Polyamine-Blocking Therapy Reverses Immunosuppression in the Tumor Microenvironment

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Abstract
Correcting T-cell immunosuppression may unleash powerful antitumor responses; however, knowledge about the mechanisms and modifiers that may be targeted to improve therapy remains incomplete. Here, we report that polyamine elevation in cancer, a common metabolic aberration in aggressive lesions, contributes significantly to tumor immunosuppression and that a polyamine depletion strategy can exert antitumor effects that may also promote immunity. A polyamine-blocking therapy (PBT) that combines the well-characterized ornithine decarboxylase (ODC) inhibitor difluoromethylornithine (DFMO) with AMXT 1501, a novel inhibitor of the polyamine transport system, blocked tumor growth in immunocompetent mice but not in athymic nude mice lacking T cells. PBT had little effect on the proliferation of epithelial tumor cells, but it increased the number of apoptotic cells. Analysis of CD45+ tumor immune infiltrates revealed that PBT decreased levels of Gr-1+CD11b+ myeloid suppressor cells and increased CD3+ T cells. Strikingly, in a model of neoadjuvant therapy, mice administered with PBT one week before surgical resection of engrafted mammary tumors exhibited resistance to subsequent tumor rechallenge. Collectively, our results indicate that therapies targeting polyamine metabolism do not act exclusively as antiproliferative agents, but also act strongly to prevent immune escape by the tumor. PBT may offer a general approach to heighten immune responses in cancer. Cancer Immunol Res 2(3); 274–85. ©2013 AACR.

Introduction
Elevated levels of polyamines (putrescine, spermidine, and spermine) have long been associated with cell proliferation in both normal and neoplastic tissues (1). Multiple oncogenes and tumor suppressors regulate tumor polyamine metabolism, resulting not only in increased polyamine biosynthesis in tumor cells but also increased cellular uptake of polyamines via an upregulated polyamine transport system (2, 3). A hallmark of tumorigenesis involves the induction of ornithine decarboxylase (ODC), the initial rate-limiting enzyme in polyamine biosynthesis (4, 5). Use of transgenic mouse models has demonstrated that increased ODC activity is sufficient to promote tumor development following low-dose exposure to carcinogens, UV irradiation, or oncogene activation and wounding (6–10). It is commonly thought that tumorigenesis is promoted by elevated polyamine levels because they stimulate cellular proliferation and angiogenesis in tumors (9, 11–14). However, besides this well-known role in proliferation, polyamines have also been reported to exert an immunosuppressive effect that could contribute to the multiple sophisticated mechanisms by which tumors evade the immune response. For instance, spermine inhibits endotoxin- or HMGB1-induced release of TNF-α, interleukin (IL)-1, IL-6, MIP-1α, and MIP-1β from human monocytes and peritoneal macrophages (15) and offers protection against lethal sepsis by attenuating local and systemic inflammatory response (16). Additional in vitro studies report that polyamines suppress lymphocyte proliferation and IL-2 production and decrease macrophage-mediated tumoricidal activity, neutrophil locomotion, and IL-2-dependent natural killer (NK) cell activity (17–21). Moreover, polyamines have been shown to suppress adaptive immune responses. Using transgenic mice in which ODC activity is targeted specifically to the epidermis, we have demonstrated that elevated polyamine levels potently suppress a T cell–mediated, hapten-induced contact allergic response (22). In all, these observations suggest that the role of polyamines as local anti-inflammatory effector molecules at sites of infection or wounds may be usurped by tumors to provide a survival mechanism to evade the immune response.

Because a common metabolic shift in many chronic inflammatory conditions and in all tumors is increased polyamine metabolism, targeting polyamine metabolism has long been an attractive approach to cancer chemotherapy. However,
treatment with α-difluoromethylornithine (DFMO), a specific inhibitor of ODC activity, has had only moderate success in treating patients with cancer (23). Subsequent studies revealed that DFMO inhibition of ODC leads to upregulation of the polyamine transporter with resulting increased uptake of polyamines derived from the diet and gut flora into the tumor cells. Thus, to polyamine-starve a tumor, both inhibition of polyamine biosynthesis as well as polyamine transport must be achieved. A novel polyamine-blocking therapy (PBT) has recently been described that includes the use of DFMO to block polyamine biosynthesis along with AMXT 1501 as an inhibitor of polyamine transport. AMXT 1501 is designed as a polyamine mimetic and consists of a lysine-spermine backbone with a C16 lipophilic substituent added to the ε-amino group of the lysine portion to optimize its ability to block cellular uptake of spermidine in the nanomolar range without crossing the cell membrane (24). Analyses of AMXT 1501 uptake into tumor cells in culture following a 24-hour incubation with 10 μmol/L of AMXT 1501 found no intracellular uptake (24). In addition, no rescue from the growth inhibitory effects of DFMO occurred when AMXT 1501 was given to cells in the absence of exogenous spermidine, suggesting that AMXT 1501 by itself or its metabolites cannot replenish cellular polyamine requirements. Together, these results suggest that AMXT 1501 inhibits the polyamine transporter at the plasma membrane and is not internalized within the cell. Initial in vivo experiments have shown that PBT causes complete or near-complete regression in the majority (88%) of carcinogen-induced squamous cell tumors in ODC transgenic mice. In addition, we investigated the effects of PBT on the immune response to tumors. Our data highlight a little-known role of polyamines as strong modulators of the inflammatory microenvironment in a tumor, and we describe a novel approach to suppress tumor growth and reverse tumor immunosuppression by targeting tumor polyamines.

