

Regulated Expression of CCL21 in the Prostate Tumor Microenvironment Inhibits Tumor Growth and Metastasis in an Orthotopic Model of Prostate Cancer

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Abstract Currently there are no curative therapies available for patients with metastatic prostate cancer. Thus, novel therapies are needed to treat this patient population. Immunotherapy represents one promising approach for the elimination of occult metastatic tumors. However, the prostate tumor microenvironment (TME) represents a hostile environment capable of suppressing anti-tumor immunity and effector cell function. In view of this immunosuppressive activity, we engineered murine prostate cancer cells with regulated expression (tet-on) of CCL21. Prostate tumor cells implanted orthotopically produced primary prostate tumors with predictable metastatic disease in draining lymph nodes and distant organs. Expression of CCL21 in the prostate TME enhanced survival, inhibited tumor growth and decreased the frequency of local (draining lymph node) and distant metastasis. Therefore, these studies provide a strong rationale for further evaluation of CCL21 in tumor immunity and its use in cancer immunotherapy.

Keywords Tumor microenvironment · CCL21 · Prostate cancer · TRAMP · T-REx system · Cancer immunosuppression

Abbreviations

DC Dendritic cell
TME Tumor microenvironment

TRAMP Transgenic adenocarcinoma of mouse prostate cancer
TCR T cell receptor
Dox Doxycycline
Tet Tetracycline
TILs Tumor infiltrating Leukocytes
TR Tet repressor
(flt-3-L) Fms-like tyrosine kinase 3 ligand
(SCID) severe combined immunodeficiency

Introduction

Prostate cancer is the most diagnosed cancer and the second leading cause of mortality from cancer among American men [1]. Surgery, hormone therapy and radiation therapy remain the treatments of choice for the early (localized) stages of prostate cancer. Despite these treatments a significant population of men have recurrent disease suggesting the presence of occult tumors in this patient group. There is currently no effective treatment for these patients with recurrent metastatic disease. In that regard, immunotherapy represents a novel and promising approach that has the potential to identify and destroy occult tumor cells.

CCL21 (secondary lymphoid tissue chemokine, exodus-2, 6Ckine) has been known as a lymphoid chemokine that is mainly and constitutively expressed by lymphatic vessels, stromal cells in the spleen and appendix, and by high endothelial venules in lymph nodes and Peyer's patches [2, 3]. CCL21 binds to the chemokine receptor CCR7 and is chemoattractant for mature DCs, naive and memory T cells [4, 5]. This chemokine as well as CCL19 are also necessary for normal lymphoid tissue organization that is essential for effective T cell-dendritic cell interac-

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tions. These properties are consistent with reports demonstrating CCL21-transfected Hepal-6 liver tumors were infiltrated with T cells and DCs and formed a new lymphoid-like tissue within the tumor mass [6]. Furthermore, expression of CCL21 in transgenic mice with islet β -cell-specific expression of CCL21 has been shown to trigger formation of lymphoid-like tissue in the pancreatic islets by recruiting T lymphocytes and DCs to this tissue [7]. Thus, these results suggest that local expression of CCL21 in the TME can co-localize essential immune cells necessary for promoting an anti-tumor immune response and tumor rejection. Although both CCL21 and CCL19 are chemotactants for T cells and DCs, CCL21 can also inhibit tumor growth independent of leukocyte recruitment because it possesses angiostatic activity [8]. For this reason we assessed the anti-tumor activity of CCL21 when secreted in the prostate tumor microenvironment.

In this study we used TRAMPC2 (*transgenic adenocarcinoma of mouse prostate*), a well-characterized orthotopic mouse prostate model to access the impact of the prostate tumor microenvironment (TME) on infiltrating DCs and T cells. TRAMPC2 tumor cells produce primary tumors with reproducible and predictable metastasis to draining peri-aortic lymph nodes in all mice and to distant organs in a subset of cohorts [9, 10]. TRAMPC2 tumors are heavily infiltrated with myeloid but not lymphoid (T and B) cells that seem to be responsible for disruption of the CD3/TCR signaling complex [11, 12]. In this study we modified the TME by inducing secretion of CCL21 from transfected TRAMPC2 to promote infiltration of DCs and T cells with minimal infiltration of myeloid cells. Expression of CCL21 was put under control of the tetracycline (tet-on) regulated expression system so that chemokine expression could be induced at specific times during tumor progression. The data presented herein suggests that local expression of CCL21 in the tumor bed represents a promising approach to induce immune-mediated regression of malignant tumors.

