

Generation of antigen-presenting cells from tumor-infiltrated CD11b myeloid cells with DNA demethylating agent 5-aza-2'-deoxycytidine

Irina Daurkin · Evgeniy Eruslanov · Johannes Vieweg ·
Sergei Kusmartsev

Received: 16 March 2009 / Accepted: 13 October 2009 / Published online: 31 October 2009
© Springer-Verlag 2009

Abstract Tumor-recruited CD11b myeloid cells, including myeloid-derived suppressor cells, play a significant role in tumor progression, as these cells are involved in tumor-induced immune suppression and tumor neovascularogenesis. On the other hand, the tumor-infiltrated CD11b myeloid cells could potentially be a source of immunostimulatory antigen-presenting cells (APCs), since most of these cells represent common precursors of both dendritic cells and macrophages. Here, we investigated the possibility of generating mature APCs from tumor-infiltrated CD11b myeloid cells. We demonstrate that *in vitro* exposure of freshly excised mouse tumors to DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (decitabine, AZA) results in selective elimination of tumor cells, but, surprisingly it also enriches CD45⁺ tumor-infiltrated cells. The majority of “post-AZA” surviving CD45⁺ tumor-infiltrated cells were represented by CD11b myeloid cells. A culture of isolated tumor-infiltrated CD11b cells in the presence of AZA and GM-CSF promoted their differentiation into mature F4/80/CD11c/MHC class II-positive APCs. These tumor-derived myeloid APCs produced substantially reduced amounts of immunosuppressive (IL-13, IL-10, PGE₂), pro-angiogenic (VEGF, MMP-9) and pro-inflammatory (IL-1β, IL-6, MIP-2) mediators than their precursors, freshly isolated tumor-infiltrated CD11b cells. Vaccinating naïve mice with

ex vivo generated tumor-derived APCs resulted in the protection of 70% mice from tumor outgrowth. Importantly, no loading of tumor-derived APC with exogenous antigen was needed to stimulate T cell response and induce the anti-tumor effect. Collectively, our results for the first time demonstrate that tumor-infiltrated CD11b myeloid cells can be enriched and differentiated in the presence of DNA demethylating agent 5-aza-2'-deoxycytidine into mature tumor-derived APCs, which could be used for cancer immunotherapy.

Keywords Tumor-infiltrated myeloid cells · 5-Aza-2'-deoxycytidine · Dendritic cells

Introduction

Previous studies demonstrated that solid tumors actively recruit bone marrow-derived CD11b myeloid cells [1–3]. Tumor-infiltrated CD11b cells support tumor growth via several distinct mechanisms, including stimulation of angiogenesis and neovascularization [4–6], stimulation of metastasis [7, 8] and participation in tumor-induced immunosuppression [9–13]. Bone marrow-derived tumor-recruited CD11b cells may also serve as stromal cells and promote tumor growth through production of various cytokines and growth factors [14, 15]. Most of the mouse tumor-infiltrated CD11b myeloid cells co-express monocyte/macrophage marker F4/80, produce M2 and pro-inflammatory cytokines and mediators (IL-10, IL-13, IL-6, IL-1β, MIP-1, MIP-2, PGE₂ and other), and also secrete pro-angiogenic factors such as VEGF and MMP-9. One typical characteristic of tumor-infiltrated myeloid cells is up-regulated expression of arginase I, which is implicated in mechanisms of the inhibition of T cell-mediated

I. Daurkin · E. Eruslanov · J. Vieweg · S. Kusmartsev
Department of Urology, Shands Cancer Center,
College of Medicine, University of Florida,
Gainesville, FL 32610, USA

S. Kusmartsev (✉)
Cancer and Genetics Research Center,
University of Florida, 1376 Mowry Rd,
Rm. 459, P.O. Box 103633, Gainesville, FL 32610, USA
e-mail: s.kusmartsev@urology.ufl.edu

anti-tumor immune response [16, 17]. Expression of arginase I in myeloid cells is dependent on expression of M2 cytokines and/or PGE₂.

On the other hand, tumor-recruited CD11b cells may play a key role in tumor destruction as cytotoxic M1 macrophages and/or via initiation of adaptive T cell-mediated immune response as antigen-presenting cells (APCs). An important characteristic of anti-tumor action in myeloid cells is a state of differentiation and Th1-cytokine orientation. It appears that the pro-tumoral (immunosuppressive, pro-angiogenic) phenotype of tumor-infiltrated CD11b myeloid cells is dictated by tumor microenvironment, which efficiently converts the newly recruited bone marrow-derived myeloid cells into immunosuppressive and tumor supporting cells.

Tumors inhibit differentiation and/or maturation of APCs and promote immunosuppressive accumulation of myeloid cells (myeloid-derived suppressor cells, MDSCs and/or tumor-associated macrophages, TAMs) via several mechanisms, including activation of Jak2-STAT3 pathway [12, 18], overproduction of VEGF [19], PGE₂ [13, 20–23] and S100A9 protein [24]. In the present study, we investigated the effect of DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (AZA) on tumor-infiltrated cells. Our data demonstrate that the addition of AZA to the whole tumor cell suspension, or to the freshly isolated tumor-infiltrated CD11b cells, results in the selective elimination of tumor cells. This allows surviving CD11b cells to differentiate into mature APCs, which co-express CD11c, MHC class II and CD86. The tumor-derived APCs secreted much lower amounts of immunosuppressive (PGE₂, IL-13, IL-6), pro-inflammatory (IL-1beta, MIP-2) and pro-angiogenic (VEGF, MMP-9) mediators than their precursors, tumor-infiltrated CD11b cells. Vaccination of naïve mice with ex vivo generated tumor-derived APCs protected mice from tumor outgrowth.

Materials and methods

Mice and tumor models

Female BALB/c and C57BL/6 female mice (all 6–8 weeks of age) were obtained from the National Cancer Institute (Frederick, MD). Murine renal carcinoma cell line Renca was kindly provided by Dr. Jennifer Smith and Dr. James Finke (Cleveland Research Institute, Cleveland, OH). The CT-26 murine colon carcinoma cell line and TRAMP-C2 murine prostate adenocarcinoma cell line were purchased from ATCC (Manassas, VA). Tumor cells were routinely maintained in vitro at 37°C in a 5% CO₂ humidified atmosphere in a DMEM/F12 culture medium supplemented with 10% FBS (HyClone). To establish subcutaneous tumors, 5 × 10⁵ of CT-26 or Renca cells were injected into left

flank of BALB/c mice. The same numbers of TRAMP-C2 cells were inoculated into C57BL/6 mice.

Reagents

5-Aza-2'-deoxycytidine (AZA) was purchased from Sigma Chemicals (St. Louis, MO). Mouse rGM-CSF was purchased from R&D Systems (Minneapolis, MN). BrdU kit, CD11b, CD11c, CD8, CD4, CD86, I-A^d antibodies and antibody against arginase I were purchased from BD Pharmingen (San Diego, CA). F4/80 antibody was from Serotec Inc. (Raleigh, NC).

