Modulators of arginine metabolism do not impact on peripheral T-cell tolerance and disease progression in a model of spontaneous prostate cancer

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**Statement of translational relevance**

Therapeutic strategies are urgently needed to overcome the immunosuppressive tumor microenvironment. Perturbation of the arginine metabolism has been proposed as one such mechanism, which also favors carcinogenesis. We have investigated the therapeutic potential of modulators of the arginine metabolism, among which sildenafil that is already used in clinical trials, in realistic mouse models of prostate cancer. We have found that these drugs, at concentration known to be effective in models of transplantable cancer, neither broke tumor-specific T cell tolerance, nor restrained spontaneous prostate cancer progression. Further investigation is needed to fully appreciate the therapeutic potential of modulators of the arginine metabolism in cancer.
ABSTRACT

Purpose. Chronic inflammation, recruitment of myeloid derived cells and perturbation of the arginine metabolism have been all proposed as mechanisms favoring prostate carcinogenesis and tumor immunoescape. Objective of this study was to evaluate whether accumulation of CD11b+Gr1+ cells, also defined myeloid derived suppressor cells (MDSC), occur in mice affected by transplantable or spontaneous prostate cancer. We also investigated whether N(G) nitro-L-arginine methyl ester (L-NAME) and sildenafil, both modulators of the arginine metabolism, restrain tumor growth and restore tumor-specific immunity.

Experimental design. Wild type C57BL/6 mice bearing TRAMP-C1 prostate cancer and transgenic adenocarcinoma of the mouse prostate (TRAMP) mice were treated with vehicle, L-NAME or sildenafil, and evaluated for CD11b+ cells accumulation in the blood, several organs and the tumor mass, and for disease progression.

Results. CD11b+Gr1high, CD11b+Gr1int and CD11b+Gr1 cells differently accumulated in different organs and especially in the tumor of the two mouse models. L-NAME and sildenafil impaired the immunosuppressive function of CD11b+ cells in both models and restrained TRAMP-C1 growth, but they did neither break tumor-specific immune tolerance nor limit tumor progression in TRAMP mice.

Conclusions. Collectively, our results emphasize substantial differences in tumor-induced alteration of myelopoiesis and sensitivity to modulators of the arginine metabolism between a transplantable and a spontaneous model of prostate cancer. They also suggest that perturbation of the arginine metabolism is dispensable for prostate cancer progression and the associated T cell tolerance.
INTRODUCTION

The pathogenesis of prostate cancer (PC), the second cause of death for neoplasia among men worldwide (1), has been linked to chronic inflammation (2). PC may also represent a site of acquired immune privilege (3), where several mechanisms suppress both locally and systemically the tumor-specific immune response (4). Among these mechanisms, myeloid derived suppressor cells (MDSC) (5) have been recently the focus of intense investigation (6). MDSC are a heterogeneous population of cells of myeloid origin that include immature macrophages, granulocytes, dendritic cells (DC) and other myeloid cells (6, 7). Recruitment of MDSC to peripheral organs under pathologic conditions is mediated by several soluble factors, among which IL-3, IL-6, IL-10, vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (5, 7, 8). In mice, MDSC are characteristically CD11b\(^+\), express the Gr-1 antigen at different levels, and may also express CD31, IL-4 receptor \(\alpha\)-chain, CD115 and CD80 (5, 9). The phenotype of human MDSC is ill defined (6, 9), and a population of CD14\(^+\)HLA-DR\(^{low/-}\) MDSC cells has been recently observed in the peripheral blood of PC patients that significantly correlated with circulating PSA levels (10).

MDSC may overexpress both inducible nitric oxide synthase (iNOS) and arginase 1 (Arg1), enzymes involved in the metabolism of arginine. As reviewed in (6), depletion of arginine from the microenvironment inhibits T cell activation and proliferation, and favors T cell apoptosis. Furthermore, iNOS produces nitric oxide (NO), which interferes with IL-2 receptor signaling, leading to cell cycle arrest. Reactive oxygen species (ROS) and peroxynitrites, bioproducts of arginine metabolism, contribute to T cell inhibition. Of relevance, T cells infiltrating either human PC or the prostate of transgenic adenocarcinoma of the mouse prostate...
(TRAMP) mice, a primary model of spontaneous PC (11), are functionally impaired and contain high level of nitrotyrosines, suggesting in loco peroxynitrites production (12).

Tumors are infiltrated also by tumor-associated macrophages (TAM), that in mice are CD11b^Gr1^, may derive from circulating MDSC and exert immunosuppressive functions (7, 9). TAM surrounding mouse prostate intraepithelial neoplasia (mPIN) and well- or moderately differentiated PC in TRAMP mice are \text{Arg1}^{\text{high}}\text{iNOS}^{\text{low}}, indicating M₂ polarization (13). A mixed M₁/M₂ infiltrate (\text{Arg1}^{\text{high}}\text{iNOS}^{\text{high}}) is characteristic of poorly differentiated PC in this model.

Interestingly, both Arg₁ (12, 14) and iNOS (12, 15) are overexpressed by epithelial PC cells, and the enzymatic activity of Arg is increased in PC patients (16), suggesting their role in sustaining the high demand of polyamines by the growing tumor tissue (14). NO is also a key signaling molecule in cancer, where it participates to cancerogenesis, angiogenesis, tumor cell proliferation and invasion (17).