Material and Methods

CCL21 Gene Expression Plasmid

The tetracycline regulated CCL21 expression vector was obtained by inserting the PCR amplified mouse CCL21 gene into the tet-on expression vector from Invitrogen (Carlsbad, CA). The vector-containing mouse CCL21 open reading frame was purchased from Invivogen (San Diego, CA). The CCL21 gene was PCR amplified with forward primer 5'-GCG CGG GAT CCC ATG GCT CAG ATG ATG AC-3' and reverse primer 5'-TCA TGT CGA GCT AGC GGG CTC CAG GCG-3' using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA).

A BamHI site (GGATCC) was inserted into the forward primer to be used for ligation to the expression vector. Amplified CCL21 gene was digested with BamHI and NheI and ligated into the T-REx expression vector digested with BamHI and XbaI. The integrity of the CCL21 expression plasmid (pcDNA4/TO/CCL21) was confirmed by sequencing.

Tumor Cell Lines, Manipulations and Implantation

TRAMPC2 cells were established from a prostate tumor from a TRAMP mouse and were kindly provided by Norman Greenberg (Baylor College of Medicine, Houston, TX). To generate stably transfected cell lines, TRAMPC2 cells were transfected with the T-REx repressor (TR) and pcDNA4/TO/CCL21 expression vectors (Invitrogen, Carlsbad, CA) using Fugene6 (Roche Applied Science, Indianapolis, IN) following the manufacturer's protocol. Cells were maintained in antibiotic containing media for at least 3 weeks before testing for tetracycline inducible expression of CCL21 by ELISA. Briefly 1×10^5 cells from each clone were seeded in 12 well plates containing 1ml of media in duplicate. The following day the media was replaced with fresh media with or without 2mg/ml of tetracycline (Invitrogen, Carlsbad, CA). The assay was performed on the third day based on the manufacturer's protocol (R and D system, Minneapolis, MN).

To establish an orthotopic tumor, mice prostate glands were surgically exposed and injected with 0.05ml of media containing 5×10^5 tumor cells. Mice were regularly monitored for tumor growth. Mice were treated with 0.02mg/ml of doxycycline (a tetracycline derivative) along with 0.5% sucrose in their drinking water when indicated. All animal protocols were conducted in accordance with National Institute of Health guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee of Eastern Virginia Medical School. Tumor infiltrating leukocytes (TILs) were isolated from palpable tumors that were excised, diced and digested enzymatically as previously reported [13]. Cells were then washed to remove enzymes and dead cells were eliminated from the preparation by Ficoll (Isolymp, Gallard-Schlesinger Industries, Carle Place, NY) gradient centrifugation [11]. Single cell suspension of spleens from normal mice and tumor bearing mice were prepared following the procedure for TILs and used as control. To detect metastatic disease in mice with TRAMP tumors, different tissues (lymph nodes, lungs, pancreas and bone marrow) were harvested aseptically and cultured as described previously [14]. In some cases prostate tumors were cultured using the same technique and cells from explanted outgrowths were expanded for re-injection into the prostate gland.

Flow Cytometry

Multiparameter flow cytometric analysis was performed as previously reported [15]. Antibodies used in this study were obtained from eBioscience (San Diego, CA). DNA content of cell lines derived from metastatic loci was determined by staining the cells with propidium iodide (PI, Sigma, St. Louis, MO) and analyzed on a BD FACScan cytometer as previously described [14].

Results

DCs Infiltrating TRAMPC2 Tumors are Phenotypically Immature

TRAMPC2 tumors grow progressively in immune competent mice suggesting that these cells induce a weak or inefficient anti-tumor immune response. This may reflect the ability of the TRAMPC2 TME to impair DC (CD11c+ cells) function. CD11c has been used here to identify DCs, although it can also be expressed by activated T and B cells as well as natural killer (NK) cells. However, intratumoral T cells remain quiescent in the TRAMP TME because they

do not express the activation antigens CD25 or CD69 (data not shown). Furthermore, T and B cells are not a major infiltrating cell types in TRAMP tumors. NK cells are typically not detected in TRAMP TILs or are present as a trace population and therefore do not contribute significantly to CD11c expression in the TRAMP TME. We observed that the majority of DCs infiltrating TRAMPC2 tumors failed to express normal levels of class II antigens (IA^b), B7.2 and CD40 molecules compared to their counterparts isolated from either normal or tumor bearing spleens (Fig. 1-b). Most of the infiltrating DCs appeared to be myeloid in origin because they did not express CD8α (B-g, h and i and C). Class I antigen (H2D^b) expression was not suppressed by the TME as equivalent levels of expression were observed on intratumoral and splenic DCs (Fig. 1-b; g, h and i). Surprisingly, CD86 expression, but not CD80, was suppressed suggesting differential regulation of B7 family members within the prostate TME (Fig. 1-c). As expected, expression of the chemokine receptor CCR7 was down-regulated relative to normal spleen (Fig. 1-c). In contrast, DC expression of PDL2 shown to inhibit the activation and cytokine production of CD4⁺ T cells [16] was elevated on intratumoral DCs relative to normal splenic DCs (Fig. 1-c). Thus, these data