Tumor digestion

CT-26 and Renca tumors were harvested on days 12–14, whereas TRAMP-C2 tumors were harvested on days 18–20 after tumor cell inoculation. Tumor-bearing mice were killed in a CO₂ chamber. Tumors were isolated from the mice and sliced into 1–3 mm³ pieces with scissors. The minced tumor tissue was incubated at 37°C for 1 h in a medium containing 2% FBS (HyClone), antibiotics (penicillin/streptomycin, HyClone), 50 U/ml collagenase I, 100 U/ml collagenase IV, 200 U/ml DNase-I and 2.5 U/ml protease XIV (Sigma, St Louis, MO). After washing cells in PBS, they were re-suspended in the complete culture medium. Viability of cells, as determined by trypan blue exclusion, was more than 90%.

Isolation CD11b cells from tumors

CD11b myeloid cells were purified from tumor cell suspension using the MACS method (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's instructions. Briefly, cells were incubated with magnetic beads conjugated with anti-mouse CD11b and positively selected on LS columns. The purity of recovered cells assessed by flow cytometry was >95%.

5-Aza-2'-deoxycytidine treatment

Whole tumor cell suspensions, or CD11b cells isolated from tumor, were cultured in the presence of rGM-CSF (20 ng/ml) and 5 μM of AZA in six-well ultra low attachment plates (Corning Inc., Lowell, MA) for 7 days. For whole tumor cell culture, fresh AZA was added every second day of culture. For CD11b cells, AZA (5 μM) in fresh medium was added on days 1, 4 and 6.

FACS analysis

Non-specific cell staining was prevented by blocking Fc receptors with anti-CD16/CD32 mAbs (Mouse BD Fc

Block™, BD Biosciences, San Jose, CA). The cells were then incubated for 30 min on ice in 100 µl of PBS with 1 µg of relevant fluorochrome-conjugated or -matched isotype control antibodies and then washed twice with cold PBS. Flow cytometry data were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and were analyzed with CellQuest software (BD Biosciences).

qRT-PCR

Total cellular RNA was purified from tumor-isolated CD11b cells using RNeasy Plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Integrity of the RNA was analyzed in 2100 Bioanalyzer (Agilent Technologies Inc). cDNA for each RNA sample was synthesized in 20 µl reactions using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). cDNA-specific TaqMan® Gene Expression Assays for 15-(NAD)PGDH (assay ID: Mm00515121_m1) and COX2 (assay ID: Mm00478374_m1) from Applied Biosystems were used in the study. A mouse ACTB (actin, beta) was used as an endogenous control (Applied Biosystems). PCRs were carried out in 50 µl reaction volumes containing TaqMan Universal Master Mix (2×), TaqMan Gene Expression Assay for target gene and optimized quantities of cDNA samples. qRT-PCR was performed using an Applied Biosystems Prism 7900HT Fast Real-Time PCR System. All samples were run in triplicate, and amplification data were analyzed using Applied Biosystems Prism Sequence Detection Software, version 2.2.1. Relative quantification was calculated according to the $\Delta\Delta C_t$ method (Applied Biosystems) using a statistical confidence of 99.9%.

Cytokine detection

Supernatants from cultured isolated CD11b cells were collected, filtered and subjected to cytokine analysis. Level of IL-10, IL-6, IL-13, IL-1beta, MIP-2 and G-CS was measured using Multiplex assay. A multiplex cytokine kit was obtained from Bio-Rad Laboratories (Hercules, CA), and the assay was performed using Luminex-200 (Luminex Corporation, Austin, TX). VEGF and MMP-9 were detected in cell supernatants using commercial quantitative sandwich immunoassay kits from R&D Systems (Minneapolis, MN).

Western blotting

Freshly isolated or cultured tumor-isolated CD11b cells were lysed with RIPA buffer in the presence of protease inhibitors. Samples (30 µg protein per lane) were subjected to electrophoresis in SDS-polyacrylamide gels, and then

blotted onto PVDF membranes. Membranes were blocked for 1 h at room temperature with 5% dry skimmed milk in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) plus 0.1% Tween 20 and then probed with primary antibodies of appropriate specificity overnight at 4°C. Membranes were washed and incubated for 1 h at room temperature with secondary antibody conjugated with horse radish peroxidase. Results were visualized by chemiluminescence detection using a commercial kit (Amersham Biosciences).

In vivo vaccination

To prepare APCs from tumor-infiltrated myeloid cells, CD11b cells were isolated from a freshly excised and digested CT-26 tumor cell suspension with magnetic beads as described above. Myeloid cells were cultured in ultra low attachment six-well plates in the presence of GM-CSF (20 ng/ml) and AZA (5 µM). Fresh AZA and GM-CSF in complete medium have been added every third day of culture. On days 7 and 8 after culture initiation, cells were collected, washed and injected (1×10^6 per mouse) subcutaneously into the right flank of naïve BALB/6 mice. Vaccination was done twice on a 2-week interval. One week after the last APC injection, BALB/6 mice were subcutaneously inoculated with 5×10^5 CT-26 or 5×10^5 Renca tumor cells. Tumor growth was monitored by regular (twice a week) measurement of tumor size using a Vernier caliper.

Statistical analysis

The statistical significance between values was determined by the Student's *t* test. All data were expressed as the mean \pm SD. Probability values ≥ 0.05 were considered non-significant. Significant values of $p \leq 0.05$ were expressed as asterisk (*). The flow cytometry data shown are representative of at least three separate determinations.

Results

In vitro exposure of freshly excised tumors to AZA results in the selective elimination of tumor cells and in the enrichment of tumor-infiltrated CD45-positive cell population

Previously DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (AZA) has been shown to induce apoptosis of both mouse and human tumor cell lines in vitro [reviewed in 25]. Here, we used AZA for the treatment of freshly excised solid mouse CT-26 colon carcinomas. In order to see effect of AZA on CT-26 colon carcinoma

tumor cells, we harvested tumors from mice, disaggregated them with collagenase cocktail, and then cultured in the presence of GM-CSF (20 ng/ml) and AZA (5 μ M) for 7 days. Cultured for 7 days, whole tumor cell suspensions were collected, washed with PBS, stained with 7-AAD and then analyzed by flow cytometry. As can be seen in Fig. 1a and b, the presence of AZA results in predominant elimination of CD45-negative cells and, in the same time, promotes a significant enrichment of the CD45-positive tumor-infiltrated cell population ($83.5 \pm 5.7\%$ in AZA-treated group vs. $18.4 \pm 4.9\%$ in control-untreated group, $p < 0.05$). Only cells negative for 7-AAD are shown in Fig. 1b. The majority of the surviving “post-AZA” CD45⁺ cell population was represented by CD11b myeloid cells (Fig. 1c). Moreover, most of these CD11b cells also co-expressed mouse dendritic cell marker CD11c. Collectively, obtained results demonstrate that exposure of whole tumor cell suspension to AZA promotes selective elimination of tumor cells, while significantly enriching the CD45-positive cell population, which consisted mostly of tumor-infiltrated CD11b myeloid cells.