Several strategies have been proposed to inhibit Arg and NOS (6). N(G)-monomethyl-L-arginine can restore the lytic function of PC infiltrating T cells in vitro (12), but its use in clinic has been discontinued due to severe toxicity (18). In vitro, N(G) nitro-L-arginine methyl ester (L-NAME) causes a significant transcriptional downregulation of eNOS that is overexpressed in endothelial cells associated with PC (19), but can also inhibit Arg activity (20). L-NAME has been reported in several transplantable models to inhibit tumor growth [e.g.,(21, 22)] and improve the endogenous tumor-specific immune response (22). Also phosphodiesterase-5 (PDE5) inhibitors (sildenafil, tadalafil and vardenafil) can modify arginine metabolism by inhibiting the degradation of cyclic guanosine monophosphate (cGMP) to GMP. Sildenafil in vivo increases cGMP and down-regulates iNOS and Arg₁ expression and
reduces the enzymatic activity in the intratumoral MDSC. Sildenafil also favors T cell immunity and delays tumor progression (22, 23). It is not known however, the in vivo effect of these treatments in models of spontaneous tumor development that more closely recapitulate the human pathology.

We investigated here whether accumulation of CD11b⁺ cells occurs in mice bearing transplantable PC and in TRAMP mice, and whether treatment with L-NAME or sildenafil restores antitumor immunity and delays tumor growth.
MATERIALS AND METHODS

Mice, cell lines and reagents. Heterozygous C57BL/6 TRAMP mice and wild type (WT) mice were housed and bred in a specific pathogen free animal facility, treated in accordance with the European Union guidelines, and with the approval of the Institutional Ethical Committee. Animals were typed for Tag expression by PCR-based screening assay, as described (24). TRAMP-C1 cells (25) were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Cambrex, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Milan, Italy), 150 U/ml streptomycin and 200 U/ml penicillin (Cambrex, Charles City, IA). B6/K-0, a kidney cell line expressing Tag (26), and B6/K-1,4,5 cells, which lack the Tag epitopes I, II/III, IV and V (27), were a generous gift of Dr. S.S. Tevethia (The Pennsylvania State University College of Medicine, Hershey). RMA is a H-2b Rauscher virus-induced thymoma (28). These cell lines were cultured in RPMI-1640 (Invitrogen) supplemented with 2 mM L-glutamine, 150 U/ml streptomycin, 200 U/ml penicillin and 10% heat-inactivated FBS. Unless specified, all chemical reagents were from Sigma-Aldrich, and monoclonal antibodies (mAb) were from BD PharMingen (San Diego, CA).

In vivo experiments. A schematic representation of the in vivo experiments is reported in Supplementary Fig.1. Six-8 wk-old C57BL/6 male mice were challenged subcutaneously (s.c.) with 2.5 x 10^6 TRAMP-C1 cells. L-NAME (added in drinking water at 1g/L) or sildenafil (0.7 g/L; Pfizer, New York, NY; a generous gift of Drs. G. Da Pozzo and A. Salonia, San Raffaele Scientific Institute, Milan, Italy) were administered starting on the day of tumor challenge. As control, groups of animals were treated with vehicle (PBS) only. Water was given ad libitum. Calculation of the dosage of the drug was based on the assumption that a mouse drinks approximately 3
ml/24h. Mice were monitored twice a wk and sacrificed after 50 days. Animals were weighted and tumor size was evaluated both by measuring its weight and two perpendicular diameters by a caliper. Cohorts of 13 or 21 wk-old TRAMP and WT mice were randomly assigned to either one of the following 3-wk treatments: L-NAME (1 g/L), sildenafil (0.13 or 0.7 g/L) or vehicle dissolved in drinking water. At the end of the second wk, all mice were vaccinated i.d. with bone marrow (BM) derived DC [5 x 10^5/mouse; (29)] pulsed with 2 μM of the immunodominant cytotoxic T lymphocyte (CTL) epitope Tag-IV [sequence 404-411; Research Genetics, Huntsville, AL; ref. (30); DC/Tag-IV]. At the end of the third and last wk, animals were killed. Animals and their urogenital apparatus (UGA) were weighted. UGA were processed for histology and immunohistochemistry, and scored on coded samples by a pathologist as previously described (31).

*Phenotypic characterization of cell populations.* Single cell suspensions of tumor draining lymph nodes (TDLN) and non TDLN (NDLN) from tumor-challenged and naïve mice, respectively, were stained with FITC-labeled anti-CD4, PE-labeled anti-CD44 and PerCP-Cy 5.5-labeled anti-CD8 mAb. Dead cells were excluded by physical parameters and/or by the addition of ToPro5 (Molecular Probes, Eugene, OR) immediately before flow cytometry analysis. For enumeration of CD11b^+ cells, blood and cells from spleen, BM, NDLN, TDLN and tumor samples of naïve and tumor-challenged mice were incubated with Fc blocking mAb and stained with FITC-conjugated CD11b and APC-conjugate Gr1 mAb. Dead cells were excluded by propidium iodide. For enumeration of CD4^+CD25^+Foxp3^+ cells, LN cells were stained with FITC-labeled anti-CD4, PerCP-Cy 5.5-labeled anti-CD8 and APC-labeled anti-CD25 (clone PC61) mAb, permeabilized and finally, stained with PE-labeled anti-Foxp3 mAb (eBioscience, SanDiego, CA) according to the manufacturer’s
instructions. In all experiments, cells were analyzed on a BD FacsCalibur® or FacsCanto®.