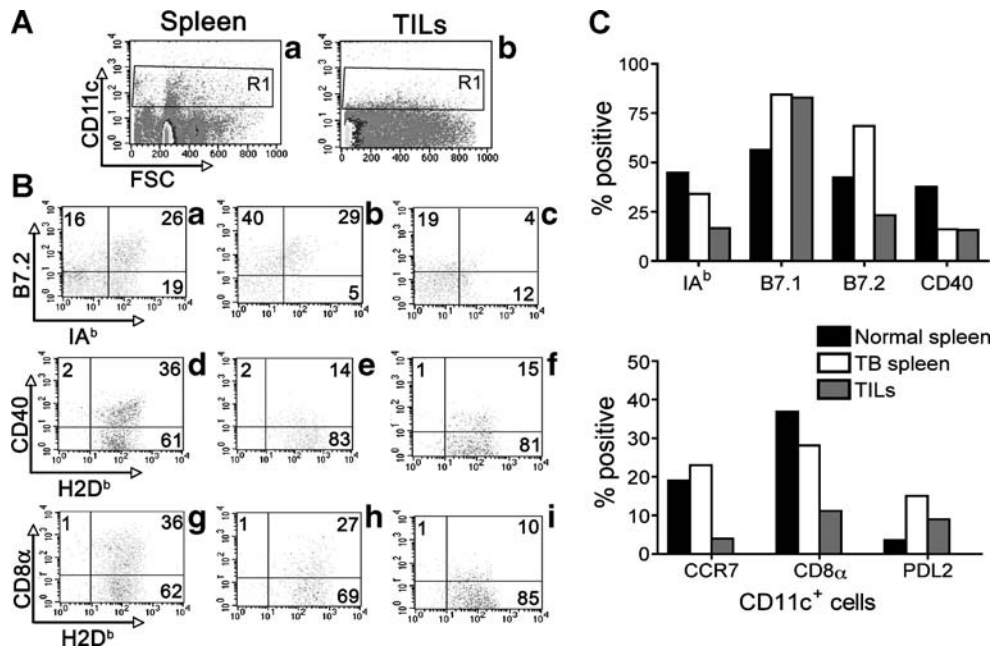


Fig. 1 Dendritic cells isolated from prostate tumors display an immature phenotype. Mice were transplanted orthotopically with TRAMPC2 tumor cells and 30 days later excised when tumor mass reached approximately 1 cm in diameter. Single cell suspension from normal and tumor bearing (TB) spleens were prepared and TILs isolated from TRAMPC2 tumors. Cells were stained with indicated mAbs and evaluated by 4-color flow cytometry. **a** Single color analysis (forward scatter vs. log fluorescent intensity) of CD11c⁺ cells of normal spleen and TILs isolated from TRAMPC2 tumors. The R1

region was set based on the appropriate isotype matched control. The background for isotype matched control was 0.07% for splenocytes and 0.04% for TILs. **b** Expression of the indicated cell surface molecules on gated CD11c⁺ cells. Values in each quadrant indicate the percentage of cells in the CD11c⁺ gate that stained with the indicated mAbs. **c** Further phenotypic characterization of splenic and tumor associated DCs displayed as bar graphs. Data are representative of 3 independent experiments with 3 mice/ group in each experiment

suggest that tumor-associated DCs are immature because they fail to express a number of cell surface markers associated with DC maturation.

