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Gene Therapy Using Tet-Repressor System to Modulate Prostate Tumor Microenvironment

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GENE THERAPY USING TET-REPRESSOR SYSTEM TO MODULATE PROSTATE TUMOR MICROENVIRONMENT

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ABSTRACT

GENE THERAPY USING TET-REPRESSOR SYSTEM TO MODULATE PROSTATE CANCER MICROENVIRONMENT

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Prostate cancer is the most commonly diagnosed malignancy in men in the United States and is projected to be the third most frequent cause of male cancer-related deaths in 2007 after lung and skin cancers. The initial treatment for prostate cancer at early stages is prostatectomy or radiation, which usually is curative. However, approximately 20% of patients are not cured by such treatments and their cancer recurs, sometimes with long latencies. In other patients prostate cancer is diagnosed only after the cancer has metastasized and there are no effective therapies at this stage. Therefore immunotherapy seems to be a promising approach to treat metastatic prostate cancer through enhancing tumor-specific T cell responses. In this regard, there is a growing interest in the generation of fully competent dendritic cells (DCs) that are known to be potent antigen presenting cells and capable of activating naïve T cells. While DCs need to acquire a mature phenotype to induce T cell activation, it is known that the microenvironment of many tumors including prostate tumors is immunosuppressive and prevents DC maturation. We used the *tr*ansgenic *a*denocarcinoma of *m*ouse *p*rostate (TRAMP) model to show that DCs infiltrating

prostate tumors are phenotypically immature and using an *in vitro* assay we showed that TRAMPC2 cells but not granulocytes are the major inducers of this phenotype. We used a well-defined orthotopic prostate cancer model to study chemokine/cytokine vaccines. Expression of secondary lymphoid tissue chemokine (SLC), granulocyte macrophage-colony stimulating factor (GM-CSF) or CD40 ligand (CD40L) in the TRAMP tumor microenvironment (TME) was chosen to induce co-localization of T cells and DCs and their interaction, expand DCs and induce their maturation. In order to make a clinically relevant model in this study we took advantage of the tetracycline inducible expression system that enabled us to control the expression of the chemokines and cytokines during the course of study. We showed that expression of SLC in the TRAMP TME inhibited tumor growth, decreased metastasis and increased survival of tumor bearing mice. Although CD40L transfected TRAMPC2 cells did not grow *in vivo* and GM-CSF transfected TRAMPC2 cells failed to grow tumors after *in vitro* passages, using *in vitro* assays we showed that these molecules reversed the inhibition of DC maturation induced by TRAMPC2 cells.

This dissertation is dedicated to my daughter, Mona Hosseini. The big smiles on her beautiful face and her love are never ending and she is the motivation that kept me going. May this dissertation and the effort required to complete it serve as motivation for her own future academic and life endeavors, whatever they may be.

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CHAPTER I

INTRODUCTION AND BACKGROUND

Metastasis is a fatal step in the progression of cancer, with death from metastases representing 90% of all human cancer mortalities (1). Most cancer patients die from metastases rather than from their primary tumors. Metastasis to lymph nodes, bone and other tissues is a drastic consequence of late diagnosis of prostate cancer. Patients usually die from metastatic disease complications and available treatments for metastatic prostate cancer are not curative. Therefore new and more effective treatments are required for late-stage prostate cancer. Immunotherapy provides a promising systemic approach to treat metastatic prostate cancer. The rationale for using the host immune system to fight prostate cancer is based upon cases of spontaneous remissions of various cancers (2). However, the existence of cancer in a host with an intact immune systems demonstrate the inefficiency of this natural defense against tumors and the ability of cancer cells to evade immune surveillance and rejection. The major objective of immunotherapeutic approaches to prostate cancer therapy is to augment the anti-tumor immune responses against malignant tissues. Studies in a well-characterized preclinical animal model will provide a valuable opportunity

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The journal model for this dissertation is the *Journal of Experimental Medicine.*

to explore the efficacy of specific cytokines to modify the tumor microenvironment (TME) and promote the induction of anti-tumor immunity.

Available therapies for prostate cancer

Prostate cancer is the second most common cancer after lung cancer in men in the United States. The American Cancer Society estimates that 218,890 men will be diagnosed with prostate cancer and 27,050 will die from the disease during 2007. Mortality rates for prostate cancer have declined since the early 1990s mostly due to improvements in detection and diagnosis through widespread use of prostate-specific antigen (PSA) testing. For localized prostate cancer, available treatments include surgery and radiotherapy. Unfortunately, up to 30%-40% of patients fail local therapy and later suffer from metastatic disease. The standard of care for patients failing primary therapy is hormone therapy but the majority of patients eventually become hormone refractory (3). Treatments in these cases are limited to aggressive chemotherapies, which can reduce serum prostate-specific antigen (PSA) levels in some patients. However, taxane- and platinum-compound-based chemotherapies produce a survival benefit of only a few months (4). Therefore, it is crucial to develop novel, well-tolerated treatment strategies.

Cancer and Immunotherapy

As a potential treatment for cancer, immunotherapy was initially experimented in the 19th century by William Cohen, who made the observation

that in rare cases of spontaneous tumor regression, the patients often suffered from episodes of infections (5). Coley had limited success in attempting to mobilize the body's immune system through the injection of bacterial extracts. However, the concept of treating cancer with the immune system seemed very appealing. This approach has the potential to generate a sustained potent immune response against cancer to prevent disease recurrence. One of the very first vaccination attempts against cancer was activation of the immune system using a bacterial infection. In a very large clinical trail, melanoma patients were treated with bacillus Calmette-Guerin (BCG). This treatment caused regression of tumors in some patients but no significant difference in overall survival was observed between BCG treated and control group (6).

The next generation of cancer vaccines used cancer cell lysates or cultured cancer cell supernatants that induced a more specific immune response against tumor antigens (7-9). Melanoma patients injected with whole cell lysate of melanoma cell lines showed higher antibody titer against a known melanoma antigen (the ganglioside GM2) than control individuals (9). This study also suggested that melanoma recurrence was delayed in patients developing higher titers of GM2 antibody (9).

It was only in the last 1-2 decades that the molecular nature of antigens recognized by T cells became known (10, 11) and many tumor antigens were identified (12). T cells recognize short peptides in association with major histocompatibility complex (MHC) molecules. The presentation of these peptides at the tumor cell surface follows the classical pathways of antigen processing:

peptides presented to cytotoxic T lymphocytes (CTLs) by MHC class I molecules are derived from intracellular proteins degraded by the proteasome, whereas, peptides presented to $CD4^+$ T cells by MHC class II molecules are derived from proteins that have been transported to an endocytic compartment (13).

In general, human tumors are poorly immunogenic and do not trigger an immune response. There are two types of tumor antigens: some tumors have unique antigens (not found on normal cells), called tumor specific antigens (TSA) (14). Tumor specific antigens are present in tumors induced by infectious agents (e.g. EBNA-1 antigen from Epstein Barr virus-induced Burkitt's lymphoma) (15) and mutated genes found only in tumor cells (e.g. mutated caspase-8 enzyme found in head and neck cancer, which is different from the normal caspase-8) (16). However, many tumors have antigens, called tumor associated antigens (TAA), found on normal cells but either modified or produced in greater quantities (17). A number of TSA recognized by melanoma reactive CTLs and T helper cells were identified and vaccines were designed using these peptides (18). Although using these peptides increased the frequency of specific T cells from 0.1% to greater than 2% of circulating $CD8^+$ T cells in many cases, low clinical response rates were observed (19). This can be due to the fact that the immune response elicited by the vaccination did not reach the quantitative capacity necessary for tumor regression (20). However, the results of another clinical trial demonstrated that standard high-dose interferon α-2b (a cytokine approved by the Food and Drug Administration (FDA) for treatment of cancer) therapy for high-risk melanoma was more effective than vaccination against the ganglioside

GM2 (21). Therefore new strategies will be needed if vaccination is to be used as an effective therapeutic route to follow for cancer treatment.

Clinical trials for cancer gene therapy

Gene therapy for cancer treatment can be broadly defined as any manipulation of DNA that results in control of growth or death of cancer cells. DNA vaccines provide a stable and persistent source of the encoded antigen leading to a permanent stimulation of the immune system and generation of long-lasting immunity (22). The construction of DNA vaccines involves cloning of the gene of interest into a plasmid under the control of a viral promoter and the most important key to the gene therapy process is the vector or the instrument by which DNA (transgene) can be transported into cells (22). Cancer vaccine trials using DNA plasmids injected by the subcutaneous or intramuscular route have been carried out in patients with metastatic melanoma and modest levels of T-cell immunity have been seen in these patients (23, 24). Recently a plasmid that encoded epitopes from the potent melanoma antigen Melan-A/melanoma antigen recognized by T cells (MART)-1 and tyrosinase were used in a phase 1 clinical trial (25). In this study the plasmid was injected into the lymph nodes of the stage IV melanoma patients to improve the immunogenicity of plasmid vaccines by directly targeting an APC-rich environment but the detection of immune responses against this specific epitope occurred only in a subset of patients and regression of melanoma was not observed (25). Therefore, it seems that DNA

vaccines have limited immunogenicity even with intranodal route of injection.

Suicide gene therapy involves the delivery of genes to tumor cells that encode enzymes (most of which are not encoded by mammalian cells) that are capable of converting prodrugs into toxic metabolites that can lead to cell cycle arrest and death. After the systemic administration of the prodrug, the neoplastic cells that express the gene are capable of transforming the drug into a toxic metabolite causing death not only of the cancer cells but also those cells in close proximity. This phenomenon, called the bystander effect, is the basis of the remarkable efficacy in tumor destruction of this system. One suicide gene therapy system is the herpes simplex virus thymidine kinase (HSV-tk) and the prodrug used in this system is ganciclovir (GVC). After extensive preclinical testing of this system *in vitro* and *in vivo*, a phase I clinical trial was conducted on prostate cancer patients by intraprostatic injection of a replication-deficient adenovirus (ADV) containing the HSV-tk gene, followed by intravenous administration of the prodrug GCV. Significant prolongation of the median serum prostate-specific antigen (PSA) doubling time from 2.9 to 6.2 months was detected. In five out of eight patients, a clear decrease of PSA values was observed. Fluorescence-activated cell sorting (FACS) analysis also showed no influence on phenotypic distribution in peripheral blood samples, except for an increasing trend of $CD8^+$ after therapy (26). However, This study only confirmed the safety profile and the possibility of clinical response to HSV-tk gene therapy for hormone-refractory prostate cancer. Therefore, new approaches in the development of effective therapeutic cancer vaccines are required. Promising

new strategies have included the use of gene modified tumor vaccines and DCs based vaccines (27).

Since the original concept of gene therapy was to create a patient specific vaccine Salgia *et al.,* conducted a phase I clinical trial using single cell suspensions prepared from resected metastases of 35 patients with non-small-cell lung cancer (NSCLC). Tumor cells were infected with a replication-defective adenoviral vector encoding granulocyte macrophage-colony stimulating factor (GM-CSF), irradiated and administered intradermally and subcutaneously. Vaccines were successfully manufactured for 34 (97%) of 35 patients. Toxicities were restricted to grade 1 to 2 local skin reactions. Nine patients were withdrawn early because of rapid disease progression. Vaccination elicited DCs, macrophage, granulocyte, and lymphocyte infiltrates in 18 of 25 assessable patients. Metastatic lesions resected after vaccination showed T lymphocyte and plasma cell infiltrates in three of six patients. This study indicated that vaccination with irradiated autologous NSCLC cells engineered to secrete GM-CSF enhanced anti-tumor immunity in some patients with metastatic NSCLC (28). However, this process proved to be expensive and time consuming, the end product was of variable quality, and the concept was not a realistic option for large-scale production.

Dendritic cell based immunotherapy is another promising approach to augment tumor antigen-specific T cell responses in cancer patients. Often malignant growth is a slow and silent process that fails to provide necessary signals for the activation of the immune system. The goal of DC vaccination is to provide these

signals with *ex vivo* and appropriately activated and loaded DCs with tumor antigens. In a study with 33 metastatic melanoma patients vaccination with autologous tumour lysate-loaded DC resulted in a slightly higher response rate compared to peptide-pulsed DC (3 versus no partial remissions, respectively) (Hersey P, Menzies, Cancer Immunol Immunother 2004;53:125–34). Although results from different phase 1 trials indicate that DC-based immunotherapy is feasible, safe, and well tolerated, clinical results are often inconclusive. The subjects enrolled in these trials had an advanced-stage disease and the lack of product standardization, results in phenotypic and functional differences in administered therapeutic DC products and therefore inconclusive clinical results.

Chemokines and Cytokines in the treatment of cancer

In spite of recent progress in understanding cancer biology and achievements in novel treatment options, the success rate for cancer therapy remains dismal. Since its initial discovery, cytokine-based immunotherapy has been extensively investigated for cancer treatment as cytokines can be easily purified and injected and therefore used as cancer treatment agents (29, 30). Growth, differentiation and function of immune cells are regulated by cytokines and chemokines that can be secreted or remain membrane bound. Cytokines regulate both the innate immune system [natural killer (NK) cells, macrophages and neutrophils] and the adaptive immune system (T cells and B cells). Cytokines have the capacity to alter the interaction between the body's immune

system and cancer cells to boost, direct or restore the body's ability to fight disease. Some cytokines have direct effect on the tumor cells like interleukin-6 that inhibits the growth of the majority of melanoma cell lines from early stages of tumors (31). Some other cytokines fight tumors through indirect mechanisms like granulocyte macrophages-colony stimulating factor (GM-CSF) that induces the differentiation and proliferation of myeloid precursor cells and eventually development of dendritic cells (DCs) (32). In some cases, cancer cells can take advantage of cytokines secreted by the host cells that promote growth, inhibit apoptosis and facilitate invasion and metastasis. For example, interleukin-1α that is secreted by macrophages stimulates the growth of breast cancer cells in nude mice (33). Interleukin-8 (IL-8) secreted from monocytes and macrophages has been shown to act as a growth factor in head and neck squamous cell carcinomas (34). Furthermore, IL-8 has also been reported to have proinflammatory and angiogenic activities that enhance tumor growth and metastatic potential of human gastric carcinoma cells and human non-small cell lung cancer (35, 36). Loberg *et al.* showed that monocyte chemoattractant protein 1 (CCL2), a chemokine expressed by human bone marrow endothelial cells, is a potent chemoattractant of prostate cancer epithelial cells (PC-3 and VCaP) and can a play role in bone metastasis in prostate cancer patients (37). PC-3 and DU145 and LnCaP prostate cancer cell lines express the receptor (CXCR4) for stromal cell-derived factor-1 (SDF1) (a chemokine expressed by osteoblasts and endothelial cells in the bone marrow) and it has been shown that SDF-1 supported the invasion of prostate carcinoma cell lines into reconstituted

basement membranes (38). This suggests the possibility that prostate cancers use the SDF-1/CXCR4 pathway during their metastasis to bone.

Cytokine therapy [e.g. IL-3, GM-CSF, interferon (IFN)-α] has been approved for treatment of specific cancers by the FDA. Disadvantages associated with cytokine therapy are frequent injections and most importantly the high rates of side effects leading to discontinued use of the treatment (39, 40). Interleukin-3 and GM-CSF have growth stimulatory effects on normal hematopoietic progenitor cells and clinical trials using these cytokines after bone marrow transplantation for various malignancies including lymphomas are frequently conducted (40). In one such a clinical trial 37 patients (20 patients with non-Hodgkin's lymphoma and 17 patients with Hodgkin's disease) were treated with IL-3 and GM-CSF before bone marrow transplantation. Side effects included nausea, fever, diarrhea, vomiting, rash, edema, chills, abdominal pain and tachycardia and three patients had to stop the treatment because of these side effects. The median time for platelet recovery was 15 days after transplantation that was about 30 days with either of the cytokines alone (40). Overall, the systemic administration of cytokines is associated with significant side effects that bear a certain resemblance to a state of overwhelming infection and has only achieved modest therapeutic benefits that perhaps reflect the failure of this approach to treat cancer (41).

Role of T cells in the treatment of cancer

All current approaches to immunotherapy have failed to match expectations

and recent studies have been directed to activation of antigen-specific T cells (42). T cells that have never encountered antigens are indicated as naïve T cells. Adaptive immunity relies on each naïve T cell bearing a unique prototype antigen receptor recognizing antigens presented to them by other cells. Cells involved in innate immunity, such as DCs, directly recognize a limited set of antigens and are responsible for the initial trapping of the antigen in the periphery and its presentation to T cells in the proximal lymph nodes (43). Upon first encounter with its specific antigen, a naïve T cell is arrested in the lymph node where it undergoes intense proliferation and acquires further differentiation markers and distinct functional capabilities.

Activated T cells fall into two major classes that have different effector functions and they are distinguished by the expression of the cell surface proteins CD4 and CD8. $CD4^+$ T cells (T helper cells) can bind to MHC class II antigens and $CD8^+$ T cells (cytotoxic T cells or CTLs) can bind to MHC class I antigens. T helper cells fall into two general classes: Th1 cells produce several characteristic cytokines, most notably IL-2 and IFN-γ, whereas Th2 cells produce a set of cytokines, most notably IL-4, IL-5, and IL-13. In turn, IL-2 and IFN-γ promote the development of strong cell-mediated immunity, whereas the type 2 cytokines promote allergic responses effective in eliminating parasites (44).

It has been suggested that the activation of naïve T cells is determined by the combination of two signals provided by antigen presenting cells (APCs) (45). The first signal functions through the antigen-specific T cell receptor complex. Foreign antigens (tumor antigens) are presented to T cells on the surface of APC

in complex with MHC molecules and are recognized by the T cell receptor (46). A small number of circulating T cells have the receptor that can recognize the specific MHC/antigen complex. The second signal is provided by interaction with the co-stimulatory molecules (B7.1 and B7.2) on APC and their receptors on T cells (CD28) (47). These interactions activate T cells to undergo a number of mitotic divisions that is referred to clonal expansion and enables a significant increase in the number of T cells that recognize the antigen.