Phenotypic characterization of APC generated from tumor-infiltrated CD11b myeloid cells

Next, we studied the effect of AZA on the GM-CSF-driven differentiation of purified tumor-infiltrated CD11b cells. First, we isolated CD11b cells from CT-26 tumors, prepared the cytopines and stained with hematoxylin–eosin. Microphotograph (Fig. 1d) demonstrates that morphology of CD11b-positive cells isolated from CT-26 colon carcinomas resembles the mixture monocyte–macrophage and immature dendritic cells. This phenotype was also confirmed by flow cytometry analysis (Fig. 2, upper row; day 0), which shows high expression of monocyte–macrophage marker F4/80 and low expression of co-stimulatory molecule and MHC class II molecule in fresh-derived tumor-infiltrated CD11b cells from CT-26 tumor.

To evaluate the effect of AZA on ex vivo differentiation of tumor-infiltrated CD11b myeloid cells, we next cultured freshly derived CD11b cells from CT-26 tumors in the presence of AZA and GM-CSF for 7 days, and then analyzed cells by flow cytometry. As shown in Fig. 2, the addition of AZA to the culture of tumor-derived CD11b cells promoted

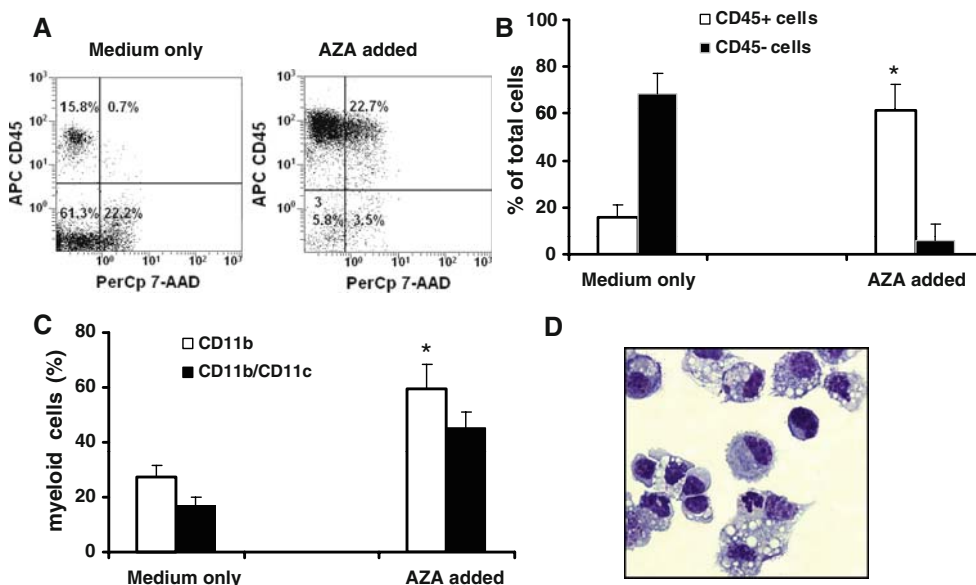


Fig. 1 AZA selectively eliminates CT-26 tumor cells and enriches the non-tumor CD45⁺ cell population. CT-26 tumors were harvested and disaggregated by treatment with collagenase cocktail as specified in “Materials and methods”. Whole single tumor cell suspensions were washed with PBS, placed in six-well plates ($2\text{--}3 \times 10^6$ per well) in complete medium, and cultured in the presence of AZA (5 μ M). Fresh AZA has been added every second day. On day 7, recovered cells were collected, washed with PBS, enumerated under a microscope with trypan blue, stained with specific antibody and analyzed by flow cytometry. **a**, **b** AZA selectively enriches tumor-infiltrated CD45⁺ cells. Whole CT-26 tumor cell suspensions were cultured for 7 days in the presence or absence of AZA, then collected, washed with PBS and stained with CD45-APC and PerCp-7AAD. Proportion of 7-AAD-positive cells and proportion of CD45-positive/negative cells was

evaluated using flow cytometry. Results of one representative experiment out of three are shown (**a**). **b** Shows proportion of CD45⁺/7-AAD-negative and CD45⁻/7-AAD-negative cells among tumor cell suspension treated with AZA. Cumulative results of three independent experiments are shown. **c** The majority of post-AZA CD45⁺ surviving CT-26 tumor-infiltrated cells consist of CD11b-positive myeloid cells. AZA-treated cells were collected on day 7. Cells were stained with CD45-APC, CD11b-PerCP and CD11c-PE antibody. Expression of CD11b and CD11c markers has been measured by flow cytometry. **d** Cytological analysis of freshly purified tumor-infiltrated CD11b cells. Tumor-infiltrated CD11b cells were purified by positive selection from CT-26 tumors as described in “Materials and methods”. Cytopines with tumor-infiltrated CD11b-positive cells were prepared and stained with hematoxylin and eosin