In vitro cytotoxicity assay. Splenocytes or magnetic bead-purified CD8+ cells (Miltenyi Biotec, Bergisch Gladbach, Germany) were re-stimulated in vitro in the presence of irradiated B6/K-0 cells (10:1 ratio). Irradiated splenocytes (1:1 ratio) were added to the CD8+ cell culture. Day-5 blasts were tested for cytolytic activity in a standard 4 h 51Cr release assay (29). 51Cr release of target cells alone was always < 25% of maximal 51Cr release (target cells in 0.25 M SDS).

CD11b+ cell purification and in vitro functional assays. CD11b+ cells purification was performed with mouse CD11b MicroBeads (Miltenyi Biotec) following the manufacturer’s instructions. Purity of the cell population was evaluated by flow cytometry and exceeded 90%. C57BL/6 splenocytes (3 x 10^5 cells/well) were stimulated in wells that had been coated with 3 μg/ml anti CD3 and 2 μg/ml anti-CD28 mAb or in the presence of CD3/CD28 beads (2 μl/8 x 10^4 splenocytes; Invitrogen, Karlsruhe, Germany). Purified splenic CD11b+ cells were added to the culture so as to constitute 1-20% of the total cells. After 3 days of incubation, cultures were pulsed with 3H-Thymidine (1 μCi/well; Amersham Corp., Milan, Italy) for the last 18 h. The incorporation of 3H-Thymidine by proliferating T cells (triplicate cultures) was measured by scintillation counting.

Arg and iNOS detection. Immunomagnetic beads-purified CD11b+ cells or tumor cells were lysed for 10 min with lysis buffer containing 0.15 mM pepstatin A, 0.2 mM leupeptin and 0.4% Triton x-100. Samples were centrifuged at 20000 x g at 4 °C for 10 min. The supernatant was used for Arg assay using QuantiChrom Arginase Assay Kit (DARG-200, Gentaur, Bruxelles, Belgium) according to the manufacturer’s instructions. Results were normalized on 10^6 cells. Alternatively, CD11b+ cells were
assessed *ex vivo* for intracellular iNOS upon stimulation with LPS (1μg/ml) for 24 h. Brefeldin A was added during the last 5 h of pulsing. Cells were fixed in 2% paraformaldehyde, permeabilized, incubated with polyclonal rabbit anti-iNOS antibody (Santa Cruz, Santa Cruz, CA) at a 1:200 dilution for 30 min at room temperature, and finally analyses by flow cytometry.

**Statistical analyses.** Statistical analyses were performed using the Log-rank, Student’s T, ANOVA and Newman-Keuls tests. Values were considered statically significant for p < 0.05.
RESULTS

Effects of modulators of arginine metabolism on myelopoiesis and growth of TRAMP-C1 tumors in vivo

We initially investigated the effect of tumor growth on myelopoiesis and recruitment of CD11b$^+$ cells to peripheral organs in the TRAMP-C1 model. TRAMP-C1 is a hormone-independent PC cell line derived from a TRAMP tumor (25), and widely used to assess novel therapeutic approaches for PC. Interestingly, TRAMP-C1 tumors in vivo are infiltrated by CD11b$^+$ cells, and iNOS mRNA is barely detectable, whereas Arg1 mRNA levels are high and persist as tumor grows (32). Hence, tumor cells were injected s.c. in C57BL/6 males, and the same day mice were randomly assigned to a treatment with either L-NAME or vehicle added to the drinking water (Supplementary Fig. 1A). We choose this treatment because it demonstrated to be highly effective in restraining growth of other subcutaneous tumors in C57BL/6 mice (22). Animals were killed 50 days later, when the tumor mass in vehicle-treated mice had reached the dimension of approximately 150 mm$^2$. Flow cytometry analysis of the blood showed a dramatic accumulation of CD11b$^+$Gr1$^{high}$ cells in vehicle-treated mice (Fig. 1B) when compared with naïve age- and sex-matched littermates (Fig. 1A). Quantification of CD11b$^+$Gr1$^{high}$ cells demonstrated a statistically significant increase in tumor-bearing mice (Fig. 1D) that was marginally restrained by L-NAME treatment (Fig. 1C and D). At difference with other transplantable tumor models (22), we did not find a relevant accumulation of CD11b$^+$Gr1$^{int}$ and CD11b$^+$Gr1$^{-}$ cells in the blood of mice affected by TRAMP-C1 tumors (Fig 1B and D). Since the blood may not be fully representative of myelopoiesis and MDSC accumulation during tumor growth, we quantified MDSC in lymphoid organs and tumors of untreated and L-NAME treated mice. An accumulation both as percentage and absolute number of CD11b$^+$Gr1$^{high}$ cells was
evident also in the spleen (Fig. 1D and Supplementary Fig. 2, respectively) and tumor samples (Fig. 1D and Supplementary Fig. 2, respectively) of tumor-bearing mice. Immunofluorescence on TRAMP-C1 tumor sections confirmed that CD11b+ cells infiltrating the tumor tissue were mostly Gr1+ (Supplementary Fig. 3). The BM was already rich in CD11b+Gr1high cells that further increased in tumor-bearing mice (Fig. 1D). A marginal increase of CD11b+Gr1high cells was also evident in TDLN, where the mature CD11b+Gr1− cells dominated and further increased in mice affected by TRAMPC-1 tumors (Fig. 1D and Supplementary Fig. 2). The CD11b+Gr1int population was clearly enriched only in the spleen (Fig. 1D and Supplementary Fig. 2). L-NAME treatment did not significantly modify MDSC accrual in the organs examined except for NDLN and TDLN, where the % and number of CD11b+Gr1int cells declined (Fig. 1 and Supplementary Fig. 2).