Cytotoxic T lymphocytes are the key components of cell-mediated immunity and activated and expanded CTLs target cells that express foreign antigens. However, a successful immune response requires the presentation of MHC class-I immunogenic peptides by both APCs and the tumor itself. Dendritic cells activate T cells by presenting them with peptide antigen in a complex with MHC molecules and then activated T cells identify target cells that express the same antigen/MHC complex. It has been shown that many human tumors have limited expression of MHC antigens and co-stimulatory molecules (48, 49). Restifo *et al.* showed that three human small cell lung carcinomas failed to transport MHC class-I antigens from the endoplasmic reticulum to the cell surface (48). Fluorescence activated cell-sorting (FACS) analysis also showed low expression of MHC class-I antigens in D7RM-1 prostate cancer cells (49). However, Goldszmid *et al.* showed that DCs co-cultured with apoptotic tumor cells induced protective immunity against B16 melanoma cells that express low levels of MHC class-I antigens. Eighty percent of the vaccinated mice remained tumor-free 12 weeks following challenge with B16 cells and the immunity was mediated by both

$CD4^+$ and $CD8^+$ T cells (50).

Recognition of the same MHC/antigen complex by activated CTLs on tumor cells sets off a cascade of events that ultimately leads to the destruction of tumor cells by programmed death cell or apoptosis (51). The destruction of target cells by CTLs occurs by two major pathways. The first pathway involves release of granules containing perforins that damage the cell membrane. Various granzymes and possibly other granules are secreted along with perforins by CTLs. These granzymes are proteases that enter the target cell through the pores generated by perforins and induce apoptosis by activation of caspases (52). The second pathway requires the interaction of Fas ligand (FasL) on CTLs and Fas molecule on the target cell that induces activation of caspases and induction of apoptosis (51). $CD8^+$ CTLs have been described to play crucial roles in host defense against malignancies in both mouse and human studies. For example, the adoptive transfer of CMS4 sarcoma specific CTLs resulted in nearly complete regression of 3-day established experimental lung metastases in a mouse model. Moreover, under conditions of extensive metastatic disease (day 10 tumor-bearing mice), the adoptive transfer of CTLs resulted in reduction of average detectable lung nodules from >150 at the time of transfer to 30-35 nodules 2 or 3 weeks post-CTLs transfer (53). They also showed that FasL pathway has a significant role in tumor regression of experimental CMS4 lung metastases by adoptive transfer of antigen-specific CTLs (53).

Tumor cells can avoid the adaptive immune response and being killed by antigen-specific $CD8^+$ T cells through impaired antigen presentation (54).

However, natural killer (NK) cells of the innate immune system kill MHC class I-deficient cells that can be missed by CTLs (55). The activity of NK cells is controlled by a balance of positive and negative signals. Engagement of inhibitory receptors by MHC class I molecules blocks activation signals (56). However, in the absence of inhibitory signals NK cell cytotoxicity must be activated by a set of triggering receptors (57). Upon activation NK cells enhance their ability to adhere to and recognize target cells, leading to a broader killing activity against tumor cells that essentially takes place as (58): 1) perforin/granzyme-dependent necrosis of target cells, involving cell adhesion and granule release (59); and 2) apoptosis of target cells, which involves cell adhesion and is mediated by surface TNF ligand family members like FasL or TNF-α that interact with specific receptors on the target cell surface (60).

Although CTLs may be important for the elimination of established disseminated tumors, studies using purified functional subsets of immune T cells have suggested that $CD4^+$ T helper cells are necessary and sufficient for tumor eradication in some cases (61). T cell deficient ATXBM B6 mice (adult, thymectomized, irradiated, T cells depleted and bone-marrow reconstituted mice) inoculated with FBL-3 tumor cells (a Friend virus-induced erythroleukemia of B6 origin) were adoptively transferred with spleen cells depleted of B6/$CD8^+$ T cells (sensitized *in vivo* by injection of irradiated FBL-3 tumor cells) were tumor free for about 80-100 days after tumor inoculation when they were sacrificed. However, mice treated with spleen cells depleted of T helper cells died after about 40-45 days (61). In another study, mice were vaccinated with irradiated B16 melanoma

cells that were transduced to secrete GM-CSF (B16-GM-CSF). Seven to fourteen days after vaccination, mice were challenged with live non-trancduced tumor cells. All vaccinated mice were tumor free for 100 days, whereas control mice died 35-40 days after tumor challenge (62). Both $CD4^+$ and $CD8^+$ T cells were required for effective vaccination, since depletion of either T-cell subset by administration of specific antibodies before vaccination abrogated the development of systemic immunity, whereas depletion of NK cells had little or no effect (62). Furthermore, Hung *et al.* showed that CD4 knockout mice vaccinated with irradiated B16-GM-CSF cells failed to prime a systemic immune response capable of rejecting live tumor cells injected 2 weeks after vaccination. However, similarly immunized CD8 knockout mice mounted a successful tumor rejection (63). Using INF-γ and IL-4 knockout mice they also showed that the protective immunity against B16 melanoma challenge was mediated mostly by INF-γ and to lesser extent by IL-4 secreted by activated Th1 and Th2, respectively (63). Therefore the optimal tumor antigen-specific vaccine should incorporate a panel of dominant tumor antigens recognized by both $CD4^+$ and $CD8^+$ T cells.

Biology and different subsets of dendritic cells and their role in T cell activation

Activation of T cells depends on tumor-associated antigen presentation to T cells by antigen presenting cells (APCs) like DCs that leads to stimulation of an immune response. Probst *et al.* used CD11c-DTR/GFP transgenic mice that allowed conditional depletion of DCs with lymphocytic choriomeningitis virus

(LCMV), which infects all types of APCs and elicits a vigorous CTLs response, to show that priming of LCMV-specific CTLs was crucially dependent on DCs (64). Norbury *et al.* provided direct evidence that virus infected DCs primed naïve $CD8^+$ T cells *in vivo* (65). They showed that following local infection, vaccinia virus infected macrophages and DCs were found in draining lymph nodes. But, only DCs presented antigen to naïve $CD8^+$ T cells as determined by direct visualization of sectioned lymph nodes using confocal microscopy. The presentation occurred about 6 hours after inoculation (65). *In vitro* and *in vivo* studies have shown that bone marrow-derived DCs are the most effective APCs at activating naïve $CD4^+$ T cells. This efficiency is thought to be related to the fact that DCs express high levels of class II MHC and co-stimulatory molecules (66). Confocal microscopy was used to show the physical interaction of DCs and naïve $CD4^+$ T cells in vivo (67). In this study fluorescent dye-labeled DCs and naïve T cell receptor transgenic $CD4^+$ T cells specific for an ovalbumin peptide/IA^d complex were shown to co-localize in the lymph nodes after adoptive transfer into syngeneic recipients. They also demonstrated that DCs caused proliferation and differentiation of $CD4^+$ T cells and production of IL-2 by these cells (67). However, other evidence has suggested that macrophages and in some cases B cells can act as APCs *in vivo*. B cells are the most potent APCs in inducing the proliferation of differentiated T helper type 2 (Th2) cells and splenic macrophages are APCs for differentiated T helper type 1 cells (Th1) (68). It has also been suggested that peritoneal macrophages are favored APCs for both Th1 and Th2 cells (69). However, B cells can function as APCs for Th1 cells, but not for Th2

cells, in an IL-1-dependent pathway (69).

The most important property of DCs is unquestionably their ability to activate naïve T cells *in vivo*. The antigen sampling and migratory capacities of DCs effectively allows naïve T cells to come into contact with peripheral antigens that they would otherwise not have encountered (70). The process that is used by DCs to sample the environment for the benefit of lymphocytes is called DC maturation. DCs have two functional states, immature and mature, with only a mature DC having the ability to prime an immune response (70). The immature state of DCs is characterized by a high phagocytic capacity and low expression of molecules such as CD40, CD83, CD80 (B7.1) and CD86 (B7.2) (71). CD40 engagement induces maintenance of high levels of MHC class II antigens and up-regulation of CD80 and CD86 molecules that provide the required signals for T cell activation (72). Both CD40 and its ligand (CD40L) belong to the tumor necrosis factor receptor (TNF-R) and TNF family (73). CD40 has no kinase domain itself, but CD40L binding to this molecule activates several second messenger systems. These include several protein tyrosine kinases (such as: lyn, syk and Jak3), phosphoinositide-3 kinase (PI-3 kinase), serine-threonine kinases and phospholipase Cγ2 (73). Inhibition of p38 stress-activated protein kinase (a PI-3 kinase) significantly reduced the LPS-induced up-regulation of CD80, CD83, and CD86 but did not significantly affect the endocytotic capacity of human monocyte-derived DCs that is a characteristic of immature DCs (74). In addition, JAK3 inhibition with a JAK3 specific inhibitor prevented the expression of co-stimulatory molecules and production of IL-12, arresting the DCs at an

immature state (75). CD40 has also been shown to interact with members of TNF-Receptor associated factors like TRAF3 that is expressed in almost all cell types. These events lead to activation of transcription factor NFκB that in monocytes eventually activates the expression of genes involved in DC maturation (76) and cytokine (such as IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α, MIP-1α) secretion by these cells (73, 77).

Depending on origin, function and localization, murine DCs are divided into at least two populations: myeloid and lymphoid DCs, which have been distinguished by the expression of CD8 (78). $CD8^+$ lymphoid DCs were first identified in the thymus of mice but DCs with the same phenotype have been identified in spleen, lymph nodes and Payer's patch (79). Myeloid and lymphoid subsets of murine DCs that have been directly isolated from spleen are different in their ability to activate T cells. The myeloid related $CD8^-$ DCs induce more intense and prolonged proliferation of naïve T cells than do lymphoid related $CD8^+$ DCs despite similar expression of MHC and co-stimulatory molecules (80, 81). However, only the $CD8^+$ DC subset, demonstrates cross-priming ability *in vivo* (82). In this cross-presentation pathway, DCs take up cell-associated antigens and present them in the context of their own major histocompatibility complex (MHC) class I molecules to $CD8^+$ T cells. This process has been shown to be important in initiating MHC class I restricted responses to peripheral self, viral, bacterial and tumor antigens (83-86). Whereas myeloid related DCs derived from monocytes produce a large amount of IL-12 and preferentially induce Th1 development, lymphoid DCs produce lower amounts of IL-12 and preferentially

induce Th2 development (87). A third minor population of DCs was originally identified in human blood. $CD11c^-$ DCs that are also called plasmacytoid DCs (pDCs) are morphologically and functionally immature (88). These DCs are found in the T cell zones of lymphoid organs and in the thymus and blood and were also described as plasmacytoid T cells or plasmacytoid monocytes (89, 90). The mouse equivalent of human plasmacytoid DCs has low expression of CD11c, CD11b and MHCII and high expression of B220 and Ly6C (detected with Gr-1 antibody). These DCs do not have high expression of CD123 and some of them express CD8 but activated mouse pDCs have high expression of CD8 and like their human counterparts, they represent *in vivo* a specialized type I interferon producing cell (91). The role of plasmacytoid DCs that are the major producer of type I interferons upon viral infection, in tumor biology is unknown (92). Human DCs do not express CD8 so a human equivalent of murine $CD8^+$ DCs has not been found. But $CD8^-$ DCs found in humans function similarly to their murine counterparts (93).

Several factors can induce DC maturation including pathogen-associated molecular patterns-containing components of bacteria, viruses and parasites, such as lipopolysaccharides, peptidoglycans and CpG motifs that induce toll-like receptor (TLR) signaling pathways (94). The mammalian TLR family consists of 10 members. Expression of TLRs by DCs is different among their subsets. Myeloid DCs express TLR1, 2, 4, 5, and 8, and plasmacytoid DCs exclusively express TLR7 and TLR9, although there are some reports that TLR7 is also expressed in myeloid DCs (95). Toll like receptors have a conserved intracellular

domain called Toll–IL-1–resistance (TIR) domain, which mediates recruitment of the TIR domain–containing adaptor molecule, myeloid differentiation factor 88 (MyD88), a critical adaptor molecule used by all TLRs (96). The recruitment of MyD88 to TIR domains of activated TLRs allows for the interaction and activation of the IL-1R–associated kinase (IRAK) family members and the subsequent activation of TNF receptor–associated factor (TRAF)-6 (97). These events, at a minimum, result in activation of transcription factor NF-κB (98). While most of the TLRs seem to be absolutely dependent on the expression of MyD88 for all of their functions, TLR3 and TLR4 are unique in their ability to activate both MyD88-dependent and MyD88-independent responses (99). A feature of MyD88-independent signaling is the induction of the type 1 interferon (IFN-β) (100). Whereas all TLRs activate NF-κB and ATF2-c-Jun transcription factors, not all TLRs induce IFN-β because not all TLRs induce interferon regulatory factor (IRF-3) activation. Thus, TLR3 and TLR4 appear to activate gene expression pathways and trigger antiviral responses by a mechanism involving the coordinate activation of NF-κB and IRF-3 (100).

The CD11c and MHC class-II (IA) molecules are expressed at high levels on all mature DCs in mice and co-expression of both markers is used to define mature DCs phenotypically (101). Mature DCs migrate to local lymph nodes and settle near the T lymphocyte rich regions. This selective migration allows the DCs to interact with vast numbers of T lymphocytes, thus enhancing T lymphocyte priming and activation (102). Hugues *et al.* showed that maturation of DCs is crucial for T cell activation and resulted in prolonged contacts between DCs and

T lymphocytes (103). Stable and prolonged contact between DCs and T cells primes T cells *in vivo*, whereas brief contacts may contribute to the induction of T cell tolerance (103). Maturation of DCs is important for the initiation of a specific anti-tumor T cell response.

Dendritic cells in cancer

Majority of both circulating and tumor infiltrating DCs from cancer patients appear to be phenotypically and/or functionally defective (104-108). Tumor infiltrating DCs from breast carcinoma have been found to be immature in all of 32 samples that were tested by immunohistochemistry and immunofluorescence, however, DCs from peri-tumoral area were mature in 20 of the samples (108). Tumor infiltrating DCs (TIDCs) in renal cell carcinoma were described as mature DCs since they express high levels of MHC antigens and the B7.2 co-stimulatory molecule (104). But the failure of these patients to mount an anti-tumor immune response despite the presence of professional APCs in the tumor tissue suggests that TIDCs are suppressed *in situ*. The suppression of DCs may be due to the expression of IL-10 in the human renal cell-carcinoma TME as IL-10 is a potent suppressor of DCs (109).

As mentioned earlier DCs are the most potent APCs capable of activation of T cells. Therefore, expansion of DCs and recruitment of DCs and T cells to the tumor site may be essential for generating sufficient and specific anti-tumor immune response. Owing to their capacity to activate and regulate T cells, DCs have been used for vaccination trails in cancer therapy (110). Mayordomo *et al.*

directly assessed the ability of vaccines consisting of DC carrying tumor peptides to elicit protective anti-tumor immune responses in 4 different murine models (111). In this study mice were injected with bone marrow-derived DCs pulsed with synthetic tumor-associated peptides that were known to be expressed by C3 sarcoma, M05 melanoma, Lewis lung carcinoma and Meth A sarcoma tumor cells. Animals vaccinated in this manner were resistant to challenge with tumor cells expressing the relevant tumor antigen in each of four different tumor models tested (111). Furthermore, in a clinical study that the readout was the expansion of $CD8^+$ T cells, 12 patients with metastatic melanoma were vaccinated with tumor antigen-pulsed DCs that led to expansion of melanoma-specific cytolytic $CD8^+$ T cell precursors in several patients. In this study the lack of melanoma-specific T cells was observed in three patients, all of which experienced early disease progression with appearance of new lesions. The results were assessed by measuring the cytotoxic function of CTLs after vaccination against multiple targets expressing melanoma antigens in a standard 4hr ^{51}Cr release assay (112). However, a major limitation for the clinical use of DCs is the availability of sufficient cell numbers. In humans, DCs represent 0.5% of peripheral blood mononuclear cells (113). Therefore, various groups have explored culture conditions to improve expansion of DCs *ex vivo*. Culturing of bone marrow-derived cells in the presence of the ligand for the receptor fms-like tyrosine kinase 3 (flt3-L) has been shown to result in a sufficient expansion of DCs (114). Flt3-L is a hemopoeitic growth factor that promotes the differentiation and expansion of hematopoeitic stem cells and progenitors. It has also been shown

that *in vivo* administration of flt3-L dramatically increases the numbers of hematopoietic progenitors in the bone marrow, peripheral blood and spleen (115, 116). Treatment of mice with human flt3-L has been shown to lead to the expansion of DCs subsets in multiple tissues and peripheral blood (117). Alterations in dendritic cell development and function are associated with tumor escape from immune-mediated surveillance (118). Therefore, using flt3-L to expand DCs to initiate and maintain antigen-specific immune responses seemed a rational choice for cancer immunotherapy. The administration of flt3-L to mice with established tumors resulted in some regression and inhibition of a variety of tumors including melanoma (119), fibrosarcoma (120), colon cancer (118, 121), lymphoma (119) and prostate cancer (122). Daily administration of flt3-L with established colon carcinoma tumors delayed tumor growth as long as the ligand was administered. However, tumor growth resumed as soon as flt3-L injections were stopped, and they grew with a similar rate that was observed in untreated animals (118).

It has been shown by us and others that flt3-L mobilized DCs *in vivo* display primarily an immature phenotype and therefore they are not capable of activating T cells (123, 124). Mosca *et al.* demonstrated that multiple signals are required to induce DCs maturation *ex-vivo* (125). They demonstrated that flt3L-mobilized DCs purified from cancer patients require a sequence of specific signals for maturation, which included initial treatment with granulocyte macrophage-colony stimulating factor followed by a combination of maturation signals such as CD40L and IFN-γ. Flt3L-mobilized DCs matured in this manner

showed higher expression of co-stimulatory molecules than DCs matured with either cytokine alone (125). Davis *et al.* showed that blood dendritic cells generated with flt3-L and matured with soluble CD40 ligand (CD40L) primed $CD8^+$ T cells efficiently in melanoma patients (126). Therefore, although flt3-L expands the number of DCs these DCs are functionally immature and need additional signals to undergo maturation that makes them capable of antigen presention.