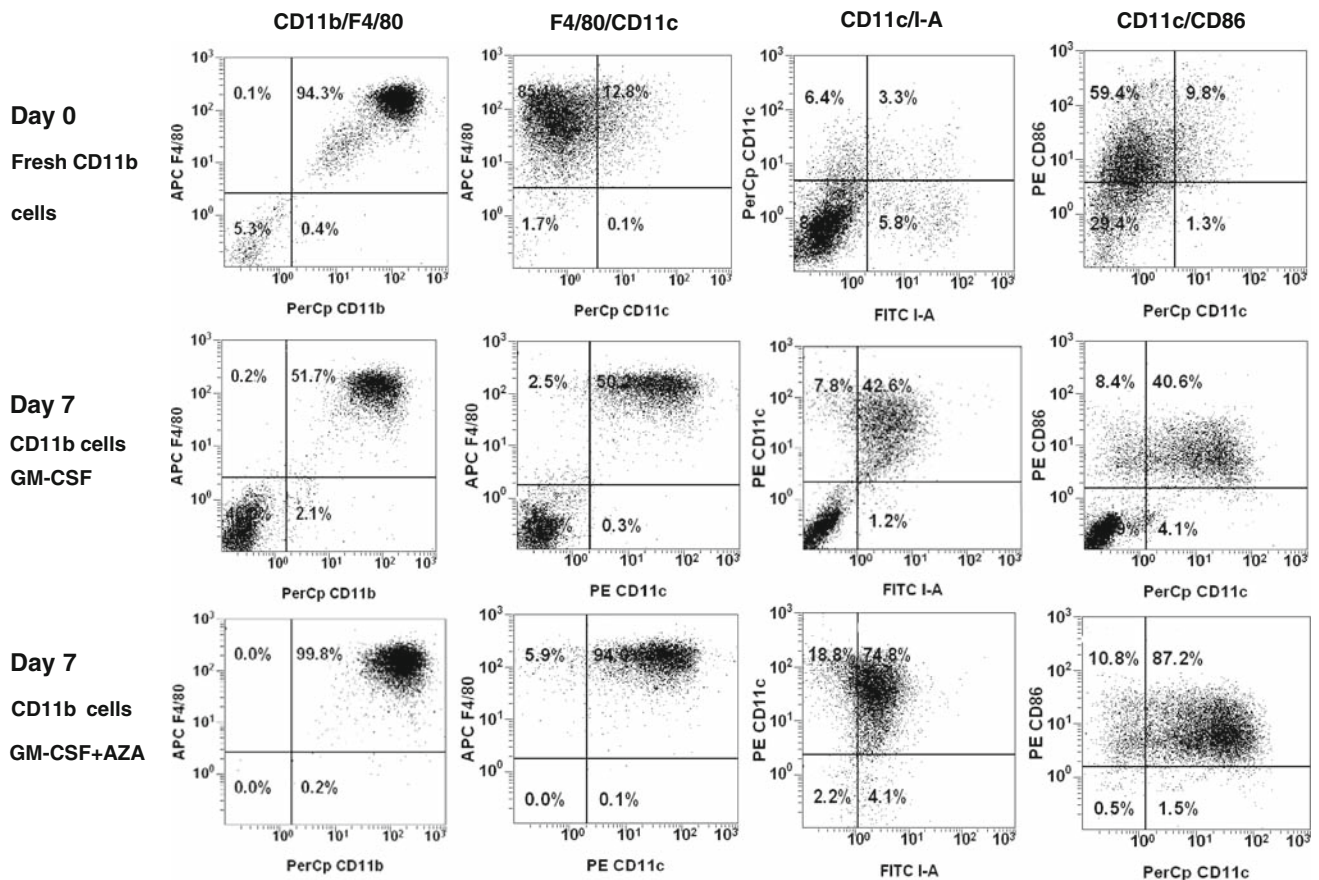


Fig. 2 AZA-mediated elimination of the tumor cells promotes differentiation of tumor-derived CD11b cells into CD11c⁺ MHC class II-positive antigen-presenting cells. CT-26 tumors were harvested from syngeneic tumor-bearing mice on days 12–14 after tumor cell inoculation. Whole tumor single cell suspension has been prepared as described in “Materials and methods”. Tumor-infiltrated CD11b cells were purified by positive selection using magnetic beads. Fresh-

isolated CD11b cells were washed with PBS, enumerated, placed in six-well plates (2×10^6 per well) in complete medium and cultured in the presence of AZA (5 μ M) and GM-CSF (20 ng/ml). On day 7, cells were collected, washed with PBS, stained with antibody against CD11b, F4/80, CD11c, I-A^d, CD86 and CD45, analyzed by flow cytometry. Results of one representative experiment out of three are shown

generation of pure CD11b⁺CD11c⁺ (>99% purity) mature APCs, which also co-express CD86 (>85%) and MHC class II (>75%). In contrast, in the absence of AZA in cultures, the proportion of myeloid cells co-expressing CD11b, CD11c, CD86 and MHC class II was markedly lower (Fig. 2). Together, obtained results suggest that AZA could significantly improve the in vitro GM-CSF-driven differentiation of tumor-derived CD11b cells into mature myeloid APCs.

Secretion of cytokines by tumor-derived APCs

It is well established that tumor-infiltrated myeloid cells, including MDSCs and TAMs, are immunosuppressive and produce a number of immunosuppressive and pro-angiogenic mediators [3, 15, 26, 27]. Importantly, that expression of mediators in tumor-infiltrated myeloid cells, including arginase I, COX-2 and PGE₂, is inducible and mediated by tumor microenvironment. Next, we evaluated whether addition of AZA to the cultures of tumor-derived CD11b cells could

affect the production of immunosuppressive and pro-angiogenic mediators. As can be seen in Fig. 3a and b, the presence of AZA in cultures promotes a potent down-regulation of arginase I and COX-2 genes. We also observed a modest increase of 15-PGDH gene expression (Fig. 3c). 15-PGDH enzyme plays a major role in degradation of PGE₂ in tumor microenvironment, and the expression of this gene is frequently down-regulated in tumor tissue [28, 29]. Figure 3d and e shows that production of the several immunosuppressive and pro-inflammatory mediators including IL-13, IL-6, IL-10, MIP-2, IL-1beta, and G-CSF has been significantly reduced in AZA-treated tumor-derived myeloid cells as compared to the fresh-derived tumor-infiltrated CD11b cells. Furthermore, tumor-derived myeloid cells cultured in the presence of AZA secreted substantially less VEGF. Together, obtained data demonstrate that tumor-isolated myeloid cells differentiated in the presence of AZA show reduced expression of arginase I, COX-2 and produce decreased amounts of pro-angiogenic, immunosuppressive and pro-inflammatory