Accumulating CD11b+ cells were indeed MDSC, since CD11b+ cells purified from the spleen of tumor-bearing mice inhibited the in vitro proliferation of splenocytes in a dose-dependent manner (Fig. 2A). Hence, MDSC accumulation is a characteristic also of subcutaneous hormone-independent PC. Of relevance, treatment with L-NAME reduced the immunosuppressive and Arg1 enzymatic activities of both CD11b+ splenocytes (Fig. 2A) and cells collected from the tumor mass (Fig. 2B).

Since, TDLN rapidly enlarge owing to the accumulation of myeloid and lymphoid cells (33), we asked whether arginine metabolism inhibitors could alter recruitment of T lymphocytes in TDLN. In C57BL/6 mice affected by TRAMP-C1 tumors, a 3-fold increase in cell number was evident in TDLN when compared with LN from naïve mice (Supplementary Figs. 2 and 4). Flow cytometry analysis of TDLN cells showed a significant increase in the number of both CD4 and CD8 cells (Supplementary Fig. 2). As seen in other transplantable tumor models (22), the
percentage of CD8 and more evidently of CD4\(^+\) T cells (Supplementary Fig. 4) in TDLN decreased in both L-NAME and vehicle-treated mice, suggesting that in TDLN a relevant perturbation of the physiologic equilibrium among the different cell populations was undergoing, that was not modified by L-NAME. Although MDSC can favor induction of Treg in tumor-bearing hosts (34), in the TDLN of these mice we found a slightly, yet not significant increase of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) cells, which was not modified by L-NAME treatment (Supplementary Fig. 4).

Finally, subcutaneous tumors were measured and compared in vehicle and L-NAME treated animals. At day 35, the tumor volume and area (Fig. 2C) in L-NAME-treated mice was half of those measured in vehicle-treated animals. To confirm this therapeutic effect, vehicle and L-NAME-treated mice were followed in a survival experiment. As reported in Fig. 2D, when all vehicle-treated mice were dead because of the tumor outgrowth, more than 50% of the tumor-bearing mice treated with L-NAME were still alive, and their overall survival was significantly increased. As a measure of potential drug-related toxicity, we monitored animal weight, and we found no differences between drug- and vehicle-treated mice (Fig. 2C). Also, as previously reported (22), this treatment did not cause macroscopic and microscopic damage to the liver, kidney, respiratory apparatus and esophagus (data not shown). All together, these data suggest that in mice bearing subcutaneous PC, L-NAME treatment is able to modulate the arginine metabolism both in periphery and at the tumor site, reduces the immunosuppressive activity of MDSC and restrains tumor growth.

C57BL/6 mice challenged with TRAMPC-1 cells were also treated with sildenafil (Supplementary Fig. 1B). This drug was initially used at 20 mg/Kg/24h (0.13 g/L) as suggested in (23). However, this treatment did not impact on TRAMPC-1 growth (data not shown). Considering that mice have a metabolic activity higher
than humans and that a dose of 100 mg/Kg/24h (0.7g/L) is needed to reach a free plasma sildenafil concentration of approximately 10 nM (35), which is well within the range found in treated humans, mice were subjected to this dose. At day 24, when the experiment was ended, the tumor volume and area in sildenafil-treated mice was half of those measured in vehicle-treated animals, whereas the body weight was comparable (Fig. 3A). This treatment regimen was associated with reduced iNOS expression by CD11b+ cells (Fig. 3B). More importantly, the effects of the drug were measurable also at the tumor site, where sildenafil treatment reduced the Arg1 enzymatic activity (Fig. 3C) and the in vitro immunosuppressive activity of the cells collected from the tumor mass (Fig. 3D).

Modification of myelopoiesis in TRAMP mice developing spontaneous PC

Several drawbacks may bias the results obtained in models of in vivo transplantation of in vitro stabilized tumor cells. Indeed, tumor cells undergo substantial genetic and epigenetic alterations during in vitro passages [e.g. ref.(36)]. Also, cells are usually engrafted without their stroma, therefore altering the natural development of a tumor mass (37). Finally, inflammation is generated at the site of injection (38), potentially induced also by contaminating bacterial products, known as being powerful inducers of MDSC (39).

Hence, similar experiments were conducted in the more realistic TRAMP model. TRAMP mice are transgenic for the SV40 early genes (Tag) expressed under the control of the rat probasin regulatory element. As a result, starting at puberty male mice invariably and progressively develop spontaneous prostate intraepithelial neoplasia (mPIN; week 6-12), adenocarcinoma (week 12-18), with lymph node and visceral metastasis (week 18-30) [wk 18-30; ref. (40)], thus recapitulating human PC
Animals were first analyzed for accumulation of CD11b+ cells during PC development and progression (Fig. 4). At 9 wk of age, when animals are affected by scattered foci of mPIN (40), the percentage of CD11b+Gr1high, CD11b+Gr1int and CD11b+Gr1- cells in the blood of TRAMP mice was substantially similar to that found in age-matched WT animals. In the following wk, although a relevant variability among animals of the same age was evident, a significant increase in the percentage of all populations of CD11b+ cells was found in TRAMP mice, and reached a plateau after wk 20. However, a similar variability and increase was found also in age-matched WT animals, as previously reported by others in 12 month-old WT mice (42).