Materials and Methods

Animals

ODC-ER transgenic mice, in which an invovcin promoter directs the expression of the inducible ODC cDNA fused in frame to a 4-hydroxytamoxifen (4-OHT)–responsive mutant estrogen receptor ligand–binding domain to the suprabasal epidermis, has been described previously (9). ODC-ER transgenic mice and their normal littermates were backcrossed into either the Balb/c or C57BL/6 background for at least 10 generations. ODC activity was induced in ODC-ER transgenic mice beginning 1 week before tumor cell injection or intradermal immunizations by topical application of 4-OHT (Sigma) dissolved in ethanol (1.0 mg/100 μL) applied each day to a shaved area of the dorsal skin. Additional mice used in tumor experiments included C57BL/6, Balb/c, and athymic nude mice obtained from Charles Rivers/NCI and FVB mice were purchased from Charles Rivers/NCI and FVB mice were purchased from Taconic Labs. Protocols using animals for this study were approved by the Institutional Animal Care and Use Committee of the Lankenau Institute for Medical Research (Wynnewood, PA) in accordance with the current U.S. Departments of Agriculture and Health and Human Services regulations and standards.

Cell culture

EL4 murine lymphoma, EG7 [an ovalbumin (OVA)-expressing EL4 variant (25)] cell lines, and murine macrophage RAW 264.7 cells, all purchased from the American Type Culture Collection (ATCC), were cultured in RPMI-1640 medium (Mediatech) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals), 100 μg/mL streptomycin, and 10 mmol/L L-glutamine; all were purchased from Mediatech. B16-F10 murine melanoma cells were purchased from the ATCC. CT26.CL25 (ATCC) is a subclone of CT26 colon carcinoma cells that have been transduced with the Escherichia coli β-gal gene, which have been shown to be equally as lethal in normal mice as the parental clone CT26.WT. Neu02 (26), a mammary carcinoma cell line derived from an autochthonous mammary gland tumor from a MMTV-Neu/FVB mouse, was generously provided by Dr. Alexander Muller (Lankenau Institute for Medical Research). B16F10, CT26.CL25, and neu02 cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Mediatech) with 10% FBS. Primary cultures of epidermal keratinocytes were prepared from 3- to 4-day-old FVB mice by a trypsin digestion method (27). Cells were plated at 3.5 × 10^5 cells per 60-mm dish in low calcium keratinocyte media (EMEM w/o calcium; BioWhittaker) supplemented with 8% chelex-treated FBS and 0.05 mmol/L calcium and grown at 35°C with 5% CO2. Keratinocytes were transduced with a replication-defective retrovirus containing the v-Ha-Ras oncogene (28) to generate Ker/Ras cells.

In vivo tumor models

Tumor models were established by s.c. injection of 1 × 10^6 EG7 cells or 5 × 10^5 B16F10 cells in C57BL/6 mice; s.c. injection of 5 × 10^5 CT26.CL25 cells in Balb/c mice; and s.c. injection of 3 × 10^6 neu02 cells in FVB mice. Mice were monitored twice a week for tumor growth, and treatment with 0.5% (w/v) DFMO in their drinking water and AMXT 1501 [intraperitoneally (i.p.), 3 mg/kg (as its 4HCl salt), twice a day Monday–Friday and once a day on weekends] was initiated when the tumors were palpable (50–100 mm^3 in size). Tumor growth was assessed morphometrically using calipers, and tumor volumes were calculated according to the formula V (mm^3) = π/6 × A × B^2 (A is larger diameter, B is the smaller diameter; ref. 29).