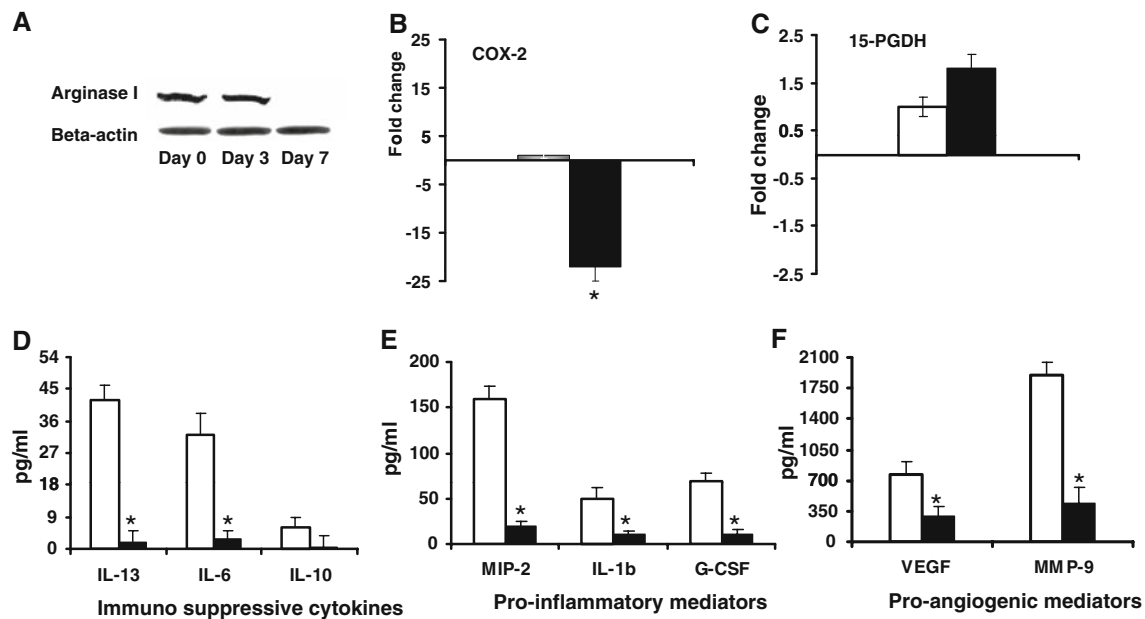


Fig. 3 Cytokine profile of freshly isolated CD11b cells and AZA-treated, in vitro differentiated tumor-derived APCs. **a** Effect of AZA on arginase I expression. Cell lysates prepared from tumor-derived CD11b cells during their in vitro differentiation in the presence of AZA (days 0 and 7). Arginase I expression was measured by Western blotting as described in “Materials and methods”. **b, c** Expression of COX-2 and 15-PGDH genes. Freshly purified (day 0, *open bars*) and in vitro differentiated AZA-treated tumor-derived CD11b cells (day 7, *filled bars*) were analyzed for expression of COX-2 (**b**) and 15-PGDH (**c**) by qRT-PCR. CD11b cells were isolated from CT-26 tumors with

magnetic beads as described in “Materials and methods”. Total RNA was isolated from purified pooled CD11b cells (3–5 mice per group). Assays were done in triplicates. Average mean \pm SD is shown. **d–f** Secretion of cytokines. To obtain cell supernatants, freshly isolated CT-26 tumor-derived CD11b cells (day 0, *open bars*) or in vitro differentiated AZA-treated cells (day 7, *filled bars*) were cultured in humidified CO₂ incubator at 37°C for 24 h. Then supernatants were collected, filtered and assayed for the presence of cytokines and growth factors using Multiplex assay. Average mean \pm SD is shown

mediators than their tumor-infiltrated CD11b myeloid cell precursors.

AZA prevents tumor outgrowth in cultures of tumor-infiltrated CD11b cells

Technically, the purity of tumor-isolated CD11b cells never reaches 100% due to minor contamination with other CD11b-negative cells, which consist mostly of tumor cells (<5%). This residual tumor cell population can be frequently observed in cultures of tumor-derived CD11b cells, and, eventually, this initially minor tumor cell population grows over time (Fig. 4, left panel) and finally takes over in most cultures. The speed of this process depends on tumor type, purity of isolated cell population and initial cell density in cultures. In contrast, the addition of AZA to the cultures effectively eliminates growth of tumor spheroids in vitro (Fig. 4, right panel) thus allowing differentiation of tumor-derived CD11b cells into mature myeloid APCs.

Vaccination of naïve mice with tumor-derived APCs induces protection from tumors

We next investigated whether vaccination of naïve mice with ex vivo differentiated tumor-derived APCs could

protect tumor outgrowth. Naïve mice were twice subcutaneously (s.c.) injected with CT-26 tumor-derived APCs in 2-week intervals. No loading with exogenous tumor-associated antigen was done before injection. A week after the last vaccination, mice were inoculated s.c. with CT-26 tumor cells in the same flank. Tumor appearance and growth were monitored twice a week. Obtained results (Fig. 5a) show that 70% of the mice vaccinated with tumor-derived ex vivo differentiated myeloid APCs (AZA-treated CD11b cells) did not develop tumors; whereas mice vaccinated with freshly derived tumor-infiltrated CD11b cells or control mice (received PBS injections instead of vaccination) developed tumors and died within 40 days after tumor cell inoculation. Importantly, this anti-tumor effect was antigen-specific, since vaccination of naïve mice with CT-26 tumor-derived APCs did not protect mice from outgrowth of irrelevant tumor: Renca mouse renal cell carcinoma (Fig. 5b). To see whether ex vivo generated CT-26 tumor-derived APCs could stimulate in vitro T-mediated immune response, we co-cultured those APCs and immune T cells isolated from tumor-free vaccinated mice. Seventy-two hours later, cells and cell supernatants were collected and analyzed for T cell proliferation and IFN- γ production, respectively. Figure 5c and d shows that APCs are able to stimulate the proliferation of T cells and IFN- γ production

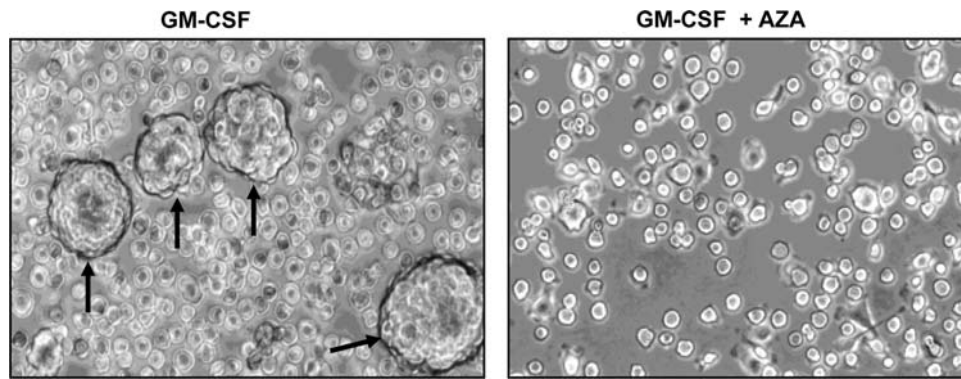


Fig. 4 AZA prevents tumor cell outgrowth in cultures of tumor-infiltrated CD11b cells. Whole CT-26 tumor cell suspension has been prepared as described in “Materials and methods”. Tumor-infiltrated CD11b cells were purified by positive selection using magnetic beads. Fresh-isolated CD11b cells were washed with PBS, enumerated, placed in six-well plates (2×10^6 per well) in complete medium and

cultured in the presence of AZA ($5 \mu\text{M}$) and GM-CSF (20 ng/ml). Microphotographs were taken on day 7. Images were visualized using Leica light microscope with original magnification $\times 100$. Arrows indicate the tumor cell clusters/spheres that appeared in untreated cultures of tumor-derived CD11b cells

in the absence of exogenous tumor-associated antigen. Taken together, these observations demonstrate that *ex vivo* manipulated tumor-derived APCs have the ability to activate the tumor-specific T cells and induce protective anti-tumor responses.

Discussion

Growth of solid tumors in both humans and mice is frequently associated with systemic expansion of bone marrow-derived CD11b myeloid cells. We and others have previously reported that the tumor growth in mice is associated with excessive accumulation of Gr-1/CD11b immunosuppressive myeloid (MDSCs) cells in the spleen, bone marrow and peripheral blood [17, 30–32]. MDSCs accumulating in tumor-bearing hosts play an important role in tumor non-responsiveness by suppressing the generation of an anti-tumor adaptive T cell-mediated immune response [33–38].