We hypothesized that variability in disease stage in TRAMP mice of the same age could account for the variability in CD11b+ cells accrual. Hence, samples from TRAMP mice were regrouped for disease score, and compared with data from WT animals of similar age. CD11b+Gr1high cells were more abundant in the early phases of tumor development, when the disease is localized to prostate lobes (Supplementary Fig. 5; disease score: 2). A significant increase in the percentage of CD11b+Gr1- cells was evident in TRAMP mice affected by advanced mPIN (Supplementary Fig. 5; disease score: 3). Curiously, both CD11b+Gr1- and CD11b+Gr1high cells appeared to be reduced in TRAMP mice affected by adenocarcinoma (Supplementary Fig. 5; disease score: 5). No differences were found for CD11b+Gr1int cells at any disease stage (Supplementary Fig. 5).

To gain better insights on MDSC accrual in TRAMP mice when the animals are affected by adenocarcinoma, male TRAMP and WT age-matched littermates were killed at 16 wk of age and CD11b+ cells were quantified in the blood, several organs and the prostate. CD11b+ cells accumulation in the blood, BM and TDLN of TRAMP
mice (Fig. 5 and Supplementary Fig. 6) mimicked although to a lesser extent what we found in mice bearing TRAMPC-1 tumors (Fig. 1 and Supplementary Fig. 2). Unlike with mice bearing TRAMPC-1 tumors however, there was no accumulation of CD11b+ cells in the spleen of TRAMP mice (Fig. 5 and Supplementary Fig. 6). Even more strikingly, spontaneous tumors were characterized by the accumulation of CD11b+Gr1- cells (Fig. 5D), whereas, TRAMPC-1 tumors were infiltrated mainly by immature CD11b+Gr1high cells and to a lesser extent by CD11b+Gr1int (Fig. 1D). Immunofluorescence of TRAMP prostate sections showed that most of the CD11b+ cells were localized in the stroma of transformed acini, and were Gr1-, therefore confirming flow cytometry analyses (Supplementary Fig. 3). These data suggest that perturbation of myelopoiesis is substantially different in mice bearing transplantable or spontaneous tumors.

To confirm that also the CD11b+ cells accumulating in TRAMP mice were “bona fide” MDSC, splenic CD11b+ cells were purified by magnetic bead sorting, and added to a culture of C57BL/6 splenocytes stimulated with anti-CD3 and anti-CD28 antibodies. Indeed, CD11b+ cells purified either from young (i.e. 9 wk) or aged (i.e. 15 wk) TRAMP mice were able to substantially reduce splenocyte proliferation (Supplementary Fig. 7).

Effects of Arg and NOS inhibitors on tumor progression in TRAMP mice

In a first set of experiments, 21 wk-old TRAMP mice were randomly assigned to the following treatments: L-NAME, sildenafil (0.13 g/L) or vehicle dissolved in drinking water (Supplementary Fig 1C). At the end of the second wk, all mice were vaccinated with DC/Tag-IV, and sacrificed one wk later. Body weight measurement (Supplementary Fig. 8A) and autopsy (not shown) did not evidence sign of drug-
related toxicity. At microscopic examination, the prostate of most of the vehicle-treated animals was characterized by enlarged acini presenting a well-differentiated microinvasive PC [ref. (33)]. The average disease score for these animals was $3.5 \pm 0.71$ (Supplementary Fig. 8B). However, treatment with L-NAME did not modify MDSC accrual (Fig. 5 and Supplementary Fig. 6), and neither L-NAME nor sildenafil caused a significant delay in disease progression. The disease score was $3.33 \pm 0.58$ and $3.00 \pm 0.00$, respectively (Supplementary Fig. 8A).

Hypothesizing that MDSC accrual and immunosuppression could have occurred earlier during tumor progression, a similar experiment was conducted starting at 13 wk of age and mice were sacrificed at wk 16. At that age, the average disease score for vehicle-treated TRAMP animals was $2.17 \pm 0.29$ (Supplementary Fig. 8B). However, also in this case, treatment with neither L-NAME nor sildenafil caused a delay in disease progression, and the disease score was $2.83 \pm 1.04$ and $3.33 \pm 0.58$, respectively (Supplementary Fig. 8B).

Finally, 13 wk-old TRAMP mice were subjected to the dose of sildenafil that demonstrated to be effective in the TRAMPC-1 model (0.7 g/L; Fig. 3). Mice followed the treatment protocol described above and were killed at wk 16. As reported in the TRAMPC-1 model (Fig. 3), sildenafil treatment inhibited the immunosuppressive activity of MDSC (Supplementary Fig. 8C). However, the average disease score for TRAMP mice treated with this dose of sildenafil was similar to that of vehicle treated mice [$3.5 \pm 0.53$ (n = 8) and $2.8 \pm 0.84$ (n = 5), respectively; data not shown].

**Modulators of arginine metabolism do not break tumor-specific tolerance in TRAMP mice**
Our last goal was to investigate the effects of Arg and iNOS inhibitors on the endogenous tumor-specific immune response. Indeed, inhibitors of arginine metabolism may increase the endogenous anti-tumor immunity in models of transplantable tumors (22, 23). We have also recently reported that in TRAMP mice Tag, whose expression in prostate epithelial cells is quantitatively similarly to other prostate-associated antigens (43), causes the loss of responsiveness of low avidity Tag-specific T cells (44), therefore mimicking the situation found for non-mutated TAA in patients with advanced PC [e.g., ref. (45)]. Hence, splenocytes recovered from 16 wk-old TRAMP and WT mice, treated either with vehicle, L-NAME or sildenafil, were specifically restimulated in vitro and tested for their ability to recognize different targets. Blasts from WT mice selectively recognized and killed syngenic RMA lymphoma cells (28) pulsed with Tag-IV and B6/K-0 cells, which endogenously express Tag (Fig. 6A). No or marginal lysis was found against unpulsed RMA cells and B6/K-1,4,5 cells that lack Tag-IV (27). Irrespective of the treatment received, splenocytes from TRAMP mice did not significantly kill any target used (Fig. 6B). Lack of response did not appear to depend on the presence of immunosuppressive cells in the culture because also CD8⁺ cells purified from the spleen of vehicle- and L-NAME-treated TRAMP mice and restimulated in vitro did not kill the relevant targets (Fig. 6C).