Characterization of TRAMPC2 Cells with Regulated Expression of CCL21

Previous studies showed that the presence of CCL21 in tumors promotes the infiltration of DCs and T cells that enhanced the anti-tumor immune response and inhibited tumor growth [6, 17]. We examined whether direct intratumoral expression of CCL21 via gene-modified TRAMPC2 cells would inhibit tumor growth and metastatic disease in this model. We therefore transfected TRAMPC2 cells with both the repressor and CCL21 tet-inducible expression vectors. Six antibiotic resistant TRAMPC2/TR/CCL21 clones were isolated that possessed low constitutive expression of the chemokine and 12- to 60-fold induction of CCL21 in the presence of tetracycline. Three out of 6 lines maintained the tet-inducible expression of CCL21 (termed TRAMPC2/TR/CCL21) after 3 and 8 additional passages although clone 6 had lower levels of inducible expression after 8 passages (Fig. 2-a). To establish a cell line that grows and maintains regulated expression of CCL21 *in vivo*, TRAMPC2/TR/CCL21 tumor cells (Fig. 2a, clone 4) were implanted into the prostate gland of nine mice. One mouse died without evidence of a palpable prostate tumor. Six mice developed palpable tumors that were excised and clonal outgrowths were obtained without selection anti-

Table 1 Distribution of inducible expression of CCL21 in clonal lines derived from TRAMC2/TR/CCL21 tumors following orthotopic tumor growth *in vivo*

Mouse	No. Clones	No. Inducible Clones	% Inducible Expression
M1	22	6	27
M2	18	1	5.5
M3	19	1	5.3
M4	11	2	18
M5	15	0	0
M6	18	0	0
Total	103	10	9.7

Nine syngeneic mice were implanted orthotopically with TRAMPC2/TR/CCL21 tumor cells (5×10^5). One mouse died without any tumor and two mice never grew tumors. After several months following implantation, 6 palpable tumors were excised, diced and explants cultured *in vitro*. Cloned lines were derived, expanded in selection media and tested for tet-induced secretion of CCL21 by ELISA. Clonal lines from six tumors isolated from individual mice were evaluated (M1-6).

biotics. Outgrowths from two tumors (M5, M6) were no longer tet-inducible and were not further studied (Table 1). Seventy clonal lines were obtained from the remaining four tumors of which ten were inducible for CCL21 expression (Fig. 2b and Table 1). Clonal outgrowths derived from mouse 1 (M1) generally had low constitutive CCL21 levels with relatively weak induction for CCL21. The remaining clones demonstrated higher tet-induced CCL21 secretion

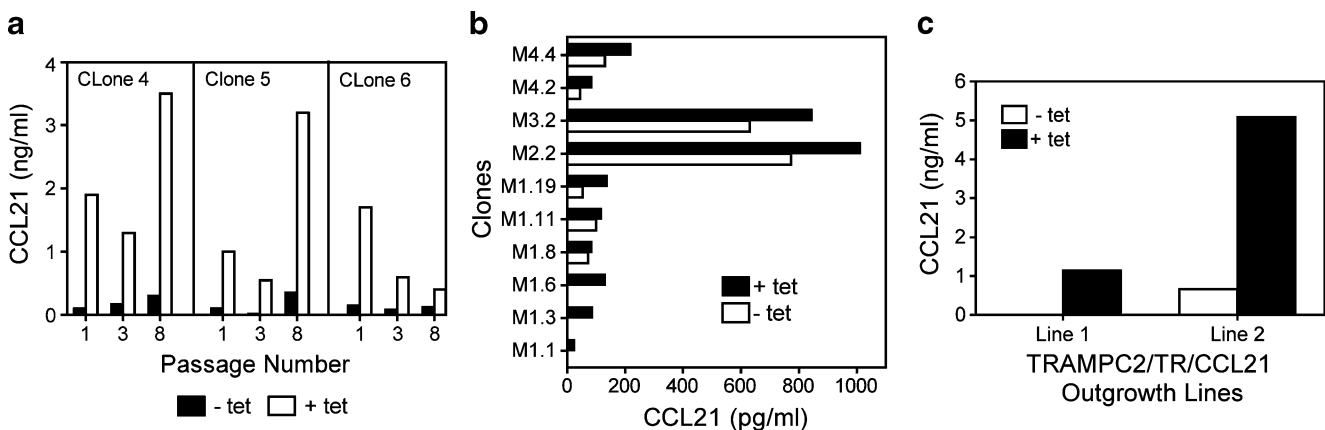


Fig. 2 Variable expression of CCL21 by TRAMPC2/TR/CCL21 cells following passage *in vitro* and tumor growth *in vivo*. **a** TRAMPC2 cells were transfected with the repressor and CCL21 expression vectors using Fugene 6 transfection reagent and selected in antibiotic-containing media. Cloned antibiotic resistant cell lines (TRAMPC2/TR/CCL21, clones 4, 5 and 6) were tested for CCL21 expression with or without 2 $\mu\text{g/ml}$ of tetracycline by ELISA. ELISA was performed after 3 and 8 passages to test whether these clones maintained inducible expression of the transgene. **b** Syngeneic mice were implanted orthotopically with TRAMPC2/TR/CCL21 tumor cells (clone 4, 5×10^5). After several months following implantation, palpable tumors were excised, diced and explants cultured *in vitro*.