Moreover, those recruited inflammatory myeloid cells play multiple roles in the regulation of tumor growth. Thus, studies demonstrate that tumor-infiltrated CD11b cells represent a major part of tumor stroma and, consequently, the targeting of tumor stroma results in tumor rejection [14]. Recent studies revealed the active involvement of CD11b cells in neovasculogenesis [5, 6], tumor angiogenesis [4], in the process of tumor cell invasion and metastasis [7, 8]. In murine tumors, most of the tumor-infiltrated CD11b cells express F4/80 which is a specific marker for monocyte/macrophages. It has been shown that in up to 70–80% of newly tumor-recruited CD11b myeloid cells in tumor microenvironment quickly acquire monocyte–macrophage marker F4/80, up-regulate expression of arginase I and become highly immunosuppressive TAMs or MDSCs [11].

But, at the same time, these cells are precursors of APCs and could be differentiated into immunostimulatory CD11c⁺ MHC class II-positive APCs. However, tumor microenvironment inhibits their differentiation and/or maturation by promoting immunosuppressive and tolerogenic function in recruited myeloid cells via induction of arginase I and M2-oriented phenotype [11, 16, 39].

In agreement with previously published studies, we show that the majority of freshly isolated tumor-infiltrated CD11b-positive myeloid cells from CT-26 colon carcinoma also co-express a F4/80 surface marker and display morphology of monocyte–macrophage cell lineage. These cells also characterized by high expression of the arginase I and COX-2. In addition, the tumor-infiltrated CD11b myeloid cells secreted high amounts of pro-angiogenic and immunosuppressive factors.

In order to modify the tumor microenvironment and improve *ex vivo* differentiation of tumor-derived CD11b myeloid cells, in the present study, we have employed the cytosine analog 5'-deoxy-2-azacytidine (decitabine or AZA), which currently is one of the most advanced drugs for epigenetic cancer therapies. AZA has been widely used as specific inhibitor DNA methyltransferase, the enzyme, which is responsible for DNA methylation in mammals. This compound functions as a DNA methyltransferase inhibitor and has shown substantial potency in reactivating epigenetically silenced (hypermethylated) tumor suppressor genes *in vitro*. Importantly, AZA has been shown to induce the death of different cancer cell lines when applied *in vitro* [25].

Our data demonstrate that the addition of AZA to the cultures of whole tumor cells or to isolated tumor-infiltrated CD11b cells results in the selective elimination of tumor cells, allowing surviving and differentiating remained CD11b cells into mature APCs, which co-express CD11c,

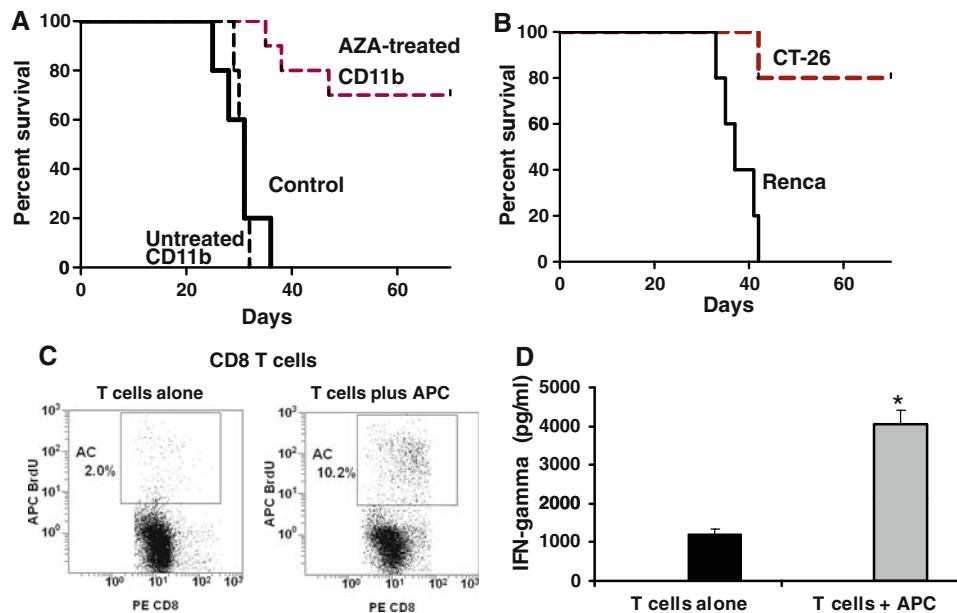


Fig. 5 Vaccination of naïve mice with ex vivo generated APCs results in protection from tumor. **a** Naïve BALB/C mice were divided into three groups. Control group in 2-week interval received two subcutaneous injections of PBS (100 μ l, in *right flank*). Mice from second group were twice injected with APC generated ex vivo from CT-26 colon carcinoma-derived CD11b cells in the presence of AZA (1×10^6 cells/mouse, s.c., 2-week interval). Third group similarly has been vaccinated with untreated tumor-derived CD11b cells. A week after the last vaccination, all mice were inoculated s.c. with CT-26 tumor cells (5×10^5 per mouse) in *right flank*. Tumor appearance and growth were monitored twice a week. Percentage of tumor-free mice over time is shown. Cumulative results derived from three independent experiments are shown. **b** Ten naïve BALB/c mice were vaccinated twice with APC generated ex vivo from CT-26 tumor-derived CD11b cells in the presence of AZA as described above (**a**). Mice were randomly

divided into two groups. One week after last vaccination, mice from the first group were inoculated with CT-26 tumor. Second group received irrelevant Renca tumor. Percentage of tumor-free mice over time is shown. Combined results from two independent experiments are shown. **c** In vitro proliferation of T cells co-cultured with tumor-derived APCs. For these experiments, we used the purified CT-26 tumor-specific T lymphocytes, which were isolated from spleens of previously vaccinated tumor-free mice. Immune T lymphocytes and ex vivo generated tumor-derived APC (CD11b cells treated with AZA) were co-cultured for 72 h (cell ratio 5:1, no exogenous tumor antigen has been added). Proliferation of CD8 T cells was assessed by incorporation of BrdU in DNA by flow cytometry. **d** IFN- γ production by T cells co-cultured with tumor-derived APC. Level of IFN- γ in cell supernatant has been measured using ELISA

MHC class II and CD86. These tumor-derived APCs secreted much lesser amounts of immunosuppressive (PGE2, IL-13, IL-6), pro-inflammatory (IL-1 β , MIP-2) and pro-angiogenic (VEGF, MMP-9) mediators than their precursors, tumor-infiltrated CD11b cells. This effect could be explained, in part, by DNA demethylating action of AZA.

To test whether ability of AZA to selectively eliminate tumor cells and enrich the tumor-infiltrated CD11b cells is true for other tumor models, we conducted additional experiments using Renca renal cell carcinoma and prostate adenocarcinoma TRAMP-C2. Freshly excised Renca and TRAMP-C2 tumors were cultured in the presence of AZA for 7 days, and then surviving cells were recovered and analyzed by flow cytometry. Obtained results (see Fig. 6) indicate that both Renca and TRAMP-C2 tumor cells are also highly sensitive to the AZA treatment, and this treatment results in enrichment of the CD45⁺CD11b⁺ tumor-infiltrated cells. These findings raise a possibility to use AZA for ex vivo enrichment of tumor-infiltrated CD11b

cells and further generation of tumor-derived mature APCs for cancer immunotherapy.