Accordingly, maturation of flt3-L expanded DCs may be induced by expression of CD40L in the TME. CD40L is expressed by activated T cells (73) and its receptor, CD40, is an integral membrane protein that is expressed throughout B-cell development. CD40 is also expressed on mature DCs, hematopoietic progenitor cells, epithelial cells and carcinomas (77). Dendritic cell CD40/CD40L interactions seem to be a critical step in the maturation of these cells into fully competent antigen presenting cells. Interaction of CD40 with CD40L has been demonstrated *in vivo* as being responsible for T cell-dependent B cell activation and class switching, facilitating germinal center reactions (77), activation of macrophages and DCs (73, 127), and establishment of memory CTLs (128). CD40 ligation of DC also has the capacity to induce high levels of the cytokine IL-12, which polarizes CD4+ T cells toward a T helper 1 (Th1) type, enhances proliferation of CD8+ T cells (129). Since CD40/CD40L interaction induces DC maturation that consequently may cause T cell activation, Cella *et al.* studied induction of DC maturation with CD40L transfected tumor cells *in vitro*. Indeed, CD40L caused up-regulation of co-stimulatory molecules B7.1 and B7.2

and adhesion molecules (CD54 and CD58) on DCs and enhanced their efficiency to present antigens to T cells (130). They also performed allogeneic mixed lymphocyte reactions to show that T cell stimulatory capacity of DCs was dramatically enhanced by CD40L (130). Significant slower tumor growth rate and less metastasis were observed following administration of CD40L plasmid into the mice with orthotopic pancreatic adenocarcinoma. Tumors of treated mice were infiltrated with T cells and DCs. Dendritic cells were mature and of myeloid origin. Tumor infiltrating lymphocytes were tumor-specific as shown by IFN-γ ELISpot assays (131). Since maturation of DCs plays an important role in the activation of T cells, expression of CD40L in the TRAMP TME infiltrated with immature DCs after flt3-L therapy can play a critical role in the induction of anti-tumor immune response.

Another cytokine that is a good candidate for expression in TME to provide required signals for CTLs activation is granulocyte-macrophage colony-stimulating factor (GM-CSF), because of its capability to cause DC expansion and maturation (132). GM-CSF is a 23-kDa glycoprotein with remarkably diverse effects on immune and non-immune cells (133). GM-CSF induces differentiation of granulocyte, macrophage, and eosinophil precursor cells. When suspensions of mouse bone marrow are cultured in the presence of GM-CSF, three types of myeloid cells expand in numbers: non-adherent neutrophils, firmly adherent macrophages and DCs that arise from cellular aggregates that are attached to the marrow stroma (132). Proliferation of monocyte, macrophages, T lymphocytes, keratinocytes, and endothelial cells is also stimulated by GM-CSF

(133). The receptors for human GM-CSF are expressed on the surfaces of myeloid cells and also non-hematopoietic cells like endothelial cells (134). Furthermore, GM-CSF can induce functional maturation of DCs. Mach *et al.* studied the expression of co-stimulatory molecules on flt3-L and GM-CSF expanded DCs. The level of B7.1 expression was dramatically increased on DCs stimulated by GM-CSF as compared with flt3-L treatment. GM-CSF also stimulated more uniform, high level expression of B7.2, CD40, and MHC class II molecules than flt3-L, although these differences were less striking (135).

In view of the fact that GM-CSF can induce DC expansion and maturation, Dranoff *et al.* studied the ability of this cytokine to stimulate systemic anti-tumor immunity *in vivo* (62). Vaccination with irradiated melanoma B16 tumor cells engineered to secrete GM-CSF stimulated the recruitment of a large numbers of antigen presenting cells to the tumor site that suggest the involvement of this cytokine in the augmentation of tumor-antigen presentation. The systemic immunity was long lasting in that the majority of vaccinated mice were subsequently challenged with non-transduced cells and remained tumor free several months after vaccination (62). Soiffer *et al.* conducted a phase I clinical trial investigating the biologic activity of vaccination with irradiated GM-CSF secreting melanoma cells in patients with metastatic melanoma. Immunization sites were intensely infiltrated with T lymphocytes, DCs, macrophages, and eosinophils in all 21 patients and metastatic lesions removed after vaccination were densely infiltrated with T lymphocytes and plasma cells and showed extensive tumor destruction (136). However, these anti-tumor immune responses

failed to induce clinical regression; rather, the necrotic tumor masses were largely replaced by inflammatory cells, edema, and extensive fibrosis (136). However, GM-CSF has the potential to cause expansion and maturation of DCs in the *tr*ansgenic *a*denocarcinoma of *m*ouse *p*rostate (TRAMP) model and induce long-term immunity through activation of T cells.

Co-localization and therefore interaction of DCs and naïve T cells is essential for activation of T cells. We have shown that TRAMP tumors are infiltrated with a very small population of T cells (137). Moreover, Bai *et al.* showed that the initial activation of tumor specific T cells takes place in the lymphoid organs and then activated T cells infiltrate the tumor and undergo clonal expansion within this site (138). Therefore secondary lymphoid tissue chemokine (SLC) that strongly attracts both mature DCs and naïve T cells through binding to its receptor chemokine C receptor 7 on these cells (139) may facilitate their interaction and promote T cell activation and tumor immunity. This chemokine is expressed by high endothelial venules and in the T cell zone of spleen and lymph nodes. Gunn *et al.* showed that in mice lacking SLC expression, homing of T cells and DCs to secondary lymphoid organs was significantly decreased (140). Dendritic cells express CCR7 upon induction of maturation (141) and CCR7 is essential for migration of naive T cells into lymph nodes and splenic white pulp cords (141). After T cell activation and differentiation, expression of CD62L and CCR7 is lost on T cells (142). Since co-localization and interaction of DCs and T cells are important factors for induction of an immune response, the ability of SLC to chemoattract both T cells and DCs

justified testing this chemokine in cancer immunotherapy (143, 144). It has been shown that SLC expression by C26 colon tumor cells caused strong recruitment of DCs that expressed an immature phenotype and were refractory to activation with a combination of stimuli like LPS, IFN-γ, and anti-CD40 agonist antibody (144). However, DCs were activated by intratumoral injection of a combination of CpG immunostimulatory sequence and IL-10 receptor antibody (144). Intratumoral injection of SLC caused a significant reduction in tumor volumes with complete tumor eradication in 40% of the mice in both line 1 alveolar carcinoma and Lewis lung carcinoma that are known to be weakly immunogenic lung cancer models (143). In these cases, SLC mediated anti-tumor responses were $CD4^+$ and $CD8^+$ T cell dependent (143). Splenocytes and lymph node-derived cells from SLC-treated tumor-bearing mice that were co-cultured with irradiated tumor cells secreted significantly increased levels of IFN-γ (13- to 28-fold), GM-CSF (3-fold, spleen only) and IL-12 (1.3- to 4-fold) compared to carrier treated animals. It is known that GM-CSF and INF-γ are secreted by activated T cells and IL-12 is secreted mainly by B cells and to a lesser extent by activated T cells. Moreover, intratumoral SLC administration led to enhanced lymph node-derived CTL activity against the parental tumor cells (143).

The expression of SLC by transfected tumor cells may be helpful for the induction of the immune response by homing DCs and T cells to the TME where there should be sufficient tumor antigen for immune recognition. We have previously shown that DCs infiltrating TRAMP tumors are phenotypically immature (124). Furthermore, we demonstrated that flt3-L therapy induced a

pronounced mixed (macrophage, DC, granulocyte) myeloid infiltrate into TRAMP tumors. It has also been shown that myeloid cells infiltrating human and mouse tumors actually helped tumor development by providing molecules and factors essential for tumor growth. For example, IL-1β that is produced mainly by activated monocytes and macrophages promotes tumor growth of Lewis Lung carcinoma by induction of angiogenic factors by stromal cells (145). Myeloid cells also exerted a profound inhibitory activity on both tumor-specific and nonspecific T cells by altering the balanced production of cytokines like IL-2 and IL-12 in the tumor microenvironment (TME) (146). We hypothesize that SLC expression in the TRAMP TME will attract a significant numbers of mature DCs and naïve anti-TRAMP T cells which will facilitate T cell/DC interaction to induce T cell activation. We further speculate that this interaction will overcome the inhibitory activity of infiltrating myeloid cells.

In summary, flt3-L therapy caused tumor regression in the mouse model of prostate cancer but this therapy was not curative. This could be due to the immature phenotype of DCs in the TRAMP TME and consequently their inability to activate T cells infiltrating these tumors. We propose that expression of SLC, CD40L or GM-CSF will provide required additional signals for maturation of DCs leading to T cell activation and anti-tumor immunity.

Tetracycline inducible mammalian expression system

The ability to control the spatial and temporal expression of a transgene, either in cell culture or in transgenic animals, is a valuable tool in gene function

studies. Constitutive over-expression of a transgene over long periods in culture or in animals may have several undesired effects that are not functional characteristics of that gene product. Therefore, regulated expression systems are invaluable for studying gene function. These systems offer advantages of dosage-dependent and temporally defined gene expression, and limit possible clonal variation when toxic or pleiotropic genes are over-expressed. Many methods have been described and used for regulated expression of transgene in mammalian cells. Some of these methods involve the use of endogenous mammalian proteins. These include the use of heat shock proteins, the glucocorticoid receptor, the estrogen receptor, the progesterone receptor and the aryl hydrocarbon receptor (147-150). In all these systems the expression of the transgene can be modulated but the problems associated with these systems are the generalized physiologic or toxic effects of the inducers and/or high basal transcriptional activity from the inducible promoter that limits their utility. The expression of *Cyp1a-1* gene, whose product, aryl hydrocarbon hydroxylase, is induced by polycyclic aromatic hydrocarbons. *Cyp1a-1* enhancer elements and promoter region have been used to inducibly express the chloramphenicol acetyltransferase (CAT) reporter gene in transgenic mice. Treatment of transgenic mice with the inducer 3-methylcholanthrene (3-MC) caused a profound increase in transgene expression (>1,000-fold) in many tissues including liver, adrenal, kidney and intestine. Very high background was observed in some tissues (spleen and bowel) (151). However, the most important problem with this system is that 3-MC is known as a carcinogen (152). The

tetracycline-mediated method is one of the most commonly used since it has several advantages over other systems. For example, mammals do not express an endogenous tetracycline repressor and systemic administration of this antibiotic to animals over long periods is harmless (153, 154). The expression of the transgene in transgenic animals is exclusively dependent on the administration/absence of tetracycline or tetracycline derivatives (155). This system provides a very tight control of gene expression in mammalian cells. The tetracycline inducible system was very efficient in regulating the oncogene HER2 expression in transfected cell lines (NIH3T3 and MCF7) both *in vitro* and when the cell lines were injected in mice (155). The function of any gene product can be studied during selected developmental windows or at critical stages of disease using tetracycline inducible system (156). For example, transgenic mice were generated by Lee *et. al* that developed spontaneous cardiac arrhythmias *in vivo* by tetracycline regulated expression of the diphtheria toxin A (DTA) gene that was targeted to the hearts of adult mice. The expression of DTA caused cell loss in hearts of these animals that may have lead to the observed arrhythmias (156). Priscilla *et. al* used the tetracycline inducible system to express a transgene (luciferase) in mice. The basal luciferase activity tested in eleven different tissues was close to basal level in most of the transgenic mice tested (total of 19 mice). Administration of tetracycline activated the transgene in most tissues (over 1000 fold induction in some cases) (157). This system was also used by Manfra *et. al* for tetracycline-dependent expression of flt3-L in transgenic mice. Tetracycline treatment induced expression of the transgene in several tissues and also

induced dramatic changes in blood levels of flt3-L, which were, on the average, 200-fold higher than the values non-treated transgenic mice. The expression of flt3-L caused the relative number of DCs in peripheral blood to increase from 8 to 40%. Cessation of flt3-L induction led to normalization of DCs numbers in blood (117).

The T-Rex system (Invitrogen, CA) has been employed in this study to control the expression of genes of interest (SLC, CD40L or GM-CSF) in TRAMPC cell lines. The T-Rex system includes two vectors: the repressor vector (pcDNA6/TR) and the expression vector (pcDNA4/TO) (Fig. 1). The repressor vector expresses the tetracycline repressor protein (TR) that binds effectively the $TetO_2$ sites on the expression vector and blocks transcription initiation. When tetracycline is added to the culture media it binds to, and changes the conformation of the TR protein, so TR can no longer bind to $TetO_2$ sites. The result is transcription initiation and eventually expression of the gene down stream from the complete CMV promoter on the expression vector.

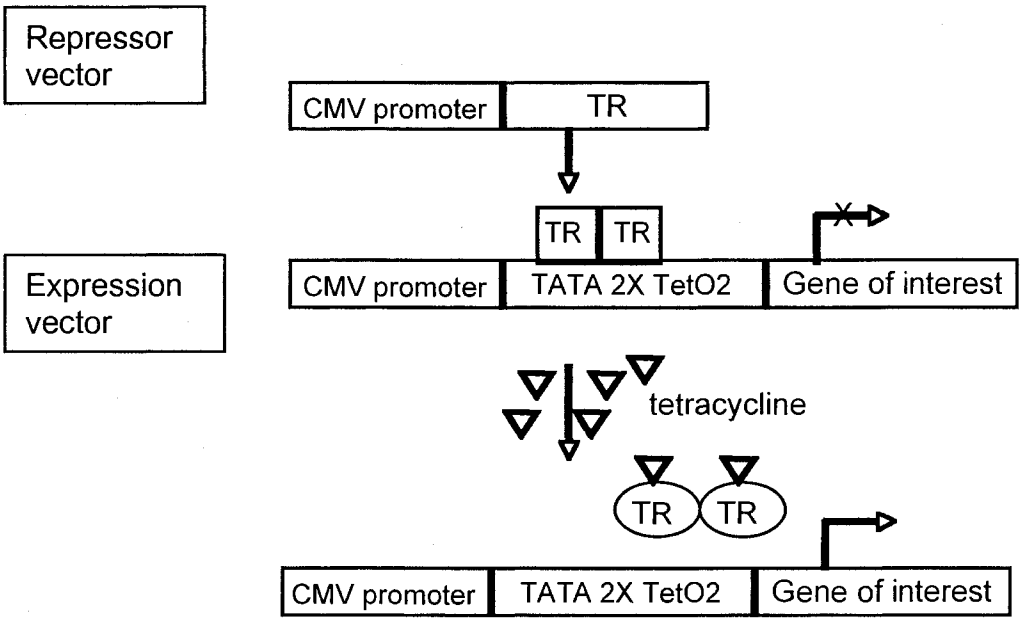


Figure 1. Tetracycline inducible expression system.
The repressor vector encodes repressor protein that binds to the operator region ($TetO_2$) of the expression vector and blocks the protein expression from this vector. Tetracycline binds to the repressor protein and induces conformational changes in this protein. Then, release of repressor protein from expression vector is followed by the protein expression from this vector.

The *t*ransgenic *a*denocarcinoma of *m*ouse *p*rostate (TRAMP) model

To study new therapeutic approaches such as gene therapy, animal models of human prostate cancer with metastatic behavior are required. In this regard the TRAMP model was developed by Greenberg *et al* (158). The initiation and progression of human prostate is a multi-step process and it develops in the secretory epithelial cells of the prostate gland. In TRAMP mice, expression of SV40 T antigen was targeted to the epithelial cells of the prostate gland of C57BL/6 mice using the rat probasin promoter. Interaction of SV40 T antigen with p53 and retinoblastoma tumor suppressor genes can cause transformation of cultured cells (159). By 12 weeks of age, TRAMP mice histologically display mild to severe hyperplasia. Severe hyperplasia and adenocarcinoma is observed by 18 weeks of age. By 24-30 weeks of age, all TRAMP mice display primary tumors and metastasis is commonly detected in the lymph nodes and lungs and less frequently in the bone, kidney and adrenal glands (158). TRAMP mice have a number of advantages over existing models. The transgene is specifically expressed in the epithelial cells of the prostate. Tumor arises in all mice and the disease progresses from mild to sever hyperplasia and adenocarcinoma that metastasizes to the lymph nodes, lungs and occasionally bones (160). The disadvantage of this model is its relative high expense, related to requirements for breeding and housing the animals. Tumors develop late in the animals' life span (24-30 weeks) so it is also relatively slow. Furthermore, Phenotypic

variability in pathologic progression has been observed in the TRAMP model. Three different cell lines (TRAMPC1, TRAMPC2 and TRAMPC3) were established from a primary tumor of a TRAMP mouse. TRAMPC1 and C2 are tumorigenic when injected subcutaneously into the flank of mice, whereas, TRAMPC3 grows *in vitro* but does not form tumors *in vivo* (160). Although the TRAMPC1 cell line is not metastatic, TRAMPC2 metastasizes to regional lymph nodes, submandibular salivary gland and lungs (161).

Orthotopic model of prostate cancer and flt3-L therapy

In order to develop a more clinically relevant orthotopic prostate cancer model, the non-metatstatic TRAMPC1 cell line was injected into the prostate and after 3 months a palpable tumor was excised and cultured *in vitro*. The cell line derived from this tumor was injected back into the prostate and after 3 passages, a metastatic cell line was derived that consistently grew in the prostate gland and produced reproducible and predictable metastases to the draining lymph nodes (DLNs). This cell line was designated as TRAMPC1P3 (122).

We had previously established that orthotopic implantation of TRAMPC1P3 cells in immunocompetent C57BL/6 mice produced clinically detectable prostate tumors within 30 days with periaortic lymph node metastasis in almost all animals (162). This model was used to assess the utility of flt3-L in tumor progression. Although some animals remained tumor free for 2-3 months, after termination of flt3-L therapy, disease recurrence was invariably observed

suggesting that flt3-L treated tumor-bearing mice did not develop sufficient anti-tumor immunity. Parental TRAMPC1 cells were initially developed by the expression of SV40 T antigen in these cells but they do not express this gene at either mRNA or protein level (160). Thus, the tumor-bearing host cannot mount an immune response to the T antigen (162). However, TRAMPC1 cells express Wilm's tumor gene encoded transcription factor (WT1) protein that has been studied as a candidate tumor antigen. Immunization with WT1 peptide did not prevent TRAMPC1 tumor growth in mice although CTLs pulsed with this peptide could lyse TRAMPC1 tumor cells *in vitro* (163). WT1 is an internal protein and should be processed and presented by MHC class I molecules on the tumor cells. The failure to protect mice by WT1 may reflect low expression of MHC class I antigen by TRAMPC1 cells. In a very recent study Fasso *et al.,* identified the first "stimulators of prostatic adenocarcinoma-specific" (SPAS) tumor antigen called SPAS-1 using T cells from mice immunized with a GM-CSF-expressing TRAMP cells in combination with *in vivo* CTLA-4 blockade. They showed that vaccination of TRAMPC2 tumor bearing mice with SPAS-1 pulsed DCs resulted in a statistically significant delay in tumor growth indicating that this protein was indeed a target for anti-TRAMP tumor T cell response *in vivo* (164). Therefore, SPAS-1 tumor antigen represents the first-identified tumor rejection antigen on TRAMP tumor cells. *Spas-1* expression was not limited to the prostate but was found in other tissues, with the highest level of expression in the heart. The expression level of *Spas-1* increased in older TRAMP tumors (27 weeks) compared to normal prostate tissue or prostate tumor from 21 week old TRAMP

mouse. The broad expression pattern of *Spas-1* found in mice raises the question of possible autoimmune side effects in the setting of active immunotherapy that remains to be determined.