For tumor rechallenge experiments, a total of 3 × 10^6 neu02 cells were orthotopically injected in the mammary fat pad of FVB mice. When tumors were 50 to 80 mm^3 in size, mice were treated with DFMO and AMXT 1501, using the protocol above, for 7 days. At that time, treatment was halted, tumor size measured, and all mammary tumors were surgically resected. After 2 weeks, the mice were orthotopically injected with the same number of neu02 cells as before (3 × 10^6) in a mammary fat pad on the opposite side. Mice were monitored for tumor growth, and tumors were measured with calipers.
Antigen-specific T-cell response detection by IFN-γ ELISpot

LacZ–replication-deficient human type 5 recombinant adenovirus (LacZ-Adeno-X; Clontech) was expanded and purified using the Adeno-X Virus Purification Kit (Clontech) and titered using the Adeno-X Rapid Titer Kit (Clontech). For immunization, ODC-ER.Balb/c mice and their normal Balb/c littermates were first topically treated once a day with 4-OHT (1.0 mg/100 μL ethanol) 1 week later, the mice were immunized via a single intradermal injection of ODC activity in ODC-ER transgenic mice, and then 1 week later, the mice were immunized using a single intradermal injection of 1 × 10^8 infectious viral particles of adenoviruses LacZ or LacZ-Adeno-X on the shaved dorsal flank (30). Two weeks later, mice were sacrificed, and spleenocytes were analyzed for IFN-γ producing cells by enzyme-linked immunosorbent spot (ELISpot) assay.

For IFN-γ ELISpot assays, multiscroll filtration plates (Millipore) were coated with 0.5 μg/mL of purified anti-mouse IFN-γ capture antibody (BioLegend) overnight at 4°C. Single-cell suspensions of spleenocytes were plated at 1 × 10^6 and 5 × 10^5 cells per well and then peptide stimulated with a known H-2d–restricted β-galactosidase peptide [TPHPARIGL; Chem-Pep] that is presented by the MHC class I L1.5–molecule on the surface of CT26.CL25 cells or with the dominant adenovirus epitope DBP121–420 (31) peptide at 20 μg/mL. For ELISpot assays with spleenocytes from EG7 tumor-bearing mice, cells were stimulated with SINFEKL peptide (AnaSpec), addition of EL4 or EG7 cells, or with the dominant adenovirus epitope DBP418–426 peptide. After 16 hours of stimulation at 37°C, the cells were removed by washing, and spots were developed with biotinylated anti-IFN-γ detection antibody and strepavidin-horseradish peroxidase (HRP) conjugate (BioLegend) followed by horseradish peroxidase (HRP) conjugate (BioLegend) followed by a chromogen solution containing diaminobenzidine and peroxidase. Apoptotic noreactive cells were localized by incubating the sections with a mouse monoclonal anti-BrdUrd antibody (Zymed Laboratories). Pictures were acquired on a BD FACS Canto II flow cytometer and analyzed using FACS Diva software (BD Biosciences) or FlowJo7 software (TreeStar). Viable cells were gated on the basis of forward and side scatter profiles. A minimum of 10^6 viable cells were analyzed for each condition.

Flow cytometry analysis of immune cell infiltrates

Tumor or spleen tissue was digested in a 0.3% collagenase/0.1% hyaluronidase solution, pressed through a nylon mesh filter, and a single-cell suspension was prepared for flow-cytometric analysis. Equal numbers of viable cells were stained with combinations of the following: CD45–ApcCy7, Gr-1–PECy7, CD11b–PE, F4/80–Apc, CD3–FITC, CD8–PECy5, and CD4–PE (all antibodies from eBioscience). Flow-cytometric data were acquired on a BD FACS Canto II flow cytometer and analyzed using FACS Diva software (BD Biosciences) or FlowJo7 software (TreeStar). Viable cells were gated on the basis of forward and side scatter profiles. A minimum of 10^6 viable cells were analyzed for each condition.

Determination of arginase activity

Arginase activity was measured in cell lysates with slight modifications, as previously described (32). Briefly, cells were lysed in 0.1% Triton X-100 and 25 mmol/L Tris–HCl, pH 8.0. To 100 μL of this lysate, 10 μL of 10 mmol/L MnCl2 was added, and the enzyme was activated by heating for 10 minutes at 36°C. Arginine hydrolysis was conducted by incubating the lysate with 100 μL of 0.5 mol/L L-arginine (pH9.7) at 37°C for 60 to 120 minutes. The reaction was stopped with 900 μL of H2SO4 (96%)/H3PO4 (85%)/H2O (1:3:7, v/v/v). The urea concentration was measured at 540 nm after addition of 40 μL α-isonitroso-propiophenone (dissolved in 100% ethanol; Sigma) followed by heating at 95°C for 30 minutes. The rate of urea production was used as an index for arginase activity.

Nitric oxide measurement

Nitric oxide (NO) production was measured as nitrite using the Griess reagent (Sigma). Culture supernatant (50 μL) was mixed with 50 μL of 1% sulfanilamide, 0.1% N- (1-naphthyl) ethylenediamine dihydrochloride, and 2.5% H3PO4. Absorbance was measured at 540 nm in a microplate reader.