In summary, treatment with either L-NAME or sildenafil neither rescued the function of tumor-specific cytotoxic T lymphocytes nor delayed tumor progression in TRAMP mice.
DISCUSSION

Strategies to overcome tumor-associated immunosuppression are essential to obtain the most rewarding clinical benefits from the application of immunotherapy to cancer patients.

Our results obtained both in a transplantable model of androgen-independent PC and in TRAMP mice at stages at which tumors are usually androgen-dependent indicate that tumor growth is associated with perturbation of myelopoiesis and accumulation of CD11b+ cells. Accrual of these cells was more noticeable in the transplantable TRAMP-C1 model, where CD11b+Gr-1high cells increased in the blood from < 10% to almost 30%. A less dramatic but yet significant increase of CD11b+Gr-1high cells was measurable in TRAMP mice. More importantly, CD11b+Gr-1high cells represented approximately 20% of the tumor infiltrating cells in TRAMPC-1 tumors, whereas, in spontaneous PC CD11b+Gr-1high cells were almost undetectable, and infiltration by more matured CD11b+Gr-1- cells and to a lesser extent by CD11b+Gr-1int cells dominated. It is tempting to speculate that accumulation of CD11b+ cells depends on the aggressiveness and pro-inflammatory activity of the tumor model used, being more pronounced in transplantable models. Indeed, mice challenged with C26-GM colon carcinoma cells producing GM-CSF or highly immunogenic RMA lymphoma cells show a far more intense accumulation of MDSC, and tumor-bearing mice have to be killed within the first two wk (22). TRAMP-C1 cells have a rather indolent growth in vivo with a doubling time of 11.3 days (25), a characteristic of human PC. Also, the local differences between a transplantable subcutaneous PC and prostate microenvironment should be taken into account. Hence, it is not surprising to find a less dramatic increase of MDSC in TRAMP mice, where tumors spontaneously
develop in several months. In addition, it might be possible that the different androgen sensitivity of tumor cells in the two models differently impacted on recruitment of inflammatory cells at the tumor site.

CD11b+Gr-1\textsuperscript{high} cells likely represent a population of immature MDSC that can differentiate both \textit{in vitro} and \textit{in vivo} into Gr1\textsuperscript{-} cells (9). Increase in the percentage of CD11b+Gr1\textsuperscript{high} cells in TRAMP mice with disease score 2 and of CD11b\textsuperscript{+}Gr1\textsuperscript{-} cells in mice with advanced mPIN (disease score: 3) and especially in the tumor supports this hypothesis, and suggests that accrual of MDSC cells is a dynamic process associated with a disease phase in which the growing tumor alters prostate architecture with stromal reaction and proliferation of smooth muscle cells. We anticipate that peripheral blood accrual of MDSC should be a characteristic of PC patients with early disease.

In the TRAMP-C1 model both L-NAME and sildenafil inhibited the immunosuppressive function of CD11b\textsuperscript{+} cells, and consequently delayed tumor growth. Reduced TRAMP-C1 growth and prolonged animal survival, although statistically significant when compared with vehicle-treated mice, were limited and all mice eventually succumbed to the disease. Hence, TRAMP-C1 cells might be less dependable on arginine metabolism for growth than C26-GM cells.

In the TRAMP model, treatment of tumor-bearing mice with modulators of arginine metabolism inhibited the immunosuppressive function of MDSC but neither modified accrual of CD11b\textsuperscript{+} cells, nor broke tumor-specific tolerance and restrained tumor growth. Although this is, to our knowledge, the first report on the \textit{in vivo} effects of modulators of arginine metabolism in PC cancer and, more importantly in a model of spontaneous tumor development, they appear at odds with several other reports showing the ability of these drugs in delaying tumor growth and increasing the
endogenous tumor-specific immune response. In addition, it has been reported that in vitro Arg and iNOS inhibitors restore tumor infiltrating lymphocyte responsiveness to PC both in humans and in TRAMP mice (12). Several considerations may reconcile this apparent discrepancy. Firstly, in transplantable models the drug is given at the time of tumor cell implantation, and may more easily diffuse among dispersed tumor cells, blocking both the pro-angiogenic and immunosuppressive effects of Arg and iNOS. Secondly, tumor cells in TRAMP mice might be less dependable on arginine metabolism for growth, and this would explain why even if MDSC accumulate also in this model, Arg and iNOS inhibitors are not effective in delaying tumor growth. At difference with the transplantable models, the drugs were administered in TRAMP mice once the tumor was already developed and well-vascularized (46). We are not aware of previous reports demonstrating that such therapy is efficacious in already established tumors. This may be even more challenging in the TRAMP model, where prostate epithelial cells are continuously exposed to products of the oncogene, and a profound state of tumor-specific immunosuppression associates with PC development.