Flt3-L therapy causes regression of TRAMPC1P3 tumor in mice

In our previous studies we evaluated the effectiveness of flt3-L therapy to inhibit prostate tumor growth (122). We injected mice orthotopically with TRAMPC1P3 cells and started flt3-L therapy one week after tumor injection to allow tumor cells to establish their immunosuppressive microenvironment. During therapy, mice were injected subcutaneously with either flt3-L or carrier. Flt3-L therapy inhibited prostate tumor growth but all the treated mice eventually died from disease after termination of the therapy. Characterization of cellular composition of TILs isolated from flt3-L treated compared to carrier treated mice showed that flt3-L did not have an impact on infiltration of natural killer cells or B cells. At the same time tumor was infiltrated by macrophages, DCs and granulocytes. By the end 30-day therapy, TRAMPC1 tumors were mainly infiltrated by $F4/80^+$, $CD11c^+$ and $Gr\text{-}1^+$ cells but not T cells (122). The population of DCs that appeared in TRAMPC1P3 tumor during flt3-L therapy was mostly $CD8^-$. $CD8^+$ DCs that are capable of antigen cross presentation (82) only appeared 20 days after the end of the therapy and by this time all carrier treated mice died. It appeared that flt3-L induced infiltration of TRAMP tumor by mostly myeloid cells (DCs, macrophages and granulocytes).

Dendritic cells are thought to be the major APCs in the host and therefore play an essential role in the anti-tumor immune response. In view of this function, TRAMPC1P3 tumor infiltrating DCs (and splenic DCs) were analyzed in order to determine their maturational status since DCs must be mature to function optimally as APCs. Flt3-L treated TRAMPC1P3 tumors were infiltrated with a substantial number of DCs ($CD11c^+$ cells, 23%). TRAMPC1C3 infiltrating DCs (TIDCs) were $CD8^-$, a subtype not capable of antigen cross presentation. TIDCs showed very low expression of MHC class II antigen (IA^b) and they expressed different levels of co-stimulatory molecule B7.2 during tumor growth but the co-expression of IA^b and B7.2 was usually very limited, whereas mature DCs of normal spleen showed high level of IA^b and B7.2 co-expression. The low expression of class II antigens on TIDCs represents the immature state of DCs that could have been induced by the TME. However, injection of TRAMPC1P3 tumor bearing mice with soluble CD40L, known to induce DCs maturation, along with flt3-L did not have any further inhibitory effect on tumor growth compared to mice only treated with flt3-L and mice treated with CD40L alone died at a comparable rate as untreated mice. These results suggested that CD40L did not increase the efficacy of flt3-L. Evaluation of draining lymph nodes immediately after termination of immunotherapy indicated that the majority of periaortic lymph nodes from carrier and CD40L treated mice had metastatic disease. Whereas DLNs obtained from mice treated with either flt3-L alone or in combination with CD40L were disease free as assessed by *in vitro* outgrowth of aneuploid cells. Analyzing the maturational status of DCs during immunotherapy showed that flt3-

L only expanded DCs that failed to express class II antigens and costimulatory molecules. Combination therapy using flt3-L and CD40L did not induce maturation of DCs in TRAMPC1P3 tumor bearing mice.

Therefore, flt3-L immunotherapy induced tumor regression of MHC^- prostate cancer implanted in the prostate. This therapy alone was not successful and did not induce long-term immunity because disease relapse occurred after the termination of treatment. Therefore, combination therapy may be required to induce DCs maturation and consequently drive clonal expansion/differentiation of tumor-specific T cells and the development of a memory response. In this regard, we propose to transfect prostate cancer cells with cytokines and chemokines and control their expression using tetracycline responsive promoter. Intra-tumoral secretion of chemokines/cytokines that are known to expand DCs and induce their maturation and also cause co-localization of DCs and T cells by transfected TRAMP cells may overcome impaired antigen presentation within the TME. In this thesis research, regulated expression of chemokines/cytokines by prostate cancer cell lines has been used to modulate the TME. It is predicted that this modification will enhance tumor immunity and inhibit primary tumor growth and metatstatic disease. It is important to evaluate immunotherapy in the animal with preexisting disease to have more clinically relevant model. The tetracycline-regulated gene expression system can provide tight control over the expression of the genes of interest (SLC, CD40L, GM-CSF). Transgenes can be expressed in established tumors and their efficacy monitored by tumor regression and

prevention of metatstatic disease. This model therefore reflects clinical realities as patients seek treatment after they were diagnosed with the disease.

CHAPTER II

SPECIFIC OBJECTIVES

Prostate cancer is the most frequent cancer in males and is the second leading cause of cancer death among men in western countries. There is no curative treatment available for patients with metastatic prostate cancer or with disease relapse after radical prostatectomy. Cancer immunotherapy aims at generating an efficient and long-lasting cytotoxic T lymphocyte (CTL) immune response for treatment of residual and metastatic disease. CTLs are activated by DCs that are professional antigen presenting cells (APCs). Mature DCs express both high levels of MHC molecules needed for specific antigenic peptide presentation to T cells, and high levels of co-stimulatory molecules for subsequent T cell activation. Previous work demonstrated that intra-tumoral DCs displayed an immature phenotype suggesting altered DC function. In view of this observation, it is anticipated that modifying the TME by the regulated expression of distinct cytokine/chemokine-expressing tumor cells will induce DC infiltration and maturation and promote accumulation of tumor-infiltrating T cells. This will fundamentally change the cellular composition of the TME and promote the development of an intra-tumoral immune response and tumor rejection.

Hypothesis

Proper interaction of immune cells and activation of host effector immune cells plays a very critical role in the induction of tumor specific immune response. We hypothesize that TRAMPC prostate tumors establish a microenvironment that suppresses the activation of immune effector cells. We further hypothesize that the TRAMPC tumor microenvironment prevents DCs maturation. Therefore, Modification of the tumor microenvironment to induce DCs maturation and their interaction with T cells can induce a sufficient immune response to enable the host to remove residual cancer cells.

Specific aim 1

To investigate the phenotype and functional status of dendritic cells infiltrating TRAMPC2 prostate tumor *in vivo* and after *ex vivo* culture.

Cytotoxic T cells are among the most characterized effector cells of the immune system. It has been shown that the presence of intra-tumoral T cells correlates with improved clinical outcome in certain human cancers during the natural immune response against tumors (158). Furthermore, DCs that are antigen-presenting cells are known to be the most powerful activators of tumor-specific cytotoxic T cells (159). Cytokine and chemokines can potentially help target tumor antigen to DCs and induce maturation of these antigen-presenting cells, attract immune effector cells expressing different cytokine/chemokine receptors and drive cellular immune response towards

activation of CTLs. The ability of SLC to co-localize T cells and DCs (136), GM-CSF to induce expansion and maturation of DCs (48, 128) and CD40 ligand (CD40L) to induce DCs maturation (48) formed the rationale to evaluate these cytokine/chemokines in the cancer immunotherapy.

Specific aim 2

To evaluate the efficacy of the SLC, CD40L or GM-CSF to induce dendritic cells maturation *in vitro* using TRAMPC cell lines expressing these genes.

Maturation is the terminal differentiation process that transforms immature DCs specialized for antigen capture into cells specialized for T cell stimulation (160, 161). Maturation results in increased expression of MHC, adhesion and co-stimulatory molecules on DCs (123, 162). Mature DCs can prime T cells in the secondary lymphoid tissues like lymph nodes and spleen to expand and differentiate into effector cells (117). We have previously shown that DCs infiltrating TRAMPC1P3 tumor microenvironment are mainly immature (163). SLC (130), CD40L (123) and GMCSF (164) are known to induce differentiation and maturation of DCs. We hypothesize that TRAMPC tumor cells induce down-regulation of MHCII and co-stimulatory molecules on DCs. Mature DCs cultured with transfected TRAMPC tumor may show down-regulation of MHC antigens and co-stimulatory molecules. Expression of SLC, CD40L or GM-CSF by TRAMPC2 cells using the inducible system that we have developed is predicted

to cause maturation of DCs and expression of MHC and co-stimulatory molecules that are required for antigen presenting function of DCs.

Specific aim 3

To characterize the tumor infiltrating leukocytes (TILs) in the TRAMPC tumor expressing SLC, CD40L or GMCSF *in vivo* and to evaluate the efficacy of expression of these genes in the tumor microenvironment to eradicate residual prostate cancer.

Our previous studies show that flt3-L therapy of mice bearing TRAMPC1P3 tumors caused tumor growth inhibition but disease relapse occurred in all the animals and eventually they died from urogenital complications within 4-5 weeks after transplantation (155). We have shown that TRAMPC1P3 tumors treated with flt3-L are infiltrated with DCs with low expression of MHCII, B7.1 and B7.2 molecules that are not capable of sufficient tumor specific T cell activation (163). We hypothesized that immature phenotype of host DCs causes the failure of the flt3-L therapy in TRAMP model. Expression of SLC in the TME after flt3-L therapy might help co-localization of DCs and T cell and hence activation of T cells. Expression of CD40L or GMCSF in the TME can cause maturation of already mobilized DCs by flt3-L. These alterations in the prostate TME may enhance the immune response mediated by T cells resulting in the eradication of residual tumor after flt3-L therapy and preventing the disease relapse after termination of the therapy.

CHAPTER III

MATERIALS AND METHODS

Cell and culture conditions

TRAMP (TRAMPC1P3 and TRAMPC2) cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) 1X with 4.5g/L glucose and sodium pyruvate and without L-glutamine (Cellgro) supplemented with 5% FBS (Hyclone), 10^{-8} Dihydrotestosterone (Sigma), 2mM L-glutamine (Cellgro), 5% Nu serum IV Culture Supplement (BD Biosciences), 2.5mg/ml insulin (Sigma) and 25µg/ml penicillin-streptomycin (Gibco BRL). Cells were cultured in a 5% CO_2 incubator at 37°C.

B16-FL cells are H-2^b mouse melanoma cells that were transfected with the trimerized form of the flt3-L gene. These cells secrete flt3-L, thus the spleens of tumor-bearing mice are enlarged and are greatly enriched for myeloid cells. B16-FL cells were cultured in 90% DMEM (Cellgro), 10% FBS (Hyclone), 25µg/ml penicillin-streptomycin (Gibco BRL) and 2mM L-glutamine (Cellgro). Cells were cultured in a 5% CO_2 incubator at 37°C.

Generation of subcutaneous and intra-prostatic tumors

To establish a subcutaneous tumor, $5x10^6$ TRAMPC1P3, TRAMPC2 or B16-FL cells were injected under the skin into the flank of 6-8 week old male C57BL/6 mice. To establish an orthotopic tumor mice prostate glands were

surgically exposed and injected with 0.05ml of media containing $5X10^5$ TRAMPC2 tumor cells. Mice were regularly monitored for tumor growth. All animal protocols were conducted in accordance with National Institute of Health guidelines and were reviewed and approved by the Institute Animal Care and Use Committee of Eastern Virginia Medical School.

Excision and processing of TRAMP tumors

After approximately 30 days, the mice were euthanized in 100% carbon dioxide, and the tumor was excised aseptically. All the skin and connective tissue was removed, and the tumor was rinsed in RPMI (Cellgro) to remove any animal hair. The tumor was cut into 2mm pieces using sterile scalpels and then the pieces were placed into a small beaker and covered with 10-15ml of digestion solution (Appendix A). The tumor pieces were stirred for 45min at 37°C. After the 45min incubation, the tumor pieces were mashed through a screen and collected in a 50ml conical tube. To remove any remaining large chunks of tumor or tissue, the supernatant was passed through a 40µm nylon cell strainer (BD Biosciences) and collected in a separate 50ml tube. To stop the digestion process, RPMI/10%FBS was added to the tube. The cells were then centrifuged at 1500rpm (280xg) for 8 minutes. After centrifugation, the media was decanted and ~10ml of RPMI was added to the tube. A cell count was obtained using the Coulter Counter.

Ficoll-hypaque gradient separation of Tumor infiltrating leukocytes (TILs)

Using a 22G needle on a 10ml syringe, ~3.0ml of Isolymph (Gallard-Schlesinger Industries, Inc) solution was added to the bottom of polystyrene tubes (Fisher Scientific). Then, $4x10^7$ cells were layered on top of the Isolymph and the cells centrifuged at 1500rpm (280xg) in an IEC Centra-8 Centrifuge for 20 minutes with no brake. After centrifugation, the layer of cells at the interface between the Isolymph and the tissue culture media was removed using a glass pipette. The layer of cells was placed in a separate 50ml conical tube with RPMI and 1% goat serum (Sigma). Finally, this tube was centrifuged at 1500rpm (280xg) for 5 minutes. A cell count was obtained using a Coulter Counter. After the gradient separation, typically ~10^7 leukocytes/tumor were recovered. Cells were kept on ice until further use.

Preparation of splenic cells

The enzymatic digestion solution for spleen cells with collagenase D (Roche Diagnostics) was prepared as follows: 400U/ml and 100U/ml of collagenase D was prepared in RPMI. Using a 22G needle on a 10ml syringe, 1ml of 100U/ml collagenase D was placed into a 100mm petri dish with RPMI. Spleen(s) were sterilely removed from mice euthanized in a CO_2 chamber and placed in the petri dish. Using the syringe and sterile forceps, 1ml of 100U/ml collagenase D was injected into the spleen. Next, sterile spatulas were used to

tear apart and tease the spleen. The supernatant was collected in a 50ml conical tube. With the remaining spleen fragments, 1ml of 400U/ml collagenase D was added to the petri dish, and the dish was placed in the CO_2 incubator for 30-90 minutes. After incubation, the spleen fragments were mashed through a screen over a petri dish. The solution that passed through the screen was collected and added to the 50ml tube containing the supernatant. To remove any remaining large chunks of spleen or tissue, the supernatant was passed through a 40µm nylon cell strainer (BD Biosciences) and collected in a separate 50ml tube. Next, the tube was centrifuged at 1500rpm (280xg) for 5 minutes. The supernatant was decanted and 3-4ml of Red Blood Cell lysis buffer (Appendix A) was added and allowed to incubate for ~5 minutes at room temperature. To stop the lysing process, RPMI/10%FBS was added to the tube. The tube was centrifuged again at 1500rpm (280xg) for 5 minutes, and the cells were counted using a Coulter Counter. Typically, ~10^7-10^8 total cells were recovered. Cells were kept on ice until further use.

Staining for flow cytometry

$2x10^6$ cells were placed into a flow cytometer tube. Cells were blocked by adding 10µl of normal goat serum (Sigma) to each tube. The tubes were incubated at room temperature for 10 minutes. Then, the recommended amount of primary antibody was added (see Appendix B for optimum antibody concentration). The tubes were incubated at 4°C in the dark for 20 minutes. After incubation, 2ml of cold wash buffer (Appendix B) was added to the cells and

centrifuged at 1500rpm (280xg) for 5 minutes. The supernatant was then decanted and the wash repeated. The pellet was re-suspended in 200µl of wash buffer. If secondary antibodies were required for the staining protocol, they were added after the primary antibody at the appropriate concentrations, with additional 4°C incubation in the dark for 20 minutes. Unbound secondary antibodies were removed by repeating the wash step above and re-suspended in 200µl of wash buffer. Typically, the cells were immediately used for flow cytometric analysis. Occasionally, the cells were fixed by addition of 500µl of 1% paraformaldehyde, stored in dark at 4°C and then were washed before flow analysis.

Isolation and purification of Gr-1$^+$ cells

Spleen Preparation:

C57BL/6 mice were subcutaneously injected with $5x10^6$ B16-FL tumor cells. After ~3 weeks, the mice were euthanized in 100% carbon dioxide. Spleen(s) were harvested using sterile technique and placed in a 100mm petri dish with RPMI. Using forceps, the spleen was gently teased apart so that the capsule was left behind. With a pasteur pipette, the clumps were broken up and placed into a 50ml conical tube. The tube was allowed to sit for 5 minutes at room temperature for the debris to settle out. After 5 minutes, the supernatant was removed and placed into a 15ml conical tube leaving the debris behind. The tube was centrifuged for 10 minutes at 1500rpm (280xg). After centrifugation, the supernatant was decanted and 3ml Red Blood Cell lysis buffer (Appendix A)

was added to the tube and allowed to incubate for 2 minutes at room temperature. Next, 15ml of RPMI /10%FBS was added to the tube. The tube was centrifuged again for 10 minutes at 1500 rpm (280xg). Then, the supernatant was decanted and 5ml of RPMI was added. Again, the clumps were allowed to settle out for 5 minutes and the supernatant was removed and placed into another 15ml tube. These cells were counted using a Coulter Counter, centrifuged and re-suspended in 200µl RPMI.

Magnetic Labeling:

Using the single cell suspension prepared from the B16-FL tumor-bearing spleen, the cells were labeled with biotin-conjugated Gr-1 antibody at the optimum concentration and allowed to incubate at 4°C in the dark for 20 minutes. After incubation, the cells were washed with 2ml MACS buffer (PBS pH 7.2 supplemented with 0.5% BSA and 2mM EDTA) and centrifuged at 1500 rpm (280xg) for 5 minutes. The wash was repeated, and the cells were re-suspended in 800µl MACS buffer. Next, 200µl anti-biotin microbeads/10^8 total cells (Miltenyi Biotec) were added and allowed to incubate at 4°C for 20 minutes. After incubation, the cells were washed with 2ml MACS buffer and re-suspended in 500µl/10^8 total cells.