Radiolabeled spermidine transport assays

Polyamine transport in tumor cells was evaluated essentially as described previously (33, 34). Cells were plated in triplicate and grown to approximately 70% confluence. After washing with PBS, 3H-spermidine (Net-522, spermidine tritiated; [3H]H3PO4, [3H]H3PO4, [3H]H3PO4, [3H]H3PO4, [3H]H3PO4, [3H]H3PO4, [3H]H3PO4, [3H]H3PO4] was added at 1.0 μCi/mL and incubated for 60 minutes at 37°C. The cells were washed with cold PBS containing 50 μmol/L cold spermidine one time followed by two washes with PBS, lysed in 500 μL of 0.1 N NaOH at 37°C for 30 minutes, sonicated for 10 seconds, and then an equal volume of 0.1 N HCl was added to neutralize pH. The 3H radioactivity in each cell lysate was measured by scintillation counting and normalized to protein.

Statistical analysis

All in vitro experiments were performed at least in triplicate, and data were compiled from two to three separate experiments. Analyses were done using SAS Version 9.2 with a two-tailed Student t test. In vivo studies were carried out using multiple animals (n = 4–8 per treatment group). Tumor growth

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curves were analyzed with a Generalized Linear model with fixed effects of treatment and time. Data were examined for the interaction between treatment group and day of observation, testing whether the slopes of the growth curves (tumor volume vs. day of observation) were significantly different for the control and treatment groups. In all cases, values of \( P \leq 0.05 \) were regarded as being statistically significant.

Results

**Elevated polyamine levels suppress antigen-specific T cells in the tumor microenvironment**

To mimic the increased polyamine biosynthesis found in tumors, we have generated an inducible ODC-ER transgenic mouse model in which the expression of a switchable form of the ODC protein fused to a modified estrogen receptor (ODC-ER) is targeted to the epidermis using an involucrin promoter (9). A single topical subthreshold exposure to a carcinogen, then induction of epidermal ODC enzyme activity by topical treatment with the inducing agent 4-OHT, leads to rapid development of SCCs in transgenic mice (9). Wound repair following skin abrasion in ODC-ER transgenic mice is prolonged and characterized by exaggerated epidermal hyperplasia, persistent inflammation, and wound-induced tumors (10).

On the basis of earlier observations of a reduced T cell–mediated contact hypersensitivity response in mice harboring elevated epidermal polyamine levels (22), we hypothesized that polyamines might contribute to T-cell immunosuppression in the tumor microenvironment. To investigate the polyamine-dependent microenvironmental effects on growth of tumor allografts, cutaneous ODC activity was induced in ODC-ER.B6 mice with 4-OHT treatment and then EG7 thymoma cells expressing OVA (25, 35) were injected intradermally into the syngeneic ODC-ER.B6 transgenic mice and normal littermates. The increased EG7 tumor growth in ODC-ER transgenic mice compared with their normal littermates was accompanied by significantly fewer IFN-\( \gamma \)-producing splenocytes as measured by ELISpot assay following ex vivo stimulation with irradiated EG7 cells or the OVA\(_{257-264}\) (SIINFEKL) peptide (Fig. 1A and B). Thus, an antigen (OVA)-specific T-lymphocyte response was suppressed in ODC-ER transgenic mice, perhaps permitting greater tumor growth of the OVA-expressing EG7 tumor cells.

Following induction of ODC activity with daily 4-OHT treatment, ODC-ER transgenic mice and normal littermates...
were then immunized by intradermal injection with adenovirus encoding β-galactosidase. The antigen-specific response of splenocytes was evaluated 14 days after immunization in an IFN-γ ELISpot assay. Following stimulation with a LacZ peptide or peptide derived from the adenovirus vector, there were significantly (P < 0.01) fewer IFN-γ–producing splenocytes from ODC-ER transgenic mice compared with normal littermates (Fig. 1C). This suppression of antigen-specific T-cell cytokine production was also seen in ODC-ER.Balb transgenic mice injected intradermally in the flank with syngeneic CT26.CL25 tumor cells that express the LacZ antigen (Fig. 1D). Interestingly, palpable CT26.CL25 tumors were only detected in ODC-ER transgenic mice and not in normal littermate mice 11 days after tumor injection when the splenocytes were assayed in the IFN-γ ELISpot.

**Antitumor efficacy of PBT relies on host immune competence**

Because increased tumor growth in ODC-ER transgenic mice was accompanied by a suppressed antigen-specific T-cell cytokine response, we tested the therapeutic efficacy of polyamine depletions via a combination of DFMO to block polyamine biosynthesis and AMXT 1501 to inhibit the polyamine transport system (PBT; Fig. 2A). In these experiments, we analyzed the responses of two different murine tumor cell lines, CT26.CL25 colon carcinoma and B16F10 melanoma cells. In both models, treatment was initiated with 0.5% DFMO in the drinking water with or without i.p. injection of AMXT 1501 (3 mg/kg, twice a day) when the tumors were 50 to 100 mm³ in size. Although treatment with either DFMO or AMXT 1501 alone retarded B16F10 tumor growth in syngeneic C57BL/6 mice, cotreatment with both DFMO and AMXT 1501 significantly inhibited tumor growth more than treatment with a single agent (Fig. 2B). High-performance liquid chromatography (HPLC) analysis of polyamine content in tumors revealed greatly elevated levels of polyamines in tumor tissue compared with non–tumor-bearing skin (Fig. 2C). Moreover, only treatment with both DFMO and AMXT 1501 significantly decreased putrescine and spermidine levels in the tumors.