Cloned lines were derived, expanded in selection media and tested for tet-induced secretion of CCL21. Clonal lines from six tumors isolated from individual mice (M1-6) were evaluated (see Table 1). This panel illustrates the expression levels achieved by tetracycline in the 10 clones (10/103) that produced CCL21. None of the lines derived from two tumors (M5 and M6) displayed inducible expression of CCL21. **c** Eight clonal lines with weak induction derived from mouse tumors 1 and 4 were pooled to generate L1. The remaining two lines (M3.2 and M4.2) were pooled to generate L2. These two pooled lines were then subjected to antibiotic selection using zeocin and blasticidin, expanded *in vitro* and then tested for inducible CCL21 production. Note different scales in panels B and C

but were “leaky” (high constitutive levels). Because the clonal outgrowths from intraprostatic tumors were isolated and grown in the absence of selection media, the relatively modest induction of CCL21 production may indicate that TRAMPC2/TR/CCL21 tumor cells lost or silenced the CCL21 gene during *in vivo* growth. To test this hypothesis and to enrich for tumor cells with stable tet-inducible expression of CCL21, the 8 weakly inducible clonal lines from mouse 1 (M1.1-1.19) and 4 (M4.2, M4.4) were pooled to generate TRAMPC2/TR/CCL21-L1. The remaining two lines were also pooled to produce TRAMPC2/TR/CCL21-L2. Both populations were then subjected to antibiotic selection. Fig. 2c demonstrates that growth in selection media enhanced CCL21 production by TRAMPC2/TR/CCL21-L1 cells (Line 1) approximately 5-fold without increasing constitutive CCL21 levels. Although TRAMPC2/TR/CCL21-L2 (Line 2) cells had higher background expression relative to Line 1, tetracycline induced much higher levels of CCL21 production. These data indicate that both these lines have the capacity to grow both *in vitro* and *in vivo* following orthotopic implantation.

Impact of Intratumoral Expression of CCL21 on Survival, Tumor Growth and Metastatic Disease

TRAMPC2/TR/CCL21 clones (L1 and L2) displayed high levels of tet-inducible expression of CCL21 and grew *in vivo*. We next wanted to test whether CCL21 expression in the TME enhances survival of mice implanted orthotopically with prostate tumor cells. Therefore, mice were randomly divided into two groups and one group received doxycycline in their drinking water starting the day after surgical implantation of TRAMPC2/TR/CCL21-L2. Mice were sacrificed and tumors explanted for *in vitro* growth when mice showed signs of morbidity. As demonstrated in Fig. 3a (left panel), prolonged survival was observed in doxycycline-treated mice. Control mice all died by day 32, whereas, doxycycline treated mice lived up to 50 days post implantation. Low levels of CCL21 were detected in the serum of 25% of tumor bearing mice treated with doxycycline and was not detected in the serum of control mice. Analysis of clonal lines derived these tumors demonstrated that <10% were capable of inducible expression of CCL21 (right panel). All these cell lines eventually lost inducible expression after a few *in vitro* passages (data not shown). Thus, tumor growth *in vivo* was associated with loss of inducible expression of CCL21. This may have contributed to the limited growth inhibition and eventual outgrowth of primary prostate tumors observed in these mice.

To determine whether prolonged survival induced by intratumoral CCL21 expression reflected inhibition of primary tumor growth and/or inhibition of metastatic

disease, we implanted TRAMPC2/TR/CCL21-L2 orthotopically into the total of 18 animals in two separate experiments (8 and 10 mice, respectively). In each experiment the mice were divided into two groups with one group receiving doxycycline in their drinking water one day after tumor implantation. Mice were sacrificed when moribund and tumors, draining lymph nodes, lungs and pancreases removed for measurements and assessment of metastatic disease. One of the mice given doxycycline in the first experiment and two from the control group in the second experiment died shortly after tumor implantation and therefore were excluded from this analysis. Tumors grew in all mice irrespective of whether they received doxycycline in their drinking water. However, Fig. 3b demonstrates that tumors excised from doxycycline-treated mice weighed less (left panel) and were smaller in size (right panel) than tumors excised from control animals. As expected, all control mice had metastases in draining periaortic lymph nodes as well as metastases in their lung in the majority of mice. A smaller subset also had disseminated disease to the pancreas (panel C). In contrast, treated mice had reduced frequency of metastasis to lymph nodes and lungs with no metastases to the pancreas. These data suggest that even limited and transient expression of CCL21 in TRAMP TME suppresses primary tumor growth as well as metastatic disease to draining lymph nodes and distant organs.