The in vitro differentiation-promoting effect of AZA on tumor-infiltrated CD11b cells could be explained by selective elimination of tumor cells, which results in significant enrichment of CD45⁺CD11b⁺ myeloid cells representing a major fraction of surviving tumor-derived cells after AZA treatment. In addition, AZA-mediated elimination of tumor cells could reduce concentrations of tumor-derived factors in cultures, thus promoting GM-CSF-driven differentiation of the tumor-derived CD11b myeloid cells into immunostimulatory APCs.

On the other hand, we also cannot rule out the possibility that AZA itself may support differentiation/maturation of tumor-infiltrated myeloid APCs. Recent reports suggest the possibility of regulating APC function and cytokine production via epigenetic mechanisms [40–42]. Other studies also suggest that low doses of AZA promote differentiation of myeloid cells [42]. Taking into account these possibilities, it is plausible that the epigenetic modifier AZA may

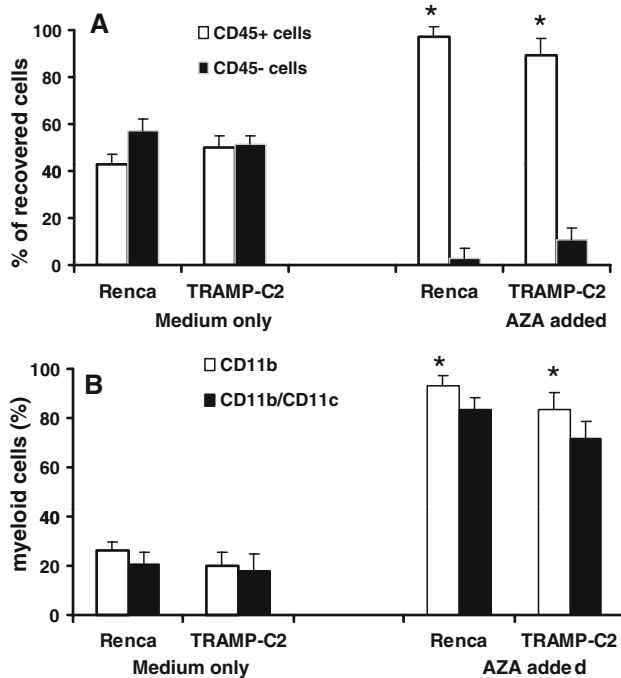


Fig. 6 Enrichment of tumor-infiltrated CD11b cell population from mouse Renca renal cell carcinoma and TRAMP-C2 prostate tumors. Renca and TRAMP-C2 tumor cell suspensions were prepared as described in “Materials and methods”. Cells were cultured in the presence of AZA (5 μ M). Fresh AZA has been added every second day. On day 7, recovered cells were collected, washed with PBS, enumerated under a microscope with trypan blue, stained with specific antibody and analyzed by flow cytometry. Cumulative results of two independent experiments are shown. **a** AZA selectively enriches CD45-positive tumor-infiltrated cells. Whole Renca and TRAMP-C2 tumor cell suspensions were cultured for 7 days, collected, washed with PBS and stained with CD45-APC. Proportion of CD45-positive/negative cells was evaluated using flow cytometry. **b** The majority of “post-AZA” CD45⁺ surviving Renca and TRAMP-C2 tumor-infiltrated cells consist of CD11b-positive myeloid cells. AZA-treated cells were collected on day 7. Cells were stained with CD45-APC, CD11b-PerCP and CD11c-PE antibody. Expression of CD11b and CD11c markers has been measured by flow cytometry

improve the differentiation of tumor-derived APC through two different mechanisms: (a) promotion of tumor cell death, which is mediated by inhibition of DNA methyltransferase and recovering of tumor suppressor genes; (b) direct stimulation of APC differentiation and possibly function via unknown mechanism.

An important feature of the DNA methyltransferase inhibitor AZA is immunomodulatory activity. The immunopotentiating effect of AZA has been reported in several studies [43–45]. Specifically, AZA induces the expression of some tumor-associated antigens such as cancer–testis antigens (CTAs) which are considered to be suitable targets for cancer immunotherapy, but its expression is epigenetically silenced. Exposure of tumors to AZA could increase immunogenicity of tumors via reactivating of silenced CTAs and up-regulating MHC class I.

It is well established that APCs derived from tumor host can exert tolerogenic effects on T cells. Tolerogenic effects of APC cells are frequently associated with a high production of IL-10 or PGE₂. Here, we show that freshly derived CD11b myeloid cells express high levels of the PGE₂-forming enzyme COX-2 and also secrete IL-10. During GM-CSF-driven differentiation in the presence of AZA, both expression of COX₂ and IL-10 production have been substantially down-regulated. Vaccination of naive syngeneic mice with ex vivo generated tumor-derived APCs resulted in protection of 70% vaccinated mice from tumor outgrowth. Together, obtained data suggest that in vitro treatment of tumor-derived myeloid cells with AZA could significantly improve both differentiation and immunostimulatory APC function. These results are consistent with a previously published study in which tumor-derived CD11c dendritic cells exerted anti-tumor effect and substantially delayed growth of tumors in vaccinated mice [46].

Taken together, our data demonstrate that immunosuppressive tumor-infiltrated CD11b myeloid cells in the presence of epigenetic modifier, DNA demethylating agent 5-aza-2'-deoxycytidine, can be enriched and further differentiated into immunostimulatory mature APCs.

Acknowledgments We gratefully thank Lisa Williams for assistance with manuscript preparation.

References

- Coussens LM, Werb Z (2002) Inflammation and cancer. *Nature* 420(6917):860–867
- Balkwill F, Charles KA, Mantovani A (2005) Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 7(3):211–217
- Murdoch C, Muthana M, Coffelt SB (2008) The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* 8(8):618–631
- Yang L, DeBusk L, Fukuda K et al (2004) Expansion of myeloid immune suppressor Gr⁺ CD11b⁺ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* 6(4):409–421
- Grunewald M, Avraham I, Dor Y et al (2006) VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 124(1):175–189
- Ahn GO, Brown JM (2008) Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: role of bone marrow-derived myelomonocytic cells. *Cancer Cell* 13(3):193–205
- Kaplan RN, Riba RD, Zacharoulis S et al (2005) VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 438(7069):820–827
- Hiratsuka S, Watanabe A, Aburatani H, Maru Y (2007) Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastases. *Nat Cell Biol* 8(12):1369–1375
- Saio M, Radoja S, Marino M, Frey AB (2001) Tumor-infiltrating macrophages induce apoptosis in activated CD8(+) T cells by a mechanism requiring cell contact and mediated by both the cell-associated form of TNF and nitric oxide. *J Immunol* 167(10):5583–5593