An additional explanation for the lack of efficacy of the Arg and iNOS inhibitors in the TRAMP model, and perhaps in human PC, might be a limited penetration of the drugs into the tumor mass, due to altered vascularization, a characteristic common to tumors of different histotype (47). This would also explain why N(G)-monomethyl-L-arginine is so effective in restoring the function of tumor PC infiltrating lymphocytes in vitro (12), where the drug is freely available. One possibility to overcome this limitation is to combine drugs that normalize tumor vessels with chemotherapy and/or immunotherapy (47). Alternatively, this treatment might be suggested as adjuvant therapy after debulking surgery. Of relevance, whereas treatment with L-NAME would likely cause severe side effects (48),
prolonged PDE-5 inhibitors have been approved for the treatment of several human pathologies and may be proposed as chronic therapy.

It might also be hypothesized that in TRAMP mice the dominant mechanism by which MDSC suppress the tumor-specific immune response is Arg and iNOS independent, therefore, explaining the lack of anti-tumor effects of L-NAME and sildenafil in these mice. MDSC may produce IL-10 and TGF-β, cytokines endowed with immunosuppressive functions (6, 9). MDSC also express the zinc-based protease ADAM17 that cleaves L-selectin on naïve T cells, therefore, decreasing T cell ability to home to sites where they would be activated (49). Although the findings that the in vitro Arg and NOS inhibitors rescue the function of PC-infiltrating T cells in TRAMP mice (12) are strongly suggestive for an altered arginine metabolism, we cannot exclude that other MDSC-mediated mechanisms are responsible for the tumor-associated T cell tolerance found in TRAMP mice (33, 44). This will be the focus of future work.

Several immunosuppressive mechanisms act simultaneously in cancer patients and contribute to immunoevasion. Hence, acting simultaneously on more than one of the known tumor-associated immunosuppressive mechanisms will likely result in more successful therapeutic effects.
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REFERENCES


LEGENDS FOR FIGURES

Figure 1. Treatment with L-NAME does not alter accrual of CD11b+ cells in C57BL/6 mice bearing subcutaneous TRAMP-C1 tumors. C57BL/6 male mice were left untreated (naïve) or challenged s.c. with TRAMP-C1 cells. The same day, tumor-challenged mice were randomly assigned to either one of the following treatments: L-NAME (1 g/L) or vehicle added to the drinking water. Recruitment of CD11b+ cells in the blood and the indicated organs of naïve mice and mice bearing a 50-day old tumor were investigated by flow cytometry. Dot plot panels depict representative results from the blood of one out of 9 naïve mice (A) and TRAMP-C1 bearing mice treated with vehicle (B; n = 11) or L-NAME (C; n = 16). The regions (R1-R3) used to define CD11b+Gr1− (R3), CD11b+Gr1int (R2) and CD11b+Gr1high (R1) cell populations, respectively, are depicted in the panels. Histograms in D report the percentage of CD11b+ subpopulations as aggregated data (average ± SD) for blood, spleen, BM, NDLN, TDLN, and tumor samples, respectively, collected from naive (white bars) and tumor-bearing animals treated with vehicle (black bars) or L-NAME (dashed bars) and analyzed as described above. Statistic analysis was performed using the ANOVA and Newman-Keuls tests: *0.01 < p < 0.05, **0.001 < p < 0.01, ***p < 0.001.

Figure 2. Treatment with L-NAME reduces the immunosuppressive activity of both CD11b+ splenocytes and cells from the tumor mass, and restrains TRAMP-C1 tumor growth. CD11b+ cells were magnetic-bead purified from the spleen of C57BL/6 mice treated as described in the legend to Figure 1 and sacrificed at day 35. CD11b+ cells were added at a final concentration that ranged from 20 to 1% to a mixed leukocyte culture set up with C57BL/6 splenocytes, as responders, stimulated with anti-CD3/CD28 beads (A, left panel). Positive and negative controls were
spleenocytes cultured in the absence of MDSC and stimulated (+) or not (-) with anti-CD3/CD28 beads. Data are expressed as the cpm mean ± SD of triplicates. A, right panel In parallel, CD11b+ spleenocytes from mice treated with vehicle (n= 4; black bar) or L-NAME (n=6; dashed bar) were analyzed for Arg1 enzymatic activity as described in the Materials and Methods section. Data are expressed as units/L of Arg1 activity of 10^6 cells ± SD. TRAMP-C1 tumor cells from the same mice were assessed for in vitro immunosuppressive a and Arg1 enzymatic activities (B, left and right panels, respectively) as described above. Data in each panel are representative of at least three independent experiments. In parallel, C57BL/6 mice were challenged with TRAMP-C1 tumor cells and treated with vehicle (n = 5) or L-NAME (n = 7) as described in the legend to Figure 1. B, Tumor dimension at day 35 is expressed as average ± SD volume (left panel) and area (middle panel). C, right panel, Treatment related toxicity was evaluated by comparing the body weight of vehicle and L-NAME treated mice. Statistic analysis was performed using the Student’s t-test: *0.01 < p < 0.05, **0.001 < p < 0.01. Data in each panel are representative of at least three independent experiments. D, Additional groups (8 animals/group) of vehicle (white circles) and L-NAME treated mice (black circles) were followed for survival and sacrificed when the tumor mass had reached the dimension of approximately 100 mm^2. Survival curves are reported in a Kaplan-Meier plot. Statistical comparison (Log-rank test): p = 0.0006.