In vivo Tumor Growth is Associated with Methylation of CMV Promoter

The data presented above demonstrated that the vast majority of TRAMPC2/TR/CCL21 tumor cells no longer displayed inducible CCL21 induction following orthotopic implantation. Two possibilities mechanisms were next considered to explain this observation: loss of the transgene or alternatively, silencing of the promoter. To test the first possibility DNA was extracted from TRAMPC2/TR/CCL21-L2 tumor pieces and cloned lines isolated and expanded to generate sufficient DNA for PCR analysis using specific primers to amplify the transfected CCL21 gene. It is apparent from Fig. 4 (panel A) that outgrowths obtained from orthotopic TRAMPC2/TR/CCL21 tumors still contained the CCL21 transgene. The absence of a product in the control mouse DNA confirmed that the primers did not amplify endogenous CCL21 gene (lane 9). To test the possibility that the promoter was silenced by methylation, we evaluated the methylation pattern of the CMV promoter. DNA isolated from tumor pieces or clonal lines were bisulfite treated and PCR reactions were performed using primers complementary to a region of CMV promoter not containing methylation sites (oligos 1) or a pair of primers complementary to a region of CMV

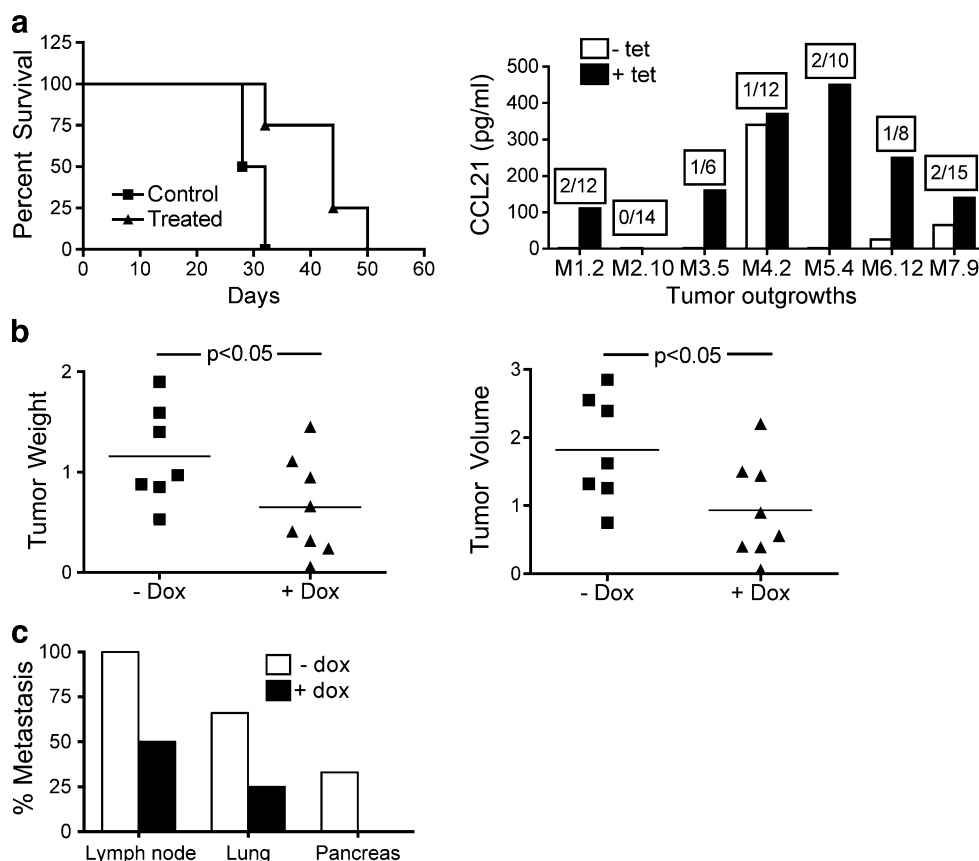


Fig. 3 Intraprostatic secretion of CCL21 prolongs survival, delays tumor growth and reduces the frequency of metastatic disease. **a** Eight mice (M1-8) were transplanted orthotopically with TRAMPC2/TR/CCL21-L2 cells. Four mice were given doxycycline in their drinking water one day after implantation. Mice were euthanized when tumors were palpable or when mice were moribund. Tumors from treated and control mice were excised, diced and cultured in tissue culture media containing antibiotics. Derived clonal lines (M1.2, M2.10, etc.) were then evaluated for inducible expression of CCL21. Enhanced survival (left panel) was correlated with induced expression of CCL21 in the prostate TME (right panel). The survival of treated mice was significantly prolonged relative to non-treated mice ($P < 0.05$). The boxed ratios represent the number of cell lines isolated with inducible CCL21 expression versus the total number of evaluated cell lines and the cell line with the highest expression of CCL21 has been shown if