10. Rodriguez PC, Quiceno DG, Zabaleta J et al (2004) Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res* 64(16):5839–5849
11. Kusmartsev S, Gabrilovich D (2005) STAT1 signaling regulates tumor-associated macrophage-mediated T cell deletion. *J Immunol* 174(8):4880–4891
12. Yu H, Kortylewski M, Pardoll D (2007) Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat Rev Immunol* 7(1):41–51
13. Eruslanov E, Kaliberov S, Daurkin I et al (2009) Altered expression of 15-hydroxyprostaglandin dehydrogenase in tumor-infiltrated CD11b myeloid cells: a mechanism for immune evasion in cancer. *J Immunol* 182(12):7548–7557
14. Zhang B, Bowerman NB, Salama JK et al (2007) Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. *J Exp Med* 204(1):49–55
15. Talmadge J, Donkor M, Scholar E (2007) Inflammatory cell infiltration of tumors: Jekyll or Hyde. *Cancer Metastasis Rev* 26(3–4):373–400
16. Kusmartsev S, Gabrilovich D (2006) Role of immature myeloid cells in mechanisms of immune evasion in cancer. *Cancer Immunol Immunother* 55(3):237–245
17. Sica A, Bronte V (2007) Altered macrophage differentiation and immune dysfunction in tumor development. *J Clin Invest* 117(5):1155–1166
18. Nefedova Y, Huang M, Kusmartsev S et al (2004) Hyperactivation of STAT3 is involved in abnormal differentiation of dendritic cells in cancer. *J Immunol* 172(1):464–474
19. Gabrilovich D, Chen HL, Girgis KR et al (1996) Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med* 2(10):1096–1103
20. Sombroek CC, Stam AGM, Masterson AJ et al (2002) Prostanoids play a major role in the primary tumor-induced inhibition of dendritic cell differentiation. *J Immunol* 168(9):4333–4343
21. Sharma S, Stolina M, Yang SC et al (2003) Tumor cyclooxygenase 2-dependent suppression of dendritic cell function. *Clin Cancer Res* 9:961–968
22. Talmadge J, Hood K, Zobel L, Shafer L, Coles M, Toth B (2007) Chemoprevention by cyclooxygenase-2 inhibition reduces immature myeloid suppressor cell expansion. *Int Immunopharmacol* 7(2):140–151
23. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S (2007) Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res* 67(9):4507–4513
24. Cheng P, Corzo C, Luetette N et al (2008) Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *J Exp Med* 205(10):2235–2249
25. Stresemann C, Lyko F (2008) Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *Int J Cancer* 123(1):8–13
26. Mantovani A, Schioppa T, Porta C, Allavena P, Antonio Sica A (2006) Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev* 25(3):315–322
27. Biswas SK, Gangi L, Paul S et al (2005) A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). *Blood* 107(5):2112–2122
28. Wang D, Dubois RN (2006) Prostaglandins and cancer. *Gut* 55(1):115–122
29. Tai HH, Cho H, Tong M, Ding Y (2006) NAD⁺-linked 15-hydroxyprostaglandin dehydrogenase: structure and biological functions. *Curr Pharm Des* 12(8):955–962
30. Kusmartsev S, Gabrilovich D (2002) Immature myeloid cells and cancer-associated immune suppression. *Cancer Immunol Immunother* 51(2):293–298
31. Sinha P, Clements VK, Miller S, Ostrand-Rosenberg S (2005) Tumor immunity: a balancing act between T cell activation, macrophage activation and tumor-induced immune suppression. *Cancer Immunol Immunother* 54(11):1137–1142
32. Fu YX, Watson GA, Kasahara M, Lopez DM (1991) The role of tumor-derived cytokines on the immune system of mice bearing a mammary adenocarcinoma. I. Induction of regulatory macrophages in normal mice by the in vivo administration of rGM-CSF. *J Immunol* 146(2):783–789
33. Young MRI, Wright MA, Matthews JP, Malik I, Prechel M (1996) Suppression of T cell proliferation by tumor-induced granulocyte-macrophage progenitor cells producing transforming growth factor- β and nitric oxide. *J Immunol* 156(5):1916–1921
34. Bronte V, Chappell DB, Apolloni E et al (1999) Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits CD8⁺ T cell responses by dysregulating antigen-presenting cell maturation. *J Immunol* 162(10):5728–5737
35. Kusmartsev S, Li Y, Chen SH (2000) Gr-1⁺ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 co-stimulation. *J Immunol* 165(2):779–785
36. Gabrilovich D, Velders MP, Sotomayor EM, Kast WM (2001) Mechanism of immune dysfunction in cancer mediated by immature Gr-1⁺ myeloid cells. *J Immunol* 166(9):5398–5406
37. Melani C, Chiodoni C, Forni G, Colombo MP (2003) Myeloid cell expansion elicited by the progression of spontaneous mammary carcinomas in c-erbB-2 transgenic BALB/c mice suppresses immune reactivity. *Blood* 102(6):2138–2145
38. Liu Y, Van Genderachter J, Brys L, De Baetselier P, Raes G, Geldhof A (2003) Nitric oxide-independent CTL suppression during tumor progression: association with arginase-producing (M2) myeloid cells. *J Immunol* 170(10):5064–5074
39. Rodriguez PC, Hernandez CP, David Quiceno D et al (2005) Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. *J Exp Med* 202(7):931–939
40. Setiadi AF, Omilusik K, David MD et al (2008) Epigenetic enhancement of antigen processing and presentation promotes immune recognition of tumors. *Cancer Res* 68(7):9601–9607
41. Villagra A, Cheng F, Wang HW et al (2008) The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. *Nat Immunol* 10(1):92–100
42. Chang YC, Chen TC, Lee CT et al (2008) Epigenetic control of MHC class II expression in tumor-associated macrophages by decoy receptor 3. *Blood* 111(10):5054–5063
43. Guo ZS, Hong JA, Irvine KR et al (2006) De novo induction of a cancer/testis antigen by 5-aza-2'-deoxycytidine augments adoptive immunotherapy in a murine tumor model. *Cancer Res* 66(2):1105–1113
44. Fonsatti E, Nicolay HJ, Sigalotti L et al (2007) Functional up-regulation of human leukocyte antigen class I antigens expression by 5-aza-2'-deoxycytidine in cutaneous melanoma: immunotherapeutic implications. *Clin Cancer Res* 13(11):3333–3338
45. Kozar K, Kamiński R, Switaj T et al (2003) Interleukin 12-based immunotherapy improves the antitumor effectiveness of a low-dose 5-Aza-2'-deoxycytidine treatment in L1210 leukemia and B16F10 melanoma models in mice. *Clin Cancer Res* 9(8):3124–3133
46. Preynat-Seauve O, Schuler P, Contassot E, Beer mann F, Huard B, French LE (2006) Tumor-infiltrating dendritic cells are potent antigen-presenting cells able to activate T cells and mediate tumor rejection. *J Immunol* 176(1):61–67