Magnetic Separation:

The MS magnetic column (Miltenyi Biotec) can be used for up to 10^7 positive cells and up to $2x10^8$ total cells or the LS magnetic column (Miltenyi Biotec) for up to 10^8 positive cells and up to $2x10^9$ total cells. The LS separation column was placed in the magnetic field. Then, the column was washed with 3ml of MACS buffer. The magnetically labeled cell suspension was added to the column. The column was washed three times with 3ml MACS buffer. The total flow through from the column was collected as the negative fraction. Finally, the column was removed from the magnet and 5ml of MACS buffer was added. A plunger was depressed forcefully through the column, washing off the cells into the collection tube. These were positively selected cells for the stained antibody, thus they were Gr-1^+ cells. The separation was repeated on a freshly prepared column to increase the purity of the positive fraction. The cells were then washed in MACS buffer and centrifuged at 1500rpm (280xg) for 5 minutes. A cell count was obtained using a Coulter Counter and re-suspended in 1ml MACS buffer. Cells were kept on ice until further use.

Dendritic cell enrichment using Nycodenz

A single cell suspension of splenocytes was prepared as explained above. Splenocytes were centrifuged and resuspended in a 13.5% (wt/vol) Nycodenz isotonic solution (Sigma). Then the cell suspension was overlaid with RPMI/2mM EDTA and centrifuged for 15 minutes at 1700g (4°C). Low-density cells were

collected from the interface, thoroughly washed in PBS/0.5% BSA and a cell count was obtained using a Coulter Counter. Cells were kept on ice until further use.

In vitro co-culture assay

The cellular composition of the *in vitro* co-culture assay employed Nycodenz enriched DCs and/or magnetic bead-enriched Gr-1^+ cells from flt3-L treated mouse spleens and TRAMPC2 cells. The purified cell types were added to tissue culture dishes according to the ratios explained in each experiment. After overnight incubation at 37°C 5% CO2 incubator the cells were collected and washed with PBS. Then the cells were labeled for flow cytometric staining according to the procedure(s) above.

Generation of tetracycline inducible expression vectors

We generated tet-inducible SLC, GMCSF and CD40L vectors (pcDNA4/TO/SLC, pcDNA4/TO/GMCSF and pcDNA4/TO/CD40L) using the T-Rex system that was purchased form Invitrogen. Plasmids containing the open reading frames for these specific cytokines were purchased from Invivogen. The cytokine genes were amplified using specific primers. The sequence of the primers used to amplify these genes is listed below. In some cases a restriction site (bold letters) was inserted into the primer to be used for cloning.

SLC primers: (*BamHI* site: GGA TCC)

Forward 5'-GCG CG**G GAT CC**C ATG GCT CAG ATG ATG AC-3'

Reverse 5'-TCA TGT CGA GCT AGC GGG CTC CAG GCG-3'

GMCSF primers: (*BamHI* site: GGA TCC)

Forward: 5'-CAT TCA **GGA TCC** ATC ACC GGT AGA GG-3'

Reverse: 5'-TAT CAT GTC GSG CTA GCT GGG CTT CC-3'

CD40L primers: (*HindIII* site: AAG CTT)

Forward: 5'-CCG CG**A AGC TT**C ATG GCC ATA GAA ACA TAC-3'

Reverse: 5'-TTA TCA TGT CGA GCT AGC GAA GAC TGC CAG-3'

The PCR reactions were performed using 2.5 units of *Pfuturbo* DNA polymerase (stratagene), 0.25mM of each dNTPs (Biorad), 250ng of each primer and 50ng of plasmid template. The following conditions were used to amplify the cytokine genes: after initial denaturation at 95°C for 2 min, 4 cycles of denaturation at 95°C for 30 seconds, annealing at 42°C for 30 seconds, extension at 72°C for 2 minutes, and then another 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 2 minutes, and final extension at 72°C for 5 minutes program.

Amplified SLC and GM-CSF genes were digested with *BamHI* and *NheI* (New England Biolabs) restriction enzymes. The DNA sequence generated by *NheI* enzyme is complementary to the sequence generated by *XbaI* that is in the multiple cloning site of the pcDNA4/TO expression plasmid. This sequence can be used for ligation of the cytokine gene into the expression vector. The amplified CD40L gene was digested with *HindIII* (New England Biolabs) and *NheI* restriction enzymes. The pcDNA4/TO plasmid was also digested with the appropriate enzymes for each ligation (in case of SLC and GM-CSF with *BamHI*

and *XbaI* and in case of CD40L with *HindIII* and *XbaI* (New England Biolabs), *XbaI* can be ligated to *NheI* as they produce compatible cohesive ends. Then SLC, GM-CSF and CD40L genes were ligated into the digested pcDNA4/TO plasmid using T4 ligase (New England Biolabs) to generate pcDNA4/TO/SLC, pcDNA4/TO/GM-CSF and pcDNA4/TO/CD40L. Then XL-1 blue competent cells were transformed with each plasmid separately. Colonies of transformed bacteria were selected and grown in ampicillin containing Luria-Bertani (LB) broth. The plasmids were extracted from the bacterial culture using plasmid miniprep kit (Qiagen) and tested for presence of the ligated gene using different restriction enzymes based on Table 1:

Table 1. Restriction enzymes used to determine the ligation of SLC, GMCSF and CD40L into the pcDNA4/TO vector.

plasmids	Enzymes used	Expected bands (base pair)
pcDNA4/TO/SLC	*NcoI*	3811, 1400, 390, 220
pcDNA4/TO/GM-CSF	*NcoI*	3440, 1810, 220
pcDNA4/TO/CD40L	*NcoI, BamHI*	3440, 1659, 514, 220

In order to confirm the integrity of the generated expression vectors, they were sequenced using the primers provided by Invitrogen in the T-Rex system.

Transfection of TRAMPC tumor cells

Different transfection reagents were tested in order to find one that shows high transfection efficiency with TRAMPC1P3 cells. The transfection reagents that included superfect (Qiagen), Lipofectin (Invitrogen), lipofectamine (Invitrogen), metafectene (Biontex), all showed efficiency of less than 1% (0.001 to 0.01%) using the green fluorescent protein expression vector. Next we tried calcium phosphate and electroporation methods. Electroporation of TRAMPC1P3 cells in the optimized condition showed about 1-2% efficiency. TRAMPC1P3 cells were transfected with the T-Rex system repressor vector using this method. TRAMPC1P3 cells that were in the log phase were harvested and incubated with the repressor vector in PBS for 10min on ice and then electroporation was performed at 800uF and 300V (low resistance) using a Biorad gene pulser (Model: 1652076). Cells were incubated on ice for 15min after electroporation and then they were cultured in fresh TRAMP media. After 48hrs, cells were passed into blasticidine containing media and antibiotic resistant clones were selected and tested for the expression of the repressor protein using immunofluorescence confocal microscopy.

Finally, we tried transfection of TRAMPC2 cells with FuGENE 6 transfection reagent (Roche) based on the protocol provided. Briefly, $2x10^5$ TRAMPC2 cells per well were seeded in a 6 well plate in 2ml of media. The

following day 5ul of FuGENE 6/well was diluted with 90ul of RPMI. After a 5-minute incubation at room temperature, 2ug of DNA/well was added to the FuGENE 6 mixture. To generate the tetracycline inducible cell lines TRAMPC2 cells were transfected with two different plasmids: the tet-inducible expression vector and the repressor vector. The ratio of the tet-inducible expression vector to repressor vector was 3:1 for transfection. After a brief vortex the mixture was incubated for 20 minutes at room temperature. This mixture was added drop-wise to the cells. After 48 hours of incubation the cells were passed into the antibiotic containing media to select for stably transfected cells. Zeocin (220ug/ml) and blasticidine (5ug/ml) are the antibiotic resistance genes on the tet-inducible expression vector and repressor vector, respectively.

Confocal Microscopy

TRAMPC1P3 cells (20000) were cultured in Lab-tek II tissue culture chambered cover glass (4 wells/slide) in 0.8ml of TRAMP media. The following day the media was removed and cells were fixed in 4% parafolmaldehyde for 15min at room temperature. Cell were washed with PBS 2 times for 5 min and then permeabilized with 0.5% Triton X-100 in PBS for 5 min. Then cells were blocked with 10% normal goat serum for 10min. Anti-Hsp70 (Stressgen) or anti-tet repressor (MoBiotech) (1ug each) or no antibody was added to each well and incubated for 30min. After 2 washes with PBS, Alexa fluor 488 goat anti-mouse IgG (Molecular Probes) was added and incubated for 30 min. After washing the secondary antibody cells were visualized using confocal microscope.

Identification of TRAMPC2/TR/cytokine clones that inducibly expressed the cytokine

Enzyme-linked immunosorbent assay (ELISA)

In order to identify TRAMPC2/TR/SLC and TRAMPC2/TR/GM-CSF clones that expressed high levels of SLC and GM-CSF respectively and had low background expression, we performed ELISA using kits from R and D systems. TRAMPC2/TR/SLC or TRAMPC2/TR/GM-CSF cells (1×10^5) were seeded in 12 well plates in duplicates in 1ml media. The following day media was replaced with fresh media with or without 2ug/ml of tetracycline and the cells were incubated for 24 hours. The assay was performed on the third day. Briefly, the wells of a 96 well plate (Costar EIA) were coated with 100ul of 4ug/ml of capture antibody and incubated overnight at room temperature. After washing the wells with wash buffer (0.05% Tween20 in PBS), the wells were blocked for 1 hour with 100ul of 1% BSA, 5% sucrose and 0.05%NaN_3 in PBS. Then the samples and the standards were added to the wells and incubated for 2 hours at room temperature. The wells were washed a few times with wash buffer before adding 100ul of horseradish peroxidase conjugated detection antibody (50ng/ml). After 2 hours of incubation the wells were washed, 100ul of 1/200 dilution of strepavidin horseradish peroxidase was added to each well and incubated for 20min. At the end of the incubation the wells were washed and 100ul of substrate solution (R and D systems) was added to each well. At the end of 20 min incubation 50ul of

2N sulfuric acid was added to each well and the optical density of each well was determined using a microplate reader set to 450nm.

Flow cytometry

We performed flow cytometry to identify TRAMPC2/TR/CD40L clones that expressed high levels of CD40L and had low background expression. TRAMPC2/TR/CD40L cells (100,000) were seeded in 12 well plates in duplicates in 1ml media. The following day the media was replaced with fresh media with or without 2ug/ml of tetracycline and the cells were incubated for 24 hours. The cells were harvested using trypsin, blocked with 5ul of goat serum and washed with wash buffer (1%goat serum in PBS solution). PE-conjugated CD40L antibody (1ug, eBiosciences) was added to each tube and incubated for 20min in dark at 4°C. Then cells were washed twice with wash buffer and analyzed with flow cytometer.

Methylation assay

To assess whether the CMV promoter had been silenced, we assessed the methylation status of the promoter using a DNA methylation kit from Zymo research. DNA was extracted from either $3X10^6$ cells or 15mg of tissue (prostate tumor) using GenomicPrep cells and tissue DNA isolation kit (Amersham Biosciences). Bisulfite conversion was performed using 20ug of DNA according to the manufacturer's protocol. Bisulfite-treated DNA (5ul) was prepared for hot-start PCR amplification, using a pair of primers complementary to a region of

CMV promoter not containing methylation sites (oligos 1) (forward: 5'-TAT TGT TAT TAT TAT GGT GAT GTG G; reverse: 5'-TTA CCC TAA AAA ATT TTA CAA CAT TA) or a pair of primers complementary to a region of the CMV promoter which contains methylation sites (oligos 2) (forward: 5'-TTA TCG TTA TTA TTA TGG TGA TGC G; reverse: 5'-GCC CTA AAA AAT TTT ACA GCA TTA T). Amplification conditions involved initial denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 2 minutes with a final elongation at 72°C for 10 minutes. An aliquot of PCR products (5ul) were run on an agarose gel and the DNA visualized using Gel Star (Lonza Bioscience).

GM-CSF ELISPOT

To estimate the frequency of TRAMPC2/TR/GM-CSF cells secreting mouse GM-CSF we performed ELISPOT assays using a kit from R and D systems. TRAMPC2/TR/GMCSF cells were placed in quadricate in the wells of a PVDF-bottom microplate in TRAMP media and incubated overnight at 37°C 5%CO_2 incubator. Tetracycline (2ug/ml) was added to two wells from four of each clone. The media was removed after overnight culture and assay was performed based on the provided protocol. Spots were quantified using a dissection microscope.

Detection of metastatic disease

To detect metastatic disease in mice with TRAMP tumors, different tissues (lymph nodes, lungs, pancreas) were harvested using sterile techniques. Tissues

were diced with scalpels into 1-2 mm^3 fragments, explanted into 6-well tissue culture dishes, allowed to attach for 5-10 min at 37°C and cultured in 0.5 ml/well of growth medium at 37°C in a humidified chamber. Growth medium was replaced every 3-4 days and cells from explanted outgrowths expanded for further analysis (155). In some cases prostate tumors were cultured using the same technique and cells from explanted outgrowths expanded for re-injection into the prostate gland.

Flow cytometric analysis of DNA content

Derived cell lines dissociated by trypsinization were fixed in cold 70% ethanol and stained with 0.5 ml propidium iodide (PI, Sigma) and RNAse A (100 U/ml) for minimum of 30min to overnight at 4°C and analyzed on a BD FACScan cytometer. DNA histograms were created using MODFIT software. Mouse splenocytes were used as an internal diploid control by spiking the samples before fixation and PI staining to ensure identical exposure to PI for both sample cells and splenocytes.

Percoll gradient centrifugation

Stock Percoll solution (GE healthcare biosciences) was prepared by mixing 9ml Percoll and 1ml 10X PBS. Then 70%, 40% and 20% Percoll was prepared from stock solution using 1X PBS. Single cell suspension of TILs and spleens were prepared as described before and resuspended in 70% Percoll. A volume of 2ml was placed at the bottom of 15ml conical tube and then layered

with 2ml 40% Percoll and 2ml of 20% Percoll and 2ml PBS on the top. Tubes were centrifuged for 45min at 1200g at 20°C. Interface cells were removed separately, washed, counted and used for further analysis.

Statistical analysis

All statistical analysis in these studies was performed using the PRISM 4 statistical software package (GraphPad Software, Inc.). This package was used to perform Student's *t*-test. The software provided P values that were statistically significant, which was when a P value was <0.05. Any significant differences were noted in the text and figures corresponding to the data.

CHAPTER IV

RESULTS

To investigate the phenotype and functional status of dendritic cells infiltrating TRAMPC2 prostate tumor *in vivo* and after *ex vivo* culture.

The function of a DC is highly influenced by its level of maturation. Immature DCs are capable of antigen uptake and processing but cannot present antigen to T cells and activate them (165). The following experiments were designed to evaluate the maturation status of DCs infiltrating TRAMP tumors. Initially TRAMP tumor infiltrating dendritic cells (TIDCs) were phenotyped using monoclonal antibodies and analyzed by flow cytometer. Since the majority of the TRAMP TIDCs were immature, we tried to identify the cell type responsible for induction of such a phenotype *in vitro* using co-culture technique. In order to study the functional status of TRAMP TIDCs we tried to purify these cells using common methods like adherence, Nycodenz and percoll gradient centrifugation.

Phenotypic analysis of dendritic cells in the TRAMPC2 tumor microenvironment

Dendritic cells play a very essential role in the generation of both innate and adaptive T cell-mediated immune responses *in vivo*; as noted previously, acquisition of APC function is a maturational process that manifests itself, at least

in part, by the up-regulation of distinct cell surface molecules essential for T cell activation. Therefore, we assessed whether intra-tumoral DCs expressed these essential molecules within the prostate TME. Figure 2 shows the phenotypic analysis of splenic and tumor infiltrating DCs using a CD11c (a marker for DCs) gate (panel Aa). The majority of DCs infiltrating TRAMPC2 tumors fail to express class II antigen (IA^b) and B7.2 co-stimulatory molecules. More than 44% of DCs from spleen of normal mice express IA^b (B-a), whereas 34% of DCs from spleen of tumor bearing mice (B-b) and only 16% of tumor infiltrating dendritic cells (TIDCs) express IA^b (B-c). Similarly, the percentage of B7.2 positive DCs was reduced from 44% in normal spleen (B-a) to 23% for intratumoral DCs (B-c). CD40 expression was also impaired by the TME as 36% of normal splenic DCs expressed this molecule (B-d) but only 16% of the tumor bearing splenic (B-e) and TIDCs expressed CD40 (B-f). Most of the TIDCs appeared to be myeloid DCs because most of these cells did not express CD8α (B-g, h and i). Class I antigen ($H2D^b$) expression did not seem to be suppressed by the TME as almost all normal spleen DCs as well as tumor bearing spleen DCs and TIDCs expressed this antigen at equivalent levels (Fig. 2, B-d, e and f). The co-expression of B7.2 and IA^b by $CD11c^+$ cells was reduced from 28% in normal spleen (B-a) to 4% in TILs (B-c). For clarity, the data presented in panel A and B has been plotted in as bar graphs in panels C and D. Surprisingly, the expression of B7.1 co-stimulatory molecule on TIDCs was not inhibited by the TRAMPC2 TME (panel C). The expression of the chemokine receptor CCR7 was also suppressed in the TME relative to normal spleen. In contrast, DC

expression of PDL2 was elevated on intra-tumoral DCs relative to normal splenic DCs (10 versus 3%, respectively). Thus, these data suggest that intra-tumoral DCs are immature because they fail to express a number of cell surface markers associated with DC maturation.

