-Arg deprivation, injection of citrulline ameliorated toxic effects after arginase therapy (20). Although our data suggest the role of l-Arg synthesis in the effects induced by peg-Arg I, we could not specifically test the effect of citrulline on T cells or MDSC from peg-Arg I–treated mice because of the lack of pharmacologic inhibitors against ASS-1 or ASL, the toxicity of ASS-1 deletion in mice (40, 41), and the unavailability of conditional ASS-1– or ASL-deficient mice.

Peg-Arg I blocked malignant T-cell proliferation through the induction of apoptosis (6), while it failed to trigger cell death in normal T cells. Survival of normal T cells to peg-Arg I was mediated by ATP production in the mitochondria. The lack of this adaptive pathway in malignant cells could explain the differences in apoptosis observed in malignant versus normal T cells treated with peg-Arg I. In fact, tumor cells are mostly dependent on aerobic glycolysis, whereas normal cells retain the ability to sense stress and maintain survival through mitochondrial-mediated pathways (42). Our results also showed that peg-Arg I did not impair activation processes or mitochondrial respiration in T cells. However, the role of T-cell activation in the low proliferation induced by peg-Arg I is still unknown, in part because of the...
difficulty to separating proliferation and activation processes. Previous research showed the importance of fatty acid oxidation and mitochondrial respiration on the proliferation of regulatory T cells (2). Therefore, it is possible that regulatory T cells are less susceptible to peg-Arg I, which could also contribute to the tolerogenic effects induced by l-Arg starvation.

We found that in addition to the potential suppression of T cells by MDSC-mediated l-Arg deprivation, l-Arg starvation also promotes accumulation of MDSC, further suppressing antitumor T-cell responses. Accordingly, peg-Arg I mimicked T-cell suppression induced by physical injury, a process also mediated by MDSC (16). Although immune suppression could be a major limitation for the use of peg-Arg I as a therapy in cancer, it may also lead to novel therapies in transplantation and autoimmune diseases. In for the use of peg-Arg I as a therapy in cancer, it may also lead to novel therapies in transplantation and autoimmune diseases. In these settings, peg-Arg I could maintain a permanent influx of MDSC to provide T-cell suppression. As such, peg-Arg I prolonged mice survival after bone marrow transplantation and prevented development of GVHD similar to mice receiving adoptive transfer of MDSC (15). With regard to the use of peg-Arg I in different cancers, peg-Arg I could be combined with chemotherapy agents that block MDSC accumulation and function, such as gemcitabine (43), 5-fluorouracil (44), and sunitinib (45). Furthermore, we found a role of GCN2 in the accumulation of MDSC in peg-Arg I-treated mice. Accordingly, previous studies indicated the role of GCN2 in the immune effects induced by l-asparaginase (30, 46). However, the intracellular mechanisms by which the depletion of l-Arg and its sensing pathways promote MDSC accumulation remain unknown. Possible pathways could include the induction of factors that promote MDSC accumulation, including VEGF, S100AA9, granulocyte-macrophage colony stimulating factor (GM-CSF), and G-CSF. Also, in agreement with the role of cellular stress in the regulation of myeloid cells function and accumulation, recent studies showed the effect of l-Arg starvation on macrophage responses to infections (41) and the specific role of stress mediators in the induction of VEGF, arginase I (47), and MDSC accumulation (48) in tumors.

In summary, our results show for the first time the role of MDSC in the suppression of T-cell function caused by l-Arg starvation. This increases the understanding of the effects of l-Arg deprivation in the regulation of antitumor immunity and suggests caution for the use of peg-Arg I in malignancies without targeting MDSC accumulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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