Similar to B16F10 tumors, CT26.CL25 tumor growth in syngeneic Balb/c mice was not significantly inhibited by DFMO treatment alone, but treatment with both DFMO and AMXT 1501 significantly inhibited tumor growth (Fig. 2D). Because T-cell responses were suppressed in ODC-ER transgenic mice with elevated polyamine levels, we hypothesized that the PBT-mediated repression of tumor growth via polyamine deprivation may be dependent on T cells. To test this hypothesis, athymic nude mice lacking T cells were injected with CT26.CL25 tumor cells, and PBT was initiated when the tumors were 50 to 100 mm³. In contrast to that seen in immunocompetent tumor-bearing Balb/c mice, combined treatment with DFMO and AMXT 1501 had no effect on CT26.CL25 tumor growth in athymic nude mice (Fig. 2E). These results suggest that PBT tumor inhibition is dependent on T-cell function.

PBT inhibition of CT26.CL25 tumor growth was accompanied by a dramatic increase in the number of apoptotic tumor cells as shown by the increased TUNEL-stained cells in tumors from DFMO- and AMXT 1501–treated mice compared with untreated mice (Fig. 3A). Mice were injected with BrdUrd 2 hours before sacrifice, and the relative proliferation index was determined by counting tumor epithelial cells that incorporated BrdUrd. DFMO treatment had no effect on the proliferation index, whereas treatment with DFMO and AMXT 1501 moderately decreased proliferation, albeit not to a statistically significant extent (Fig. 3B).

We expanded the above studies to a genetically defined tumor model by using primary keratinocytes transformed via v-Ha-ras retroviral infection (Ker/Ras cells). Using a similar methodology, Ker/Ras cells were used to generate orthotopic skin tumors by intradermal injection in the flank of syngeneic FVB mice. PBT treatment was initiated 13 days after injection of Ker/Ras tumor cells when the tumors were between 50 and 100 mm³ in size. Cotreatment with both DFMO and AMXT 1501 significantly suppressed Ker/Ras tumor growth compared with that in untreated mice, demonstrating better therapeutic efficacy than treatment with either DFMO or AMXT 1501 alone (Fig. 4A). There was no significant difference in tumor growth in mice treated with AMXT 1501 alone as compared with that in control-treated mice. Notably, DFMO alone was a better inhibitor of Ker/Ras tumor growth compared with its tumor-suppressive ability with B16F10 or CT26.CL25 tumors. These results underscore that PBT inhibits two main sources of polyamines for tumors. Some tumors, such as the Ker/Ras tumors, may be more dependent on polyamine biosynthesis, and DFMO is sufficient to significantly retard tumor growth. However, growth of tumors with an upregulated polyamine transporter will be suppressed only with the addition of an inhibitor of polyamine transport, such as AMXT 1501. The relative spermidine transport activity in each of these different tumor cells was measured using an in vitro assay of tumor cells for uptake of 3H-spermidine. As expected, treatment of cells with AMXT 1501 completely blocked uptake of 3H-spermidine (Fig. 4B). With no addition of a polyamine transport inhibitor, B16F10 tumor cells showed the greatest polyamine transport activity compared with CT26.CL25 cells or Ker/Ras tumor cells (Fig. 4B). These data reflect our observed in vivo effect that treatment with AMXT 1501 alone reduced the growth of B16F10 tumors but not that of Ker/Ras tumors.

After 17 days of treatment, Ker/Ras tumors were analyzed for inflammatory infiltrates by immunohistochemical staining and by flow cytometry analysis. Although F4/80⁺–stained macrophages were detected in all tumors regardless of treatment, very little infiltration of macrophages was seen in the epithelial compartment of tumors from untreated mice compared with tumors from mice treated with DFMO and AMXT 1501 (Supplementary Fig. S1). Following PBT treatment, F4/80⁺–stained macrophages were no longer marginalized outside the tumor epithelial compartment (Supplementary Fig. S1). Flow cytometry analyses of tumor-infiltrating cells revealed significant differences in the subpopulations of CD45⁺ immune cells between mice that were treated and untreated. Although no significant differences were shown in the numbers of CD45⁺ inflammatory cells in disaggregated and viable tumor cells, treatment with DFMO and AMXT 1501 (PBT) increased the percentage of CD3⁻ T cells...
and decreased the percentage of F4/80+ macrophages and Gr-1+ (neutrophil/monocyte) subpopulations in CD45+ tumor-infiltrating cells compared with that in CD45+ tumor-infiltrating cells from untreated mice (Fig. 4C–H). In particular, PBT significantly decreased the population of Gr-1+ CD11b+ cells, which have been characterized as myeloid-derived suppressor cells (MDSC) in many tumor types. Although PBT decreases the numbers of tumor myeloid cells, the increased tumor infiltration of both myeloid cell populations and T cells suggests a phenotypic change in the tumor myeloid population with PBT treatment.