there were more than one cell lines expressing CCL21. **b** Mice (total of 18, two separate experiments) were given an orthotopic injection of 5×10^5 TRAMPC2/TR/CCL21-L2 cells. One cohort was given doxycycline in their drinking water after surgery and one group served as control. Tumor growth was monitored by palpation and approximately 2 months after implantation when the control mice were morbid, tumors were excised. Weight (left panel) and volume (right panel) of the tumors was then measured. **c** Lymph nodes, lungs and pancreases of mice from one of the experiments described in panel B were also removed, diced and cultured as described previously. Tumor cells grew out of the organs with metastases and generated cell lines that were expanded *in vitro*. Aneuploid DNA content validated that the outgrowths represented TRAMPC2/TR/CCL21 tumor cells [12]

promoter which contains methylation sites (oligos 2). If the promoter is not methylated, a PCR product forms with both primers, whereas, a single product is only detected with oligos 1 if the promoter is methylated [18]. Fig. 4b demonstrates that when the pcDNA4/TO/CCL21 plasmid was tested following bisulfite conversion, PCR reactions with both primers produced a product indicating that the original plasmid DNA was not methylated. In contrast, when DNA was extracted from two excised TRAMPC2/CCL21-L2 tumors (M1 and M2, Fig. 3a), both promoters appeared to be methylated, however, when a clonal outgrowth derived from tumor M1 was tested, PCR products formed with both primers suggesting that the section of tumor excised for clonal expansion had a

functional promoter (not methylated). These data indicate that during tumor growth in the prostate gland, the promoter is variably methylated. Thus, in some sections of the tumor, the promoter may still be functional. This may explain why we detected some low-grade induction of CCL21 in some clonal lines derived from explants of TRAMPC2/TR/CCL21-L2 tumors (Fig. 3a, right panel).

Discussion

In this report we showed that TRAMP tumors were infiltrated with small population of DCs. Although expression of CD11c on intratumoral DCs was low relative to

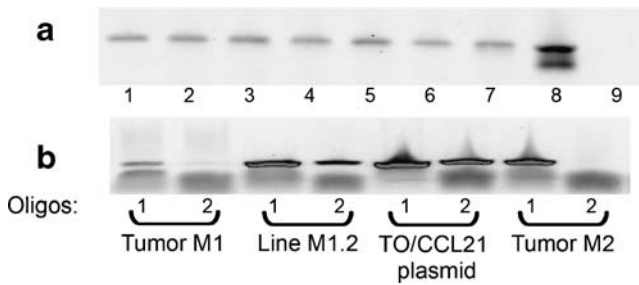


Fig. 4 The CCL21 transgene is retained but the CMV promoter is methylated in TRAMPC2/TR/CCL21 tumor cells following progressive tumor growth *in vivo*. **a** DNA extracted from cloned cell lines derived from TRAMPC2/TR/CCL21-L2 tumors were tested for the transgene by PCR using primers specific for CCL21 transgene. Lanes 1–7 represent PCR products obtained when DNA was extracted from cell lines derived from 7 different TRAMPC2/TR/CCL21-L2 tumors (Fig. 3a-left panel). PcDNA4/TO/CCL21 plasmid used for transfection was included as a positive control (lane 8) and mouse DNA was used as negative control (lane 9). **b** DNA extracted directly from TRAMPC2/TR/CCL21-L2 tumors (M1 and M2) and a cell line derived from tumor M1 (line M1.2, see Fig. 3a-right panel) were tested for methylation status of CCL21 transgene promoter (CMV). TO/CCL21 plasmid was used as negative control. Extracted DNA was bisulfite treated and then was used in two different PCR reactions using oligos 1 (directed against a region of the CMV promoter not containing methylation sites) or oligos 2 (directed against a region of the CMV promoter which contains methylation sites)