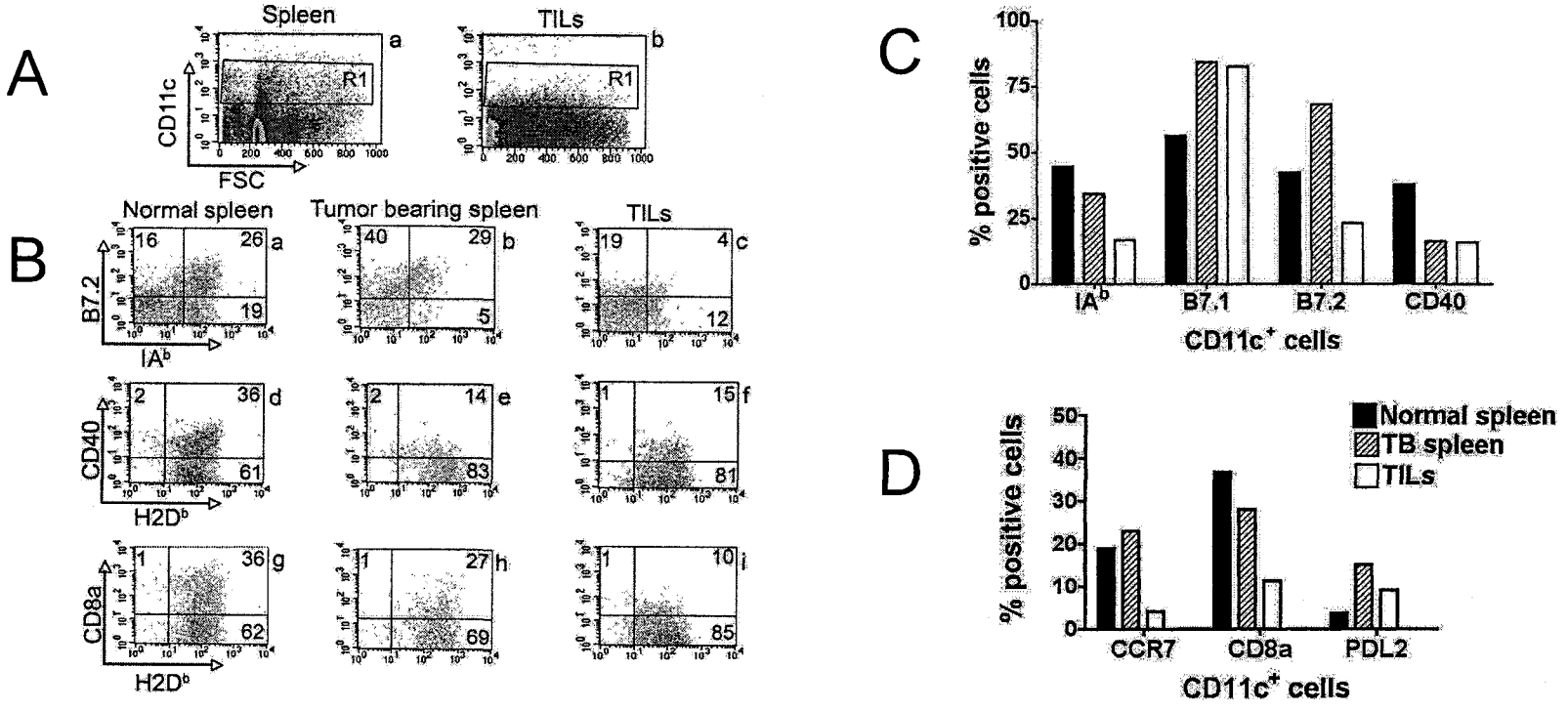


Figure 2. Phenotypic characterization of TRAMPC2 tumor infiltrating DCs.
Mice were transplanted with TRAMPC2 tum or cells and tumors were excised about 30 days later when tumor reached about 1cm in diameter. Single cell suspension from normal and tumor bearing (TB) spleens and TILs were prepared. Normal, TB spleen cells and TILs were stained with indicated mAbs and evaluated by 4-color flow cytometry. A: A single color analysis (forward scatter vs. log fluorescent intensity) of $CD11c^+$ DCs of normal spleen (a) and TILs isolated from TRAMPC2 tumors (R1 shows the $CD11c^+$ gate) (b). B: Gated $CD11c^+$ cells from each population were assessed for expression of B7.2, IA^b, $H2D^b$, CD40 and CD8a molecules. The values in each quadrant indicate the percentage of cells in the $CD11c^+$ gate that stained with the indicated mAbs. Panel C and D: Further phenotypic characterization of TIDCs displayed as bar graphs. These results are representative of 3 independent experiments.

Enrichment of dendritic cells through adherence

Although TRAMP TIDCs are phenotypically immature, the functional status of these cells is unknown. Purification of TIDCs is an important step for performing functional assays. We therefore tested the efficacy of several different methods to isolate intra-tumoral DCs. The most common procedure to enrich for DCs is based on the selective adherence of DCs to tissue culture plates (166). Dendritic cells will initially adhere to plastic but unlike macrophages, become detached after incubation overnight. Panels in Figure 3 show the gates for splenic and tumor infiltrating $CD11c^+$ DCs. This method resulted in about two-fold enrichment of splenic DCs from 5% to 9% as shown in Figure 3, panels a and b. However the same method only enriched TIDCs from 4% in the unfractionated sample (3-c) to 5% in the non-adherent cells (3-d).

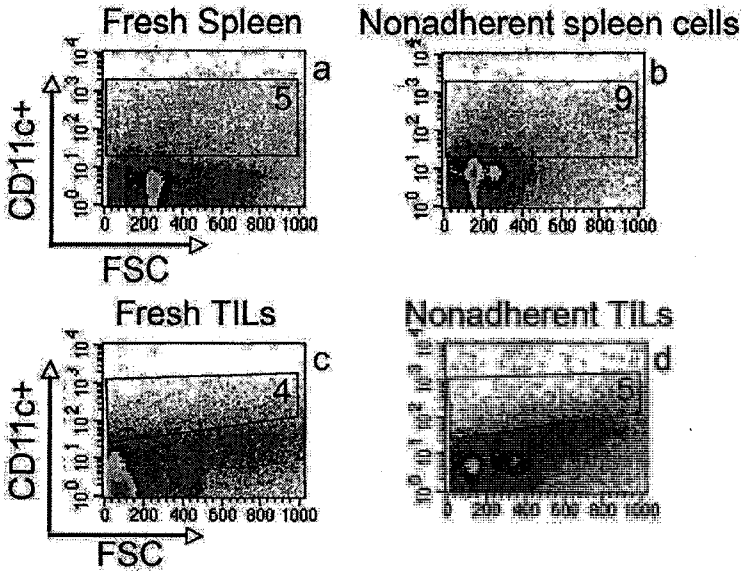


Figure 3. Enrichment of DCs through adherence.
Single cell suspension of normal spleens and TILs isolated form TRAMPC2 tumors were prepared as discussed in the Material and Methods. Fresh spleen cells and TILs were stained with CD11c antibody for comparison (panels a and c). Cells were cultured and allowed to adhere for about 4-5 hours. Then non-adherent (lymphocytes) cells were decanted and cells were cultured overnight with fresh media. The following day non-adherent cells isolated from spleen (panel b) and TRAMPC2 tumors (panel d) were harvested and stained with CD11c mAb and analyzed by flow cytometry. The numbers in each box shows the percentage of $CD11c^+$ DCs in each population.

Enrichment of dendritic cells using Nycodenz gradient centrifugation

In view of the failure of adherence to enrich for DCs, we next evaluated another commonly used procedure to purify DCs, namely, Nycodenz gradient centrifugation. Single cell suspensions of spleen and TRAMPC2 tumor were therefore prepared and subjected to Nycodenz gradient centrifugation as explained in the Material and Methods. The cells in the interface were stained with CD11c antibody to identify DCs. Figure 4 (panels a and b) shows that using this procedure DCs isolated from spleens of B16FL tumor bearing mice were enriched from 34% to 82%. Performing the same procedure did not result in enrichment of TIDCs (panels c, d).

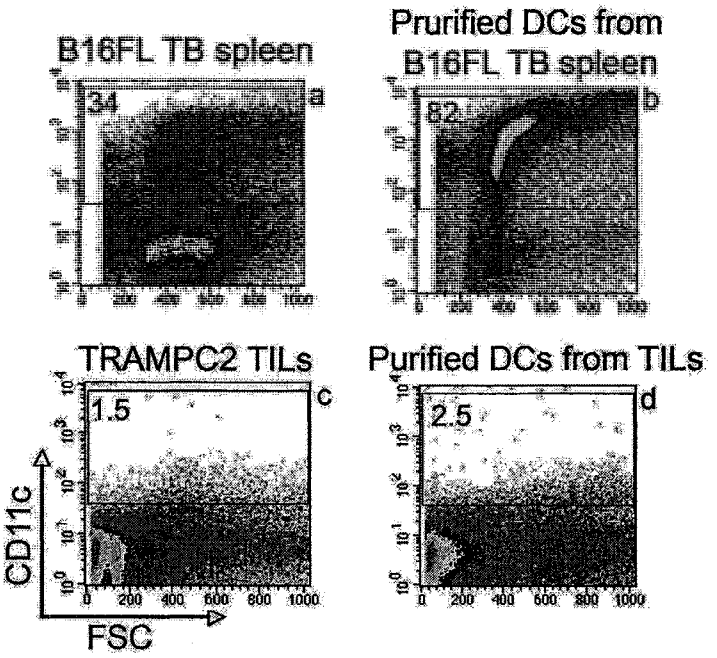


Figure 4. Nycodenz gradient centrifugation enriched for splenic but not tumor-infiltrating dendritic cells.
A single color analysis (forward side scatter vs. log fluorescent intensity) of $CD11c^+$ cells of B16Fl spleen (a and b) and TILs isolated from TRAMPC2 tumors (c and d) is shown. Single cell suspensions were prepared of spleen from B16Fl bearing mice and TRAMPC2 tumor. Cells were stained with anti-CD11c mAb to determine the percentage of DCs before enrichment in spleen (a) and in TILs (c). Then, the remaining cells were centrifuged on a Nycodenz gradient. The interface cells were harvested and stained with the same mAb to determine the purity of DCs in B16Fl spleen (b) and TILs (d). The values in each panel show the percentage of $CD11c^+$ DCs in each population.

Enrichment of dnedritic cells using Percoll gradient centrifugation

As a final approach to enrich for intratumoral DCs, we evaluated discontinuous Percoll gradient centrifugation that has been used to enrich leukocytes. Spleen cells and TILs were re-suspended in 70% Percoll, topped with layers of 40%, 20% and 0% Percoll in PBS and then centrifuged. As shown in Figure 5 panels A and B almost all of the leukocytes (other than T cells) from the spleen floated to the 70/40 interface. T cells were mostly found in the 40/20 interface and were enriched from 20% in un-fractionated spleen cells to 40% after Percoll gradient. There were almost no cells in the 20/0 interface in the spleen. On the other hand, most of the leukocytes of TIL fraction including DCs were found in the 40/20 interface. Although DCs were slightly enriched in the 40/20 interface of TILs, this fraction still contains major contamination by other cells like macrophages ($CD11b^+$) and granulocytes ($Gr\text{-}1^+$). There were almost no cells in the 70/40 and 20/0 interface in TILs. This experiment clearly shows that leukocytes and specifically DCs isolated from the TME behave abnormally relative to their splenic equivalents.

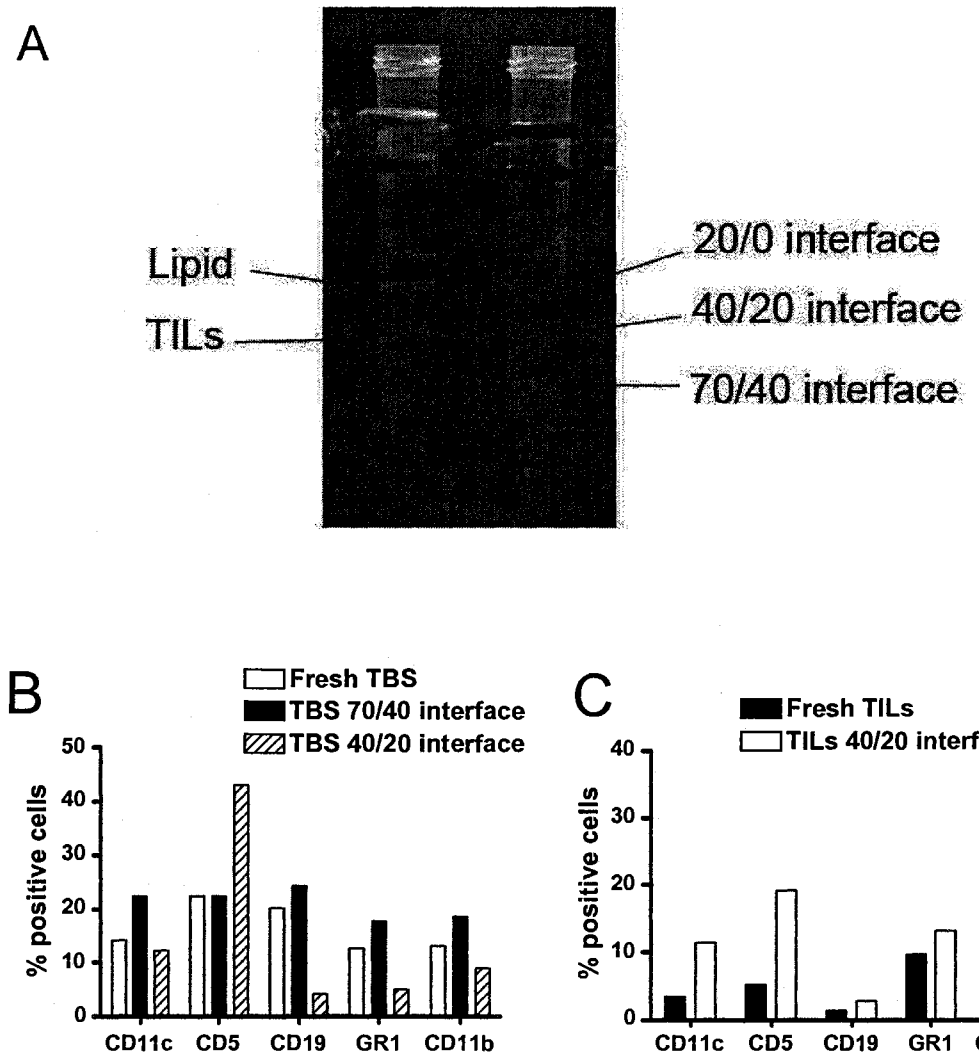


Figure 5. Efficacy of dendritic cell enrichment by discontinuous Percoll gradient centrifugation.
Single cell suspensions were prepared from spleen of tumor bearing mice and TRAMPC2 tumors. Cells were resuspended in 70% Percoll and placed at the bottom of the tubes and layered on top with 40%, 20% and 0% of Percoll in PBS and centrifuged. Some cells were stained with indicated antibodies before fractionation of TILs and splenocytes for comparison. A: distribution pattern of spleen cells and TILs in a discontinuous Percoll gradient. B and C: percentage of different cell types found in the indicated interfaces when spleen cells (panel B) or TILs (panel C) were fractionated on Percoll.

TRAMPC2 cells mediate dendritic cells immature phenotype *in vitro*

Dendritic cell maturation is essential for the generation of an effective anti-tumor immune response. As demonstrated above and in agreement with findings by other groups, we showed that DCs isolated from TRAMPC2 tumors display an immature phenotype that may contribute to the ability of this tumor to grow progressively in the immunocompetent host. Bell *et al.* observed numbers of immature DCs in the tumor microenvironment of breast carcinoma to be much higher than in normal breast epithelium, suggesting increased homing and infiltration (101). High expression of HLA-DR class II (human leukocyte antigen-DR class II) and low expression of B7.1 and B7.2 molecules on TIDCs in colon carcinoma sections have been described (98). Gabrilovich *et al.* showed that mature DCs from 3T3 fibroblast tumor bearing mice are not effective antigen presenting cells (167). Furthermore, We showed that DCs in the TRAMP TME do not behave as expected as we tried to purify them using different methods. Condensation of cytoplasm and shrinkage of the cell, membrane blebbing, chromatin condensation and nuclear fragmentation that are typical features of apoptosis has been reported when DCs were co-cultured with different tumor cells (168). Although causes of defects in the morphology and function of DCs are unknown it has been shown that granulocytes from cancer patients co-purify with low density peripheral blood mononuclear cells (PBMCs) on a density gradient rather than sediment, as expected, to the bottom of the gradient (169). Since we were unable to purify DCs from TRAMP tumors we performed a series

of co-culture studies in an attempt to identify the cell type responsible for induction of this immature phenotype displayed by DCs infiltrating TRAMPC2 tumors. Purified DCs from spleen of B16FL tumor bearing mice were stained fresh with specified antibodies and after overnight culture alone, with 5X or 10X TRAMPC2 cells. As shown in Figure 6, 14% of fresh DCs were IA^b positive. The percentage of IA^b positive cells increased to 38% after overnight culture. The addition of TRAMPC2 cells to the culture seemed to inhibit the expression of IA^b *in vitro* as the presence of 5X and 10X TRAMPC2 caused the IA^b expression to drop to 29 and 19% respectively. Interestingly the reduction of IA^b expression seemed to be dose response effect in the presence of tumor cells. Fresh DCs were essentially devoid of B7.1 expression but overnight culture resulted in a strong up-regulation of this co-stimulatory molecule. This induction was suppressed in the presence of TRAMPC2 tumor cells in a dose-dependent fashion. A similar pattern of induction and inhibition was observed for B7.2 and CD40 with the exception that basal levels of these molecules were higher than B7.1. As shown in panel B when a CD11c gate was defined the co-expression of IA^b/B7.1 and IA^b/B7.2 increased when DCs were cultured (from 8 and 18% to 25 and 44%, respectively). Co-culture of DCs with TRAMPC2 cells prevented the up-regulation of these molecules to some extent compared to DCs cultured alone. The co-expression of $H2D^b$/CD40 molecules increased only about 5% when DCs were cultured alone; however the presence of TRAMPC2 cells inhibited the up-regulation of these molecules. These studies suggest that the immature phenotype of DCs in the TRAMP TME could be induced at least

partially by tumor cells.