PBT limits induction of an M2 macrophage phenotype associated with tumor immunosuppression

Multiple subpopulations of tumor-infiltrating immune cells, including Gr-1+ CD11b+ MDSCs, granulocytes, immature dendritic cells, and regulatory T cells, have been shown to profoundly suppress T-cell functions via arginase-mediated arginine depletion (36–38). Inhibition of myeloid cell–associated arginase activity eliminates tumor-associated immune dysfunction and suppresses tumor growth (37, 38). Arginase hydrolyzes L-arginine to the products urea and L-ornithine, which is further metabolized via ODC to polyamines (Supplementary Fig. S2).

Figure 2. Tumor growth inhibition by DFMO + AMXT 1501 is dependent on T cells. A, structural representations of the inhibitor of the polyamine transport system, the D-C16-acyl-Lys-spm conjugate, AMXT 1501, and the ODC inhibitor, a-DFMO. B, C57BL/6 mice were injected s.c. with 5 × 10^5 B16F10 tumor cells. When the tumors were 50 to 100 mm^3 in size, treatment was initiated with either saline, 0.5% DFMO (w/v) in the drinking water, AMXT 1501 (i.p., 3 mg/kg, twice a day Monday–Friday and once a day on weekends), or both DFMO and AMXT 1501. Graph shows tumor growth rate and volume in mice bearing B16F10 tumors under different treatments (n = 6–7 mice/treatment group; mean volume per time point ± SEM). C, upon sacrifice, non–tumor-bearing normal skin and tumor tissues (n = 6–7 mice/treatment group) were frozen in liquid nitrogen, ground to a fine powder, homogenized in 0.2 N perchloric acid, and incubated at 4°C overnight. Dansylated polyamines were separated on a reversed-phase C18 HPLC column (52). Polyamine values were normalized for the amount of DNA in the tissue extracts and are expressed as nmol/mg DNA. Values are mean ± SEM, with an asterisk marking significant differences between polyamine levels in control mice and treated mice with P < 0.02. D, Balb/c mice were injected subcutaneously with 5 × 10^5 CT26.CL25 tumor cells. When the tumors were 50 to 100 mm^3 in size, treatment was initiated with either saline, 0.5% DFMO (w/v) in the drinking water, or cotreatment with 0.5% DFMO and AMXT 1501 (i.p., 3 mg/kg, twice a day Monday–Friday and once a day on weekends). Graph shows tumor growth rate and volume in mice bearing CT26.CL25 tumors under different treatments (n = 5 mice/treatment group; mean volume per time point ± SEM). E, athymic nude mice were injected s.c. with 5 × 10^5 CT26.CL25 tumor cells. When the tumors were 50 to 100 mm^3 in size, treatment was initiated with either saline or cotreatment with 0.5% DFMO in the drinking water and AMXT 1501 (i.p., 3 mg/kg, twice a day Monday–Friday and once a day on weekends). Graph shows tumor growth rate and volume in mice bearing CT26.CL25 tumors under different treatments (n = 5 mice/treatment group; mean volume per time point ± SEM). * P < 0.01; ns, not significant.
addition to induction by Th2 cytokines, we have observed that arginase activity in tumor myeloid cells may also be induced by increased tumor levels of polyamines. Figure 5A shows that arginase activity is significantly increased in primary keratinocytes with elevated ODC activity and polyamine levels, potentially explained by an increased metabolic utilization of L-ornithine. Conversely, arginase activity is decreased with DFMO treatment. Although DFMO itself has been described as an arginase inhibitor (39), an alternative explanation for the decreased arginase activity upon DFMO treatment of these cells could be the feedback inhibition of arginase by the increased levels of ornithine resulting from DFMO inhibition of ODC.

Because macrophages have been shown to polarize to an arginase-expressing M2 phenotype following exposure to Th2 cytokines such as IL-4 or IL-10 (40), we tested the effect of inhibition of polyamine biosynthesis using DFMO on the M1/M2 polarization of cultured murine RAW264.7 macrophage cells. As expected, IFN-γ or lipopolysaccharide (LPS) induction of a proinflammatory M1 phenotype was accompanied by increased nitric oxide synthase (NOS) activity as indicated by increased NO production, and IL-4 induction of a M2 phenotype was marked by an induction of arginase activity (Fig. 5B and C). DFMO treatment significantly blocked IL-4 induction of arginase activity (Fig. 5B). However, the same concentrations of DFMO had no significant inhibitory effect on LPS induction of NOS activity in RAW264.7 cells (Fig. 5C). Together, these data suggest that inhibition of polyamine biosynthesis reverses the immunosuppressive tumor microenvironment by skewing myeloid cell polarization from an M2 progrowth phenotype to a M1 proinflammatory phenotype.