splenic DCs, it still exceeded the isotype control (Fig. 1). We also demonstrated that DCs infiltrating TRAMPC2 tumors had low levels of MHCII, B7.2 and CD40 expression compared to their normal splenic counterparts. Most of the intratumoral DCs were myeloid-derived because they displayed a CD8 α ⁻ phenotype. In addition to DC infiltrate, TRAMP tumors were infiltrated primarily by macrophages and immature (Gr-1⁺) myeloid cells but few T and B cells. Because myeloid cells have been shown to be immunosuppressive in several tumor models [19, 20], we transfected TRAMPC2 tumors with CCL21, a chemo-attractant for DCs and T cells. We speculated that inducible expression of this chemokine should promote DC and T cell infiltration and promote anti-tumor immunity and tumor rejection. To test this paradigm we generated transfected TRAMPC2 tumor cells with inducible expression of CCL21 so that we could regulate chemokine production at discrete times during tumor growth. We isolated several lines with stable and inducible expression of CCL21 *in vitro* and derived two cell lines that also grew reproducibly in mouse prostate glands. Mice implanted orthotopically with one of these lines (TRAMPC2/TR/CCL21-L2) and treated with doxycycline had reduced primary tumor growth, decreased frequencies of metastatic disease and enhanced survival. The inability of CCL21 to cure mice of prostate tumors may have been related to low levels of CCL21 expression. Thus, <10% of the transfected cells cloned from prostate tumors still had inducible expression

of this chemokine and at levels well below that obtained from the parental line. The failure of transfected cells to secrete CCL21 was not due to loss of the transgene but rather methylation of the CMV promoter that drives expression of this chemokine.

Previous work demonstrated that the chemotactic activity of CCL21 for DCs and T cells could be used to augment anti-tumor immune responses [21–23] and all of these reports indicated that the anti-tumor activity of CCL21 was mediated by enhancing the infiltration of mature DCs and CD8⁺ T cells to the tumor. These data also suggested that modification of the TME could lead to effective T cell priming and the generation of functional anti-tumor effector cells without interaction of DCs and T cells in lymphoid organs. Consistent with these studies we found that the expression of CCL21 in TRAMPC2 TME inhibited tumor growth (Fig. 4a). We did not detect any major difference in the composition of the tumor infiltrate in tumors removed from moribund mice. Differences as a result of CCL21 expression may have existed at earlier times during tumor growth, a hypothesis that is currently being evaluated. The inability of CCL21 to induce infiltration of CD8 α ⁺ DCs may have also contributed to the limited growth inhibition observed in these studies. The TME represents a potential rich source of tumor antigen and this DC subset is capable of cross-presentation to CD8⁺ T cells [24].

Although CCL21 is important in recruiting DCs and T cells and is classified as a CC chemokine (binds to CCR7 receptor), murine CCL21 has been shown to bind to mouse CXC chemokine receptor CXCR3 [25]. This is a property that CCL21 shares with two other angiostatic chemokines, interferon-inducible protein 10 (IP-10) and monokine induced by interferon- γ (MIG) [26]. CXCL3 is expressed on human microvascular endothelial cells under normal and pathological conditions and engagement of this receptor by these ligands inhibits endothelial cell proliferation *in vitro* [27]. Therefore anti-tumor activity of CCL21 can also be associated with its angiostatic activity through binding to CXCR3 receptor. Consistent with this view, Arenberg *et al.*, showed that injection of CCL21 into the A549 human lung tumors in the severe combined immunodeficiency (SCID) mice inhibited tumor growth and reduced metastasis when the number and size of metastatic loci was compared to control mice [28]. It has also been shown in some studies that expression of CCR7 by tumor cells is involved in directing lymph node metastasis [29]. However, TRAMP tumor cells do not express CCR7 and therefore other mechanisms must be responsible for the reproducible lymph node metastasis of these cells. Potential candidates include basic fibroblast growth factor (bFGF) and IL-8 which can promote tumor growth and spontaneous lymph node metastasis in bladder cancer [30]. Further studies will be required to identify the signal(s) responsible for

metastatic spread in this tumor model. Inactivation of the transgene in the prostate TME, limited expression of CCL21 is sufficient to inhibit prostate tumor growth and metastatic disease. We previously reported that Fms-like tyrosine kinase 3 ligand (flt-3-L) therapy of established TRAMP tumors, in both ectopic and orthotopic settings, suppressed tumor growth and inhibited metastatic disease [13, 14]. Although neither of these therapies is curative, the combination of two treatment strategies may overcome the immunosuppressive properties of the prostate tumors and be more effective than either treatment strategy alone. Current studies are designed to test this paradigm and to identify promoters that resist inactivation (methylation) *in vivo*.

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