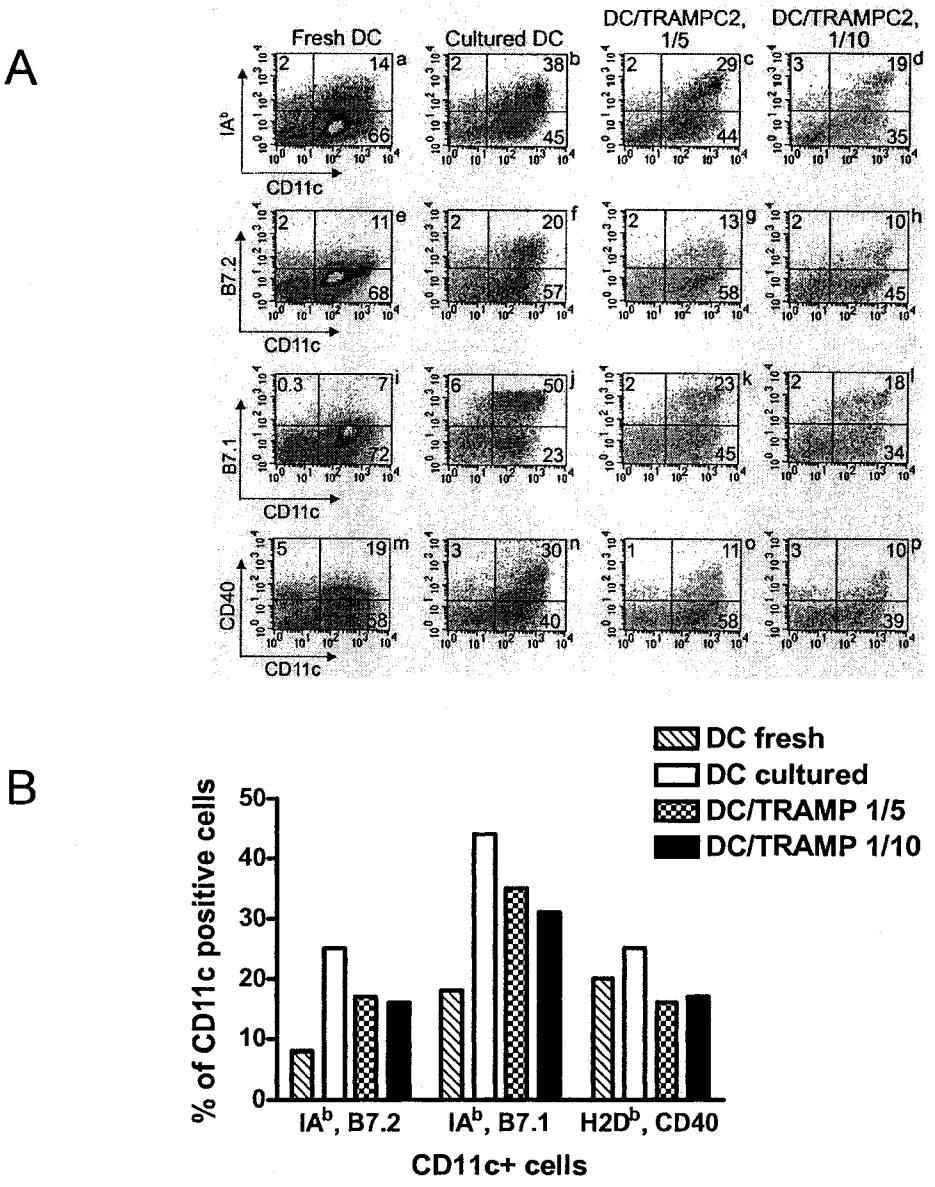


Figure 6. TRAMPC2 cells partially induce the immature phenotype of tumor infiltrating DCs.

Dendritic cells were purified from spleen of B16Fl tumor bearing mice by Nycodenz gradient centrifugation. Purified DCs were stained fresh with indicated antibodies for comparison (a, e, I, m). Purified DCs (X) were cultured alone (b, f, g, n), with 5X (c, g, k, o) and 10X (d, h, I, p) TRAMPC2 cells overnight. The following day DCs were harvested and stained with indicated antibodies and analyzed using 4-color flow cytometry. Panel A: The co-expression of CD11c and IA^b (pane a-d), B7.1 (panels e-h), B7.2 (i-l) or CD40 (m-p). Panel B: A $CD11c^+$ gate was defined and the co-expression of IA^b/B7.1; IA^b/B7.2 and $H2D^b$/CD40 by DCs in each condition was plotted. These are the results of 3 independent experiments.

Immature phenotype of dendritic cells induced by TRAMPC2 cells is contact mediated *in vitro*

TRAMPC2 cells prevent DCs to express maturation markers *in vitro*. This effect could be via direct contact or through the release of soluble factors such as IL-10 (170) and TGF-β (171). In order to address this issue, DCs were cultured with or without TRAMPC2 cells in tissue culture dishes with transwells to prevent cell-cell contact. As shown in Figure 7 the inhibitory effect of TRAMPC2 cells on DCs seemed to be mainly contact mediated. For example, no more than 11% of fresh DCs (panel a) were IA^b positive and that increased to 71% when DCs were cultured alone in media (b). When 10X TRAMPC2 cells (panels c) were added to the cultured DCs, the expression of class II antigen was inhibited as the percentage of IA^b positive DCs was only 44%. However, when TRAMPC2 cells were not in contact with DCs (panel d) the expression of IA^b was 72% that was similar to DCs cultured alone. Similarly, fresh $CD11c^+$ cells were 3% $B7.1^+$ (panel e) and this increased to 55% when $CD11c^+$ cells were cultured alone (panel f). TRAMPC2 cells inhibited the expression of B7.1 molecule by DCs (panel g) as only 20% of cultured $CD11c^+$ cells in the presence of TRAMPC2 cells were B7.1 positive, however, 34% of DCs cultured with TRAMPC2 cells in transwell expressed B7.1. Similar results were obtained for the expression of B7.2 molecules by DCs and the expression of this molecule was inhibited when DCs were in contact with TRAMPC2 cells (panels j, k and i). However, CD40 expression seemed to be inhibited through soluble factors as its expression by

DCs was inhibited whether DCs were in contact with TRAMPC2 cells or not (panel o and p). As shown in Figure 7 panel B the percentage of IA^b/B7.1, IA^b/B7.2 and $H2D^b$/CD40 molecules co-expression decreased compared to DCs cultured alone when DCs were cultured in contact with TRAMPC2 cells. The percentage of IA^b/B7.1, IA^b/B7.2 molecules co-expression did not decrease when DCs were cultured in transwells separated from TRAMPC2 cells. The co-expression of $H2D^b$/CD40 on the other hand decreased both when DCs were in contact or not with TRAMPC2 cells compared to DCs cultured alone which means that the expression of CD40 molecules by DCs could be inhibited by TRAMPC2 cells through soluble factors.

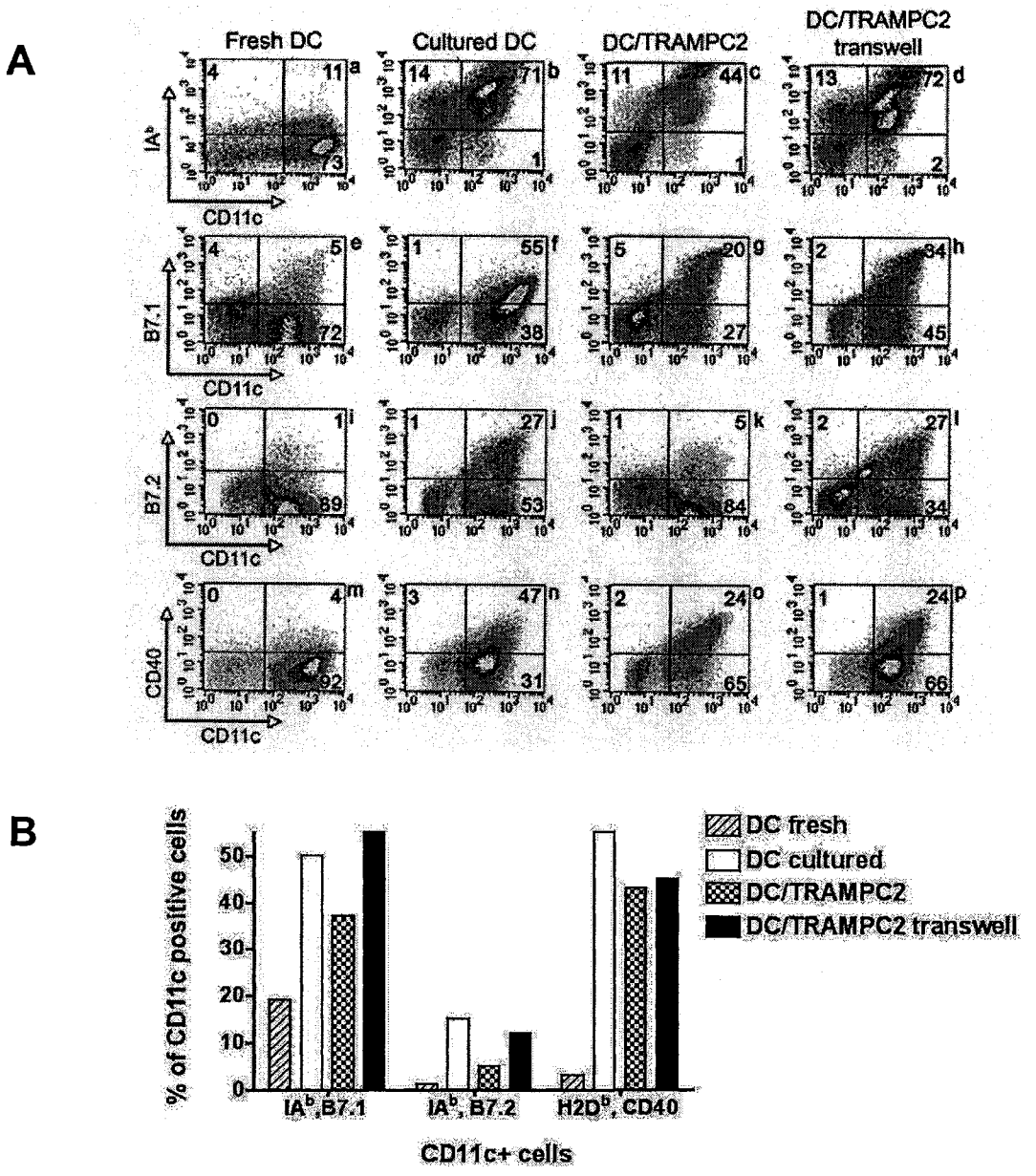


Figure 7. TRAMPC2 cells prevented maturation of DCs *in vitro* mainly through direct contact.

A: Dendritic cells were purified from spleen of B16FI tumor mice by Nycodenz gradient centrifuge. Fresh DCs were stained with indicated antibodies to establish basal levels of expression (a, e, I, m). $2X10^6$ (X) DCs were placed in the upper chamber of transwells. 5X (c, g, k, o) or 10X (d, h, l, p) or 0 (b, f, g, n) TRAMPC2 cells were added to the lower chamber in TRAMP media and cultured in 37°C, 5% CO_2 incubator overnight. Dendritic cells were harvested the following day, stained with indicated antibodies and analyzed by four-color flow cytometer.

B: A $CD11c^+$ gate was defined and the co-expression of IA^b/B7.1, IA^b/B7.2 or $H2D^b$/CD40 molecules by DCs in each condition has been plotted. These are the results of 2 independent experiments.

Granulocytes do not induce the immature phenotype of dendritic cells

Although TRAMPC2 cells can inhibit culture-induced DC maturation, large numbers of tumor cells are required for this effect *in vitro*. This raises questions about the physiological relevance of these observations. As noted previously, TRAMPC2 tumors are infiltrated by large numbers of myeloid cells that could express immunosuppressive factors. For example, granulocytes are a major cell type found infiltrating TRAMPC2 tumors (130) and have been shown to have immunosuppressive activity (172). Therefore, we used an *in vitro* system to investigate whether Gr-1$^+$ cells play any role in the inhibition of DC maturation in the TRAMPC2 TME. In this experiment we cultured DCs purified from spleen of B16FL tumor bearing mice alone, with purified Gr-1$^+$ cells or with both Gr-1$^+$ and TRAMPC2 cells. As shown in Figure 8 (panel A) 27% of fresh DCs (a) expressed IA^b but when they were cultured in just media class II induction was evident (panel b). The addition of GR-1$^+$ cells inhibited this maturation modestly (panel c), whereas, IA^b expression was more dramatically inhibited in the presence of both tumor and Gr-1$^+$ cells (panel d). The addition of Gr-1$^+$ cells did not appear to inhibit the expression of any of the other maturation markers used in this study (panels e-p). As shown in panel B, co-expression of IA^b/B7.1; IA^b/B7.2 and H2D^b/CD40 was down-regulated by Gr-1$^+$ cells but the mixture of Gr-1$^+$ and TRAMPC2 cells caused down-regulation of IA^b, B7.1 molecules compared to the co-expression of these molecules by DCs cultured alone.

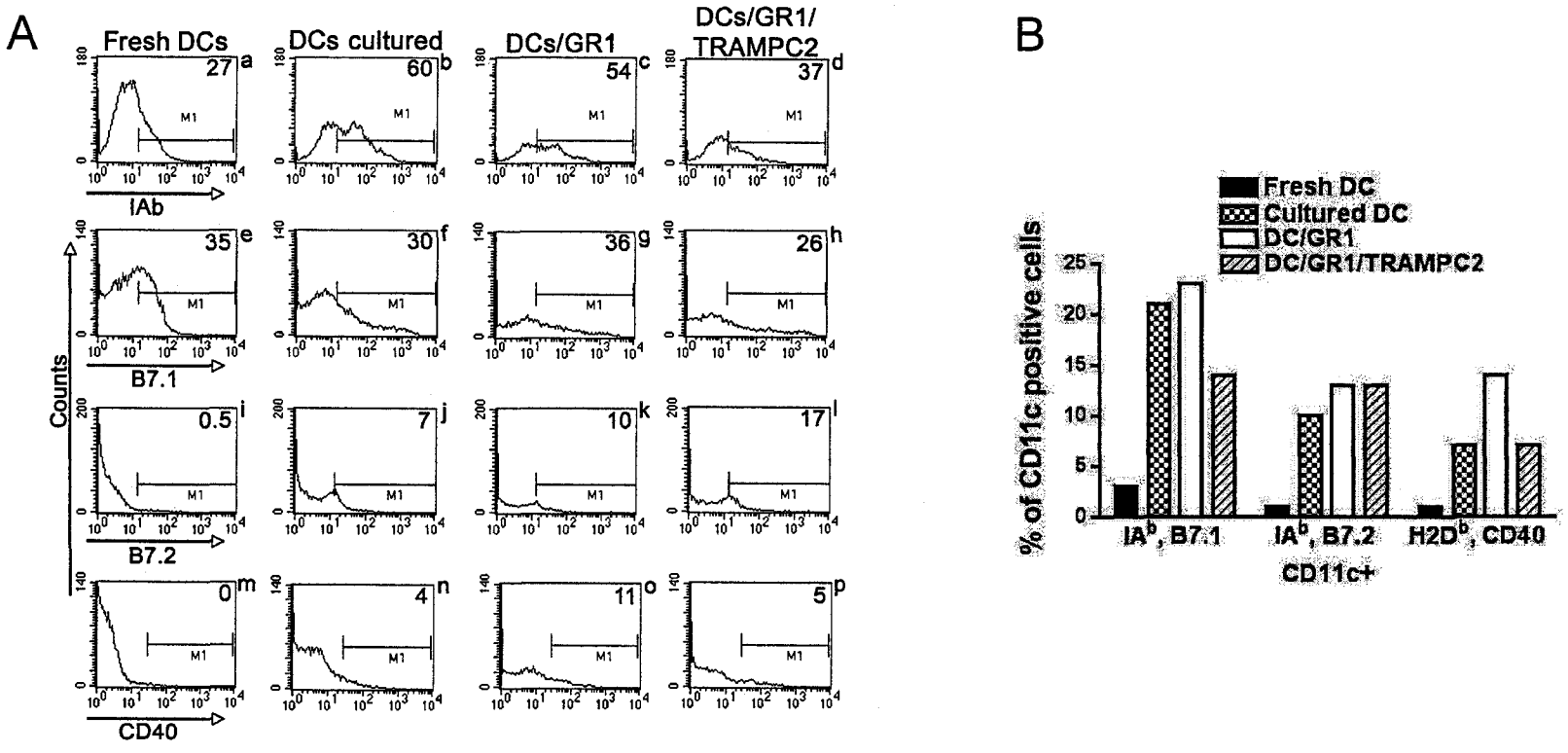


Figure 8. Granulocytes did not induce immature phenotype of DCs *in vitro*.
A: Dendritic cells were purified from spleen of B16Fl tumor mice by Nycodenz gradient centrifuge. Fresh DCs were stained with indicated antibodies for comparison (a, e, i, m). Granulocytes were purified from spleen of B16Fl tumor bearing mice using magnetic beads. $2X10^6$ (X) DCs were cultured alone overnight (b, f, j, n). Dendritic cells were also cultured with 5X Gr-1^+ cells overnight (c, g, k, o) or both with 5X Gr-1^+ and 2.5X TRAMPC2 cells (d, h, l, p). Dendritic cells were harvested the following day, stained with indicated antibodies and analyzed by four-color flow-cytometer. Panel B: A $CD11c^+$ gate was defined and the co-expression of IA^b/B7.1; IA^b/B7.2 and $H2D^b$/CD40 by DCs in each condition was plotted. These are the results of 2 independent experiments.

The data presented here demonstrate that DCs infiltrating TRAMPC2 tumors have an immature phenotype defined by low levels of expression of class II antigens and co-stimulatory molecules. Furthermore we showed that TIDCs do not behave normally in a variety of fractionation protocols and this prevented purification for functional analysis. Based on studies utilizing an *in vitro* co-culture protocol, TRAMPC2 cells appeared primarily responsible for the immature phenotype displayed by TIDCs. Moreover, this activity appeared to be contact-dependent because expression of MHC class II and co-stimulatory molecules was not inhibited by TRAMPC2 cells when tumor cells were separated from DCs in transwell plates.

Evaluation of SLC, GMCSF and CD40L to induce dendritic cell maturation *in vitro* using TRAMPC2 cell lines expressing these chemokine/cytokines

It has been shown that TIDCs in several transplantable and transgenic mouse tumor models uniformly had an immature phenotype and were refractory to activation with a combination of microbial and T cell-derived stimuli (137). Therefore experiments were designed to first establish that maturation of purified immature DCs isolated from spleen of B16FL tumor bearing mice can be induced using bacterial stimuli and then using co-culture method to study whether maturation of the immature DCs can be induced in the presence of TRAMPC2 tumor cells. Furthermore, we wanted to study whether the cytokines that are

known to induce DC maturation were able to do so when expressed by TRAMPC2 cells and whether these cytokines were able to reverse the inhibitory effect of TRAMPC2 tumor cells on DCs. Therefore, we generated prostate cancer cell lines that inducibly could express specific cytokine and chemokines and these cell lines were used to perform these studies.