PBT promotes durable protection against tumor rechallenge
If reversal of a polyamine-dependent immunosuppressive tumor microenvironment contributes to PBT inhibition of tumor growth, then it is possible that PBT generates a protective T-cell memory to protect against tumor recurrence. We used a model of HER2/neu-mediated breast cancer to functionally test whether short-term treatment with DFMO and AMXT 1501 before surgical excision of a primary tumor can afford protection against rechallenge with the same tumor. Neu02 mammary tumor cells isolated from a mammary adenocarcinoma in a transgenic MMTV-Neu mouse (26) were orthotopically injected in a right-side mammary fat pad of syngeneic female FVB mice. When the tumors were between 50 and 80 mm3 in size, half the mice were treated with both DFMO and AMXT 1501 for 7 days, whereas the other half were treated with tap water and injected with control vehicle. After 7 days of treatment, the tumors were surgically resected, and treatment was discontinued. After 2 weeks, the mice were orthotopically reinjected with Neu02 cells in a left-side mammary fat pad. As expected, Neu02 tumors grew significantly bigger in naive FVB mice than in mice previously challenged with Neu02 tumor cells with surgical removal of their first Neu02 tumor. Tumors grew only in surgically resected mice that had not received prior PBT treatment (Fig. 6). In contrast, there was no tumor growth in any mice that had been previously treated with PBT before surgery (Fig. 6). Overall, these results revealed that PBT may elicit a durable immune memory when administered before surgical resection of the primary tumor.
Discussion

Tumors have evolved a variety of survival mechanisms to suppress the innate and the effector arms of the immune system, thus compromising most cancer immunotherapy and vaccine therapies in clinical trials. An exception is the recent targeting of the immune checkpoint molecules, CTLA-4 or PD-1, a strategy that holds great promise by unleashing the inherent immune reactivity to the tumor (41). The development of additional, complementary therapeutic approaches to defeat the tumor’s immune checkpoint mimicking ability is urgently needed. The availability of broad-based immunochemotherapies that reverse the immunosuppressive microenvironment inherent in growing tumors offers profound clinical implications. For instance, inhibition of the pathways controlled by indoleamine 2,3-dioxygenase (IDO; ref. 42), adenosine (43), and arginase (44), all shown to impede T cell-mediated tumor elimination, is being vigorously pursued. Our data raise the possibility that tumor production and release of polyamines into the tumor microenvironment may contribute to immune editing or sculpting of tumor cells and therefore represent an important mechanism for selection of immune-cloaked cells inherent to the immunosuppressive tumor microenvironment. On the basis of the data discussed here, we propose adding polyamines to the list of immunosuppressive small-molecule metabolites. Our study is significant because it highlights this immunosuppressive function of tumor polyamines as an important factor contributing to tumor development and survival in...
addition to the commonly held view that polyamines are needed for tumor cell proliferation. Importantly, PBT-mediated reduction of tumor polyamine levels dramatically retards tumor growth by reversing this immunosuppression.

In this study, we observed several intriguing phenomena using multiple murine tumor models: (i) elevated polyamine-mediated suppression of an antigen-specific immune response creates a more permissive microenvironment for tumor growth; (ii) inhibition of tumor growth by polyamine depletion in tumors via cotreatment with DFMO and AMXT 1501 is T cell dependent; and (iii) treatment with DFMO and AMXT 1501 before surgical removal of the primary tumor leads to protective immunity to tumor rechallenge. These data are consistent with earlier reports (15, 16, 19, 20) that polyamines inhibit induction of proinflammatory cytokines in monocytes, protect against lethal sepsis, and suppress immune cell activity. Polyamine levels are elevated in tumors and are released by dying cancer cells found in hypoxic and necrotic areas of the tumor following chemo- or radiotherapy. Our data provide compelling in vivo evidence that the anti-inflammatory effects of polyamines released into the tumor microenvironment contribute to the immunosuppressive milieu commonly found in most tumors. Furthermore, it is intriguing to speculate on the immunosuppressive capabilities of each individual polyamine.

The majority of earlier research explored the potency of spermine as an immunosuppressive metabolite; our data support a profound biologic outcome following predominantly putrescine and spermidine depletion from the tumor microenvironment (Fig. 2C). Only minor changes in the levels of spermine were observed with any of the treatments described. Clearly, subtle changes in the balance of individual polyamines have a significant impact on the immune biology of the tumor microenvironment. Furthermore, the potential for toxic consequences of drastic spermine depletion and associated off-target effects should also be considered when designing a polyamine antimetabolite approach.