Figure 3. Treatment with sildenafil is able to restrain TRAMP-C1 tumor growth. C57BL/6 mice were challenged with TRAMP-C1 cells. The same day, tumor-challenged mice were randomly assigned to either one of the following treatments: vehicle (n =10; black bars) or sildenafil (0.7 g/L; n = 9; dotted bars) added to the drinking water. A, Tumor dimension at day 24 is expressed as average ± SD volume
(left panel) and area (middle panel). Treatment related toxicity was evaluated by comparing the body weight of vehicle and L-NAME treated mice (right panel). B, CD11b⁺ splenocytes from TRAMP-C1 bearing mice treated with vehicle or sildenafil were stimulated in vitro with LPS for 24 h and analyzed for intracellular expression of iNOS by flow cytometry. Data are expressed as mean fluorescence intensity (MFI). C, Arg1 enzymatic activity of cells collected from the tumor mass of the mice described above. D, Cells from the same tumor samples where also investigated for immunosuppressive activity as described in the Legend to Figure 2. Positive and negative controls were splenocytes cultured in the absence of MDSC and stimulated (+) or not (-) with anti-CD3/CD28 beads. Data are expressed as the cpm mean ± SD of triplicates. Statistic analysis was performed using the Student’s t-test: *0.01 < p < 0.05,**0.001 < p < 0.01. Data in each panel are representative of at least two independent experiments.

**Figure 4. CD11b⁺ cells accumulate in the blood of both aged TRAMP and WT mice.** Blood cells from naïve WT and age-matched TRAMP male littermates were stained for CD11b and Gr1 markers and analyzed by flow cytometry. A, Dot plot panels depict results from one out of 9 WT and 15 TRAMP mice of 16-20 wk of age (SSC-H, side scatter channel; FSC-H forward scatter channel). The regions (R1-R3) used to define CD11b⁻Gr1⁻, CD11b⁻Gr1int and CD11b⁻Gr1high cell populations, respectively, are depicted in the right panels. Panel B reports the data from 8 independent experiments aggregated for mouse age (week) at the time of killing, in which blood samples collected from WT (white bars; 9 wk: 3, 16-20 wk: 9, and 24-36 wk: 26 mice) and TRAMP animals (black bars; 9 wk: 5, 16-20 wk: 15, and 24-36 wk: 33 mice) were analyzed as described above. Data are expressed as percentage of cells
within the selected population. Statistic analysis was performed using the Student’s *t-*test: *0.01 < p < 0.05, **0.001 < p < 0.01, ***p < 0.001.

**Figure 5. Treatment with L-NAME does not alter the accrual of CD11b+ cells in TRAMP mice.** Thirteen wk-old TRAMP mice were randomly assigned to either one of the following 3-wk treatments: L-NAME, or vehicle dissolved in drinking water. At the end of the second wk, all mice were vaccinated i.d. with DC/Tag-IV. At the end of the third and last wk, animals were killed. Dot plot panels depict representative results from the blood of one out of five WT (A) and TRAMP mice treated with vehicle (B; n = 6) or L-NAME (C; n = 6). The regions (R1-R3) used to define CD11b+Gr1- (R3), CD11b+Gr1int (R2) and CD11b+Gr1high (R1) cell populations, respectively, are depicted in the panels. D, Histograms in panels report the percentage of CD11b+ subpopulations as aggregated data (average ± SD) for blood, spleen, BM, TDLN, and prostate samples, respectively, collected from naive (white bars) and TRAMP mice treated with vehicle (black bars) or L-NAME (dashed bars) and analyzed as described above. Statistic analysis was performed using the ANOVA and Newman-Keuls tests: *0.01 < p < 0.05, **0.001 < p < 0.01.

**Figure 6. Modulators of arginine metabolism do not cause break of tolerance in TRAMP mice.** Thirteen wk-old TRAMP and WT mice were randomly assigned to either one of the following 3-wk treatments (3-5 animals/group): L-NAME, sildenafil or vehicle dissolved in drinking water. At the end of the second wk, all mice were vaccinated i.d. with DC/Tag-IV. At the end of the third and last wk, animals were killed and their splenocytes (A and B) or magnetic bead-purified CD8+ cells (C) were stimulated in vitro with irradiated B6/K-0 cells, and tested 5 days later for cytotoxic activity (measured as 51Cr release); unpulsed (white diamonds) or Tag-IV-pulsed (black squares) RMA, B6/K-0 (black circles) and B6/K-1,4,5 (white circles) cells
were used as targets. Each panel of the figure is representative of at least three independent experiments, which gave similar results.
Rigamonti N. et al. Fig. 3
Figure 4

A

B

CD11b+Gr1high

% Cells

0 10 20 30

9 16-20 24-36

CD11b+Gr1int

% Cells

0 5 10 15

CD11b+Gr1−

% Cells

0 5 10 15 20

Week

0 5 10 15 20 25

* ** ***
Rigamonti N. et al. Fig. 5

Naïve TRAMP
Vehicle                           L-NAME

D

- Naive
- TRAMP Vehicle
- TRAMP L-NAME

% Cells

** Blood

% Cells

Spleen

% Cells

BM

% Cells

TDLN

% Cells

Prostate

CD11b+Gr1high

CD11b+Gr1int

CD11b+Gr1−
Rigamonti N. et al. Fig. 6

A

WT

% Lytic Activity

E:T ratio

RMA

RMA + Tag-IV

B6/K-1,4,5

B6/K-0

B

TRAMP SPLENOCYTES

% Lytic Activity

E:T ratio

Vehicle

L-NAME

Sildenafil

C

TRAMP CD8+ CELLS

% Lytic Activity

E:T ratio

Vehicle

L-NAME
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