PBT suppresses tumor growth in several in vivo tumor models by, at least in part, stimulating an immune antitumor effect that is T cell dependent. Our results demonstrate that PBT antitumor efficacy is dependent on T cells and perhaps decreases M2 macrophage/monocyte cells that have protumor growth properties. One possible mechanism is via inhibition of tumor myeloid cell arginase activity. We have shown that increased polyamine levels induce arginase activity in epithelial cells and may also contribute to the induction of immunosuppressive arginase-expressing tumor myeloid cell populations. Inhibition of polyamine biosynthesis with DFMO blocks the IL-4–induced arginase activity (marker of M2 polarization)

Figure 5. DFMO blocks both ODC induction of arginase activity in primary keratinocytes and M2 polarization of IL-4–treated macrophages. A, primary keratinocytes were isolated from the skin of K6/ODC transgenic mice and their normal littermates. Half of the keratinocytes were cultured in medium containing 2 mmol/L DFMO, and after 24 hours the cell lysates were assayed for arginase activity. All cell cultures were assayed at least in triplicate. B and C, murine RAW264.7 macrophage cells were treated with IL-4, IFN-γ, or LPS for 24 hours in the presence or absence of DFMO (0.1 or 0.5 mmol/L). B, cell lysates were assayed for arginase activity (urea production), and C, the cell supernatants were assayed for NO production using the Griess reagent. All results are given as mean ± SD; *, P < 0.01; OD, optical density.
Tumor Polyamines Promote Immune Escape

Targeting polyamine metabolism has long been an attractive approach to cancer chemotherapy. However, treatment with DFMO has had only moderate success in treating patients with cancer (23). Subsequent studies highlighted that DFMO inhibition of ODC leads to upregulation of the polyamine transporter with resulting increased uptake of polyamines from the diet and gut flora into the tumor cells (47, 48). Thus, to polyamine-starve a tumor, both inhibition of endogenous polyamine biosynthesis as well as uptake of exogenous polyamines must be achieved. Interestingly, the therapeutic efficacy of DFMO was reported to be much greater in mice bearing mutant L1210 leukemia cells deficient in their polyamine transport system than in mice injected with parental L1210 tumor cells (49). Subsequently, Moulinoux and colleagues (20, 50) reported a 40% inhibition in tumor progression and metastases and an antitumor immune stimulation in animals treated with DFMO and fed a polyamine-deficient diet and neomycin in the drinking water to partially reduce gut bacterial polyamine production. The discovery of an effective, nontoxic inhibitor of the polyamine transport system (AMXT 1501) offers a direct strategy to block the polyamine transport system that is upregulated in many malignant tumors. PBT is a novel approach to selectively starve tumors via the combined use of a key inhibitor of polyamine biosynthesis and a potent inhibitor of the polyamine transport system. Previous studies with PBT have shown complete regression of carcinogen-induced SCCs in ODC transgenic mice (24). Although these skin tumors were completely dependent on polyamine biosynthesis driven by a strong ODC transgene, we demonstrate here the power of PBT to suppress malignant tumor growth in wild-type, immunocompetent animals. Our expectation for increased tumor association of AMXT 1501 following polyamine biosynthesis inhibition by DFMO in animals will be the subject of future studies.

The capacity of PBT to target both polyamine biosynthesis as well as the polyamine transport system effectively deprives tumor cells of polyamines necessary for their growth and survival. Treatment with DFMO alone is cytostatic, not cytotoxic in tumors (23, 51). We have shown that PBT inhibition of tumor growth was accompanied by increased apoptosis but no significant decrease in the proliferation index in tumor cells, indicating that the antitumor efficacy of PBT depends more on its ability to recondition the tumor microenvironment than its activity as an antiproliferative agent. With the use of PBT that more effectively starves tumors of polyamines compared with DFMO alone, the essential function of tumor polyamines as immunosuppressive modifiers can for the first time be appreciated along with the commonly viewed proproliferation role of polyamines in tumor development. The higher polyamine levels found in most tumor types reflect their common requirement for polyamines to mold a tumor microenvironment that suppresses antitumor immune responses and stimulates tumor proliferation, survival, and angiogenesis. Thus, PBT immunochemo therapy offers a much needed general approach to treat multiple types of cancer. Polyamine deprivation via PBT offers exciting potential as an adjunct cancer treatment both with conventional chemotherapeutic...
agents and in stimulating antitumor immune responses in tumor immunotherapies.

Disclosure of Potential Conflicts of Interest

MR Burns is employed as President & CEO of Aminex Therapeutics, Inc. and also has an ownership interest (including patents) in the same. S.K. Gilmour is a coinventor for a patent describing the therapeutic use of PBT. No potential conflicts of interest were disclosed by the other authors.

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