Induction of dendritic cell maturation by LPS

Lipopolysaccharide (LPS) is the major component of gram-negative bacterial cell wall and is known to induce DC maturation both *in vivo* and *in vitro* (134, 173). As mentioned in the background section SLC, GM-CSF and specifically CD40L are also known to cause up-regulation of DC maturation markers. Lipopolysaccharide-induced DC maturation would be considered the maximum achievable under our experimental conditions. Immature DCs were therefore cultured with or without (2 μg/ml) LPS overnight. Lipopolysaccharide caused a very modest up-regulation of IA^b from 23 to 36% (Fig. 9, panels a, b), but a more pronounced induction of B7 family members of B7.1 (Fig. 9, panels c, d), and B7.2 (Fig. 9 panels e, f). A significant induction was also observed for CD40 expression (Fig 9, panels g, h).

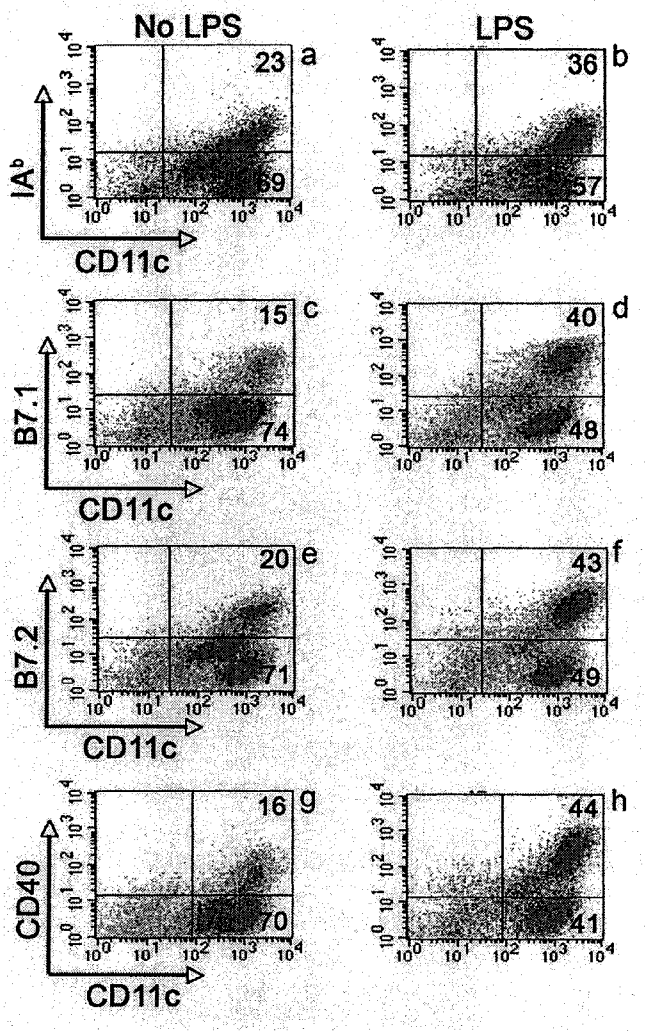


Figure 9. Induction of DC maturation by lipopolysaccaride *in vitro*.
Immature DCs purified from spleen of B16FL tumor bearing mice were cultured alone (panels a, c, e, and g) or with 2 μg/ml LPS (panels b, d, f and h) overnight. Dendritic cells were harvested the following day, stained using optimal concentration of indicated antibodies for 20 min at 4°C and analyzed by 4-color flow cytometer. A forward and side scattered gate has been set up. Panels a through h show flow cytometric dot plots demonstrating co-expression of the indicated molecules on $CD11c^+$ cells.

We showed here that DCs infiltrating TRAMP tumors were phenotypically immature and majority of them did not express the molecules required for T cell activation. The ultimate goal of this study is to induce maturation of DCs using different cytokines and chemokines *in vivo*. Therefore, first we wanted to determine whether immature DCs could be activated by LPS in the presence of TRAMP tumor cells. Purified immature DCs were cultured alone, with 2ug/ml of LPS and with 10 times more TRAMPC2 cells along with 2ug/ml LPS overnight (Fig. 10). Dendritic cells were harvested and stained with indicated monoclonal antibodies and analyzed by flow cytometer. Cultured DCs were 47% IA^b positive while addition of LPS caused up-regulation of IA^b to 74%. In the presence of both TRAMPC2 cells and LPS immature DCs still up-regulated IA^b (51%) although not to the extent that LPS had caused in the absence of TRAMPC2 tumor cells (Fig. 10, panels a, b and c). While only 1% of cultured DCs expressed B7.1, DCs cultured with LPS were 7% B7.1 positive and addition of TRAMPC2 cells caused higher percentage of DCs to express B7.1 (20%) (panels d, e and f). The same results were observed with B7.2 and CD40 molecules. These data showed that although TRAMPC2 cells prevented DC maturation but the immature DCs that were exposed to tumor cells were still capable of up-regulation of class II antigens and co-stimulatory molecules.

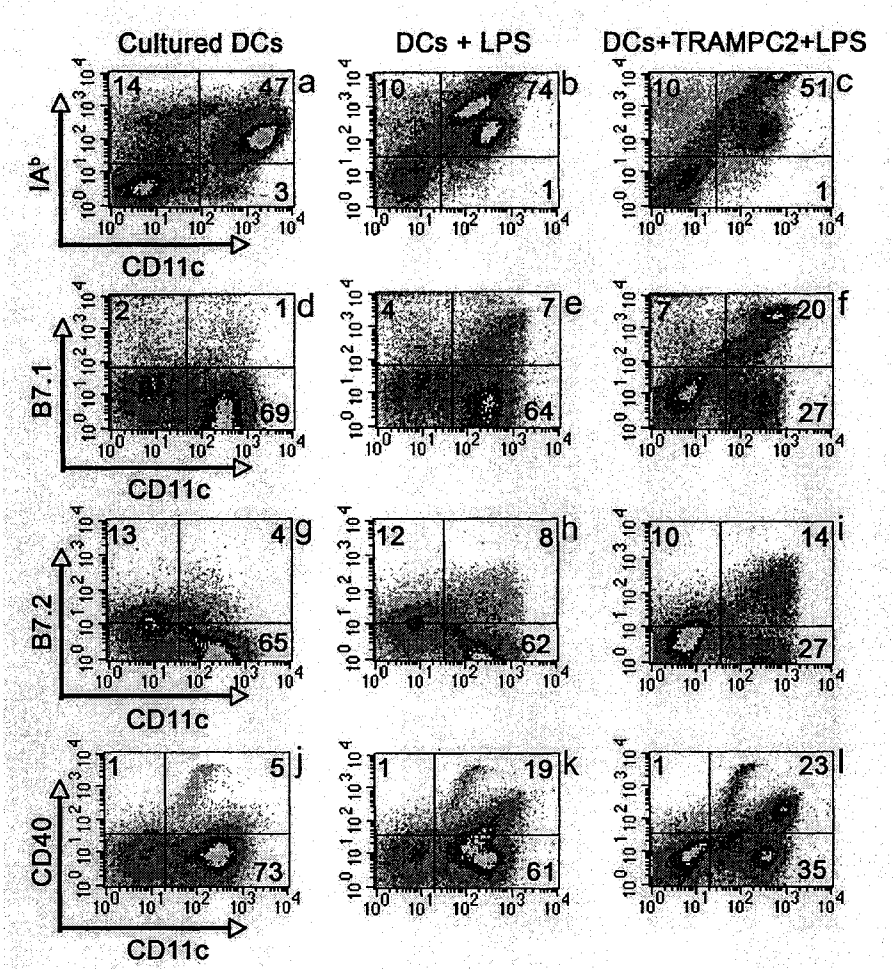
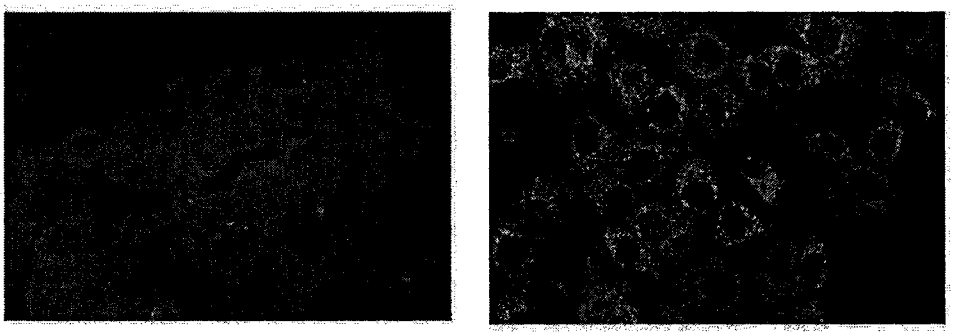


Figure 10. Immature DCs in contact with TRAMPC2 cells can still undergo maturation.
Purified immature DCs (X) were cultured alone, with LPS (2ug/ml) and with LPS (2ug/ml) in the presence of TRAMPC2 (10X) overnight. Dendritic cells were harvested the following day, stained using optimal concentration of indicated antibodies for 20 min at 4°C and analyzed by 4-color flow cytometer. Panels a through h show flow cytometric dot plots demonstrating co-expression of the indicated molecules on $CD11c^+$ cells.

Generation and characterization of stably transfected TRAMPC1P3 cells with regulated transgene expression

To generate TRAMP cell lines with regulated expression of cytokines of interest, TRAMPC1P3 cells were first transfected with the repressor protein by electroporation. After about 3 weeks of selection in blasticidine, antibiotic resistant clones were screened for the expression of the repressor protein by confocal microscopy using a monoclonal antibody against the tet repressor protein (TR) protein. Figure 11A shows that ~80% of the cells displayed constitutive expression of TR protein in the cytosol and nucleus of TRAMPC1P3 cells. In Figure 11B a mAb against heat shock protein 70 was used as positive control.



A B

Figure 11. Expression of tetracycline repressor protein in transfected TRAMPC1P3 tumor cells.
TRAMPC1P3 tumor cells were transfected with the pcDNA6/TR vector by electroporation. Cells were subsequently stained with mAbs against either TR (A) or murine heat shock protein 70 (B).

In the course of maintaining transfected cells for further transfection with different expression vectors, we observed that with continuous passage, expression of the repressor protein diminished (data not presented). We therefore considered the possibility that TR protein expression may have reflected episomal activity of the vector. To minimize this possibility, we performed several additional transfection experiments using linearized pcDNA6/TR DNA. However, transfection with linearized vector did not prevent diminished TR protein expression during maintenance of this cell line *in vitro* (data not shown). One way to explain our inability to detect repressor protein in transfected clones after *in vitro* passages was very low expression of the repressor protein. This could happen through different mechanisms like losing the gene or silencing the gene's promoter.

Generation and characterization of stably transfected TRAMPC1P3 cells with the repressor and SLC expression vectors

Previous experiments showed that when TRAMP cells were transfected with the repressor vector the repressor protein could be detected by confocal microscopy using a mAb against TR. However, after a few *in vitro* passages it seemed that these cells did not have high enough concentration of the repressor protein to be detected by confocal microscopy. However the amount of the repressor protein could be enough to prevent the expression of the gene of interest from the expression vector. As an alternative approach we tried to co-

transfect TRAMPC1P3 tumor cells with TR vector and SLC expression vector (pcDNA4/TO/SLC) and screen for the expression of SLC protein in the absence and presence of tetracycline. Therefore, we co-transfected TRAMPC1P3 cells with the repressor vector and SLC expression vector using Fugene 6 that showed higher transfection efficiency for TRAMP cells than electroporation. Cells were passed into media containing antibiotics (zeocin and blasticidine) and antibiotic resistant clones were maintained for 3-4 weeks before they were tested for the expression of SLC. We performed an ELISA using the supernatant of TRAMPC1P3/TR/SLC cells when they were cultured with or without 2ug/ml of tetracycline for 24hr. Figure 12 shows two different TRAMPC1P3/TR/SLC clones that were tested for inducible expression of SLC. Clone #3 when it was initially tested (passage 1) showed a relatively high SLC expression with tetracycline (about 180pg/ml) and low background (30pg/ml). After three additional *in vitro* passages (passage 4) SLC expression by clones #3 was lower (131 pg/ml) and had higher background (58 pg/ml). When Clone #4 was tested for the first time it expressed 130 pg/ml of SLC when induced with tetracycline and had no detectable background expression. However, clone #4 expressed about 84 pg/ml of SLC without tetracycline induction at passage 4 and expressed 90 pg/ml when induced. It seemed that *in vitro* passages resulted in increased "leakiness" of the clones amd lowered expression levels following induction by tetracycline (Fig. 12).

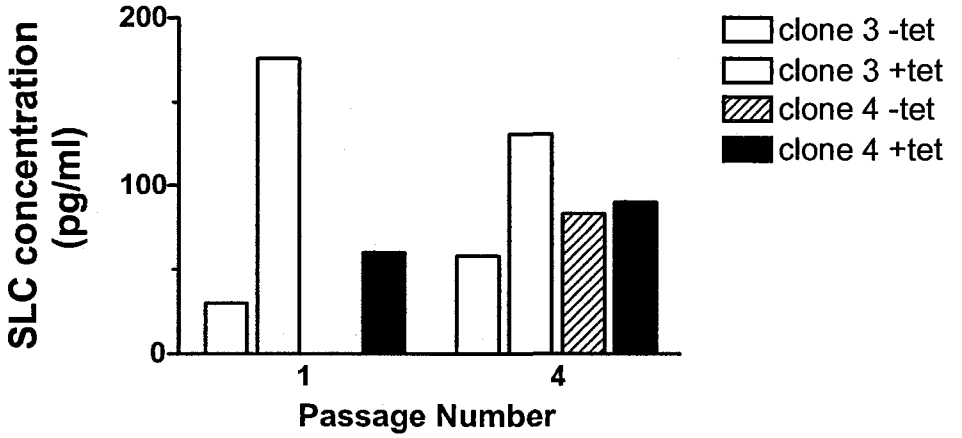


Figure 12. Expansion of TRAMPC1P3/TR/SLC cells *in vitro* is associated with loss of tet-inducible expression of SLC.
TRAMPC1P3 cells were co-transfected with TR and SLC expression vectors and two tet-inducible clones expanded *in vitro* in the presence of antibiotics. The concentration of SLC in the supernatant was determined after 24 hr of induction in the presence of 1ug/ml of tetracycline. After 4 passages the same clones became "leaky" and were less inducible by tetracycline.

Next we sub-cloned TRAMPC1P3/TR/SLC clones that showed some level of SLC expression in order to isolate cells that expressed higher levels of SLC with low background expression. In another series of transfection experiments, two representative tet-inducible clones were sub-cloned by limiting dilution in an attempt to isolate stable transfectants. Two out of 62 sub-clones had negligible background and relatively high induction level; however, after 3 passages the induction level diminished and then became undetectable after several additional passages (Fig. 13 and data not presented). TRAMPC1P3/TR/SLC 2-19 (Fig. 13), a sub-clone derived from clone 2, had no background expression with an induced level of SLC expression of 206 pg/ml when it was first tested. After three additional passages, the same cell line had higher background (64 pg/ml) and lower tetracycline inducible level of SLC expression (123 pg/ml). When TRAMPC1P3/TR/SLC clones were tested again after a few more passages, no SLC expression was detected (data not shown).

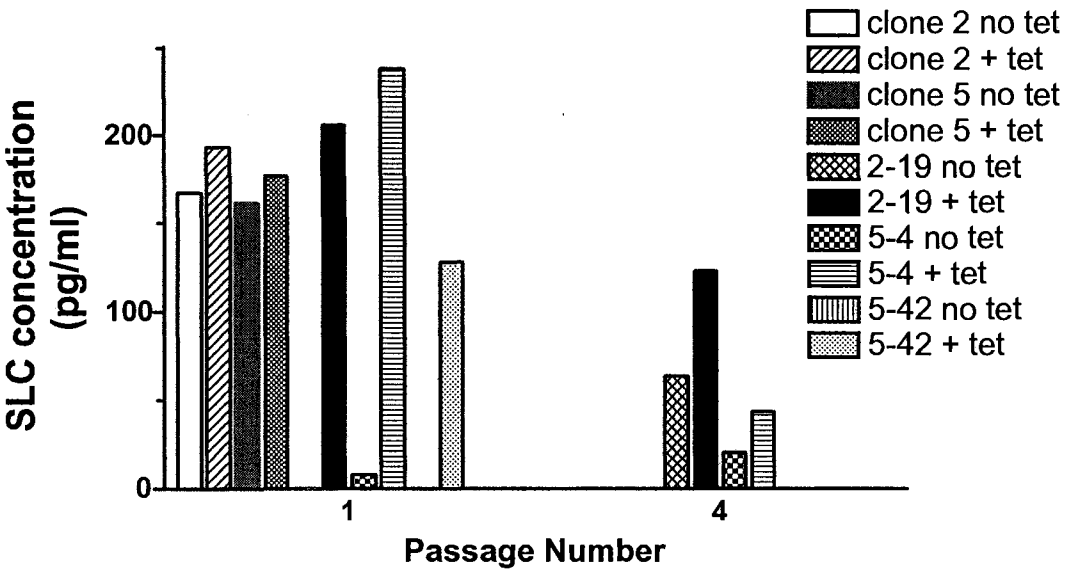


Figure 13. Loss of tet-inducible expression in subclones derived from TRAMPC1P3/TR/SLC cells.
TRAMPC1P3 cells were transfected with TR and SLC expression vectors and two positive clones (2 and 5) were identified by ELISA. After several passages, both lines showed high background expression and possessed minimal induction with tet. These lines were sub-cloned by limiting dilution method and then screened for SLC induction. Three positive sub-clones derived from either clone 2 (2-19) or clone 5 (5-4, 5-42) that had low background and high initial SLC induction. After three additional passages, tet induction either diminished or became undetectable.

The fact that all TRAMPC1P3/TR/SLC clones shown in Figure 13 lost the expression of SLC after passages *in vitro* may reflect loss of the SLC gene. To assess this possibility, PCR primers were designed that could amplify the transfected SLC gene but not endogenous SLC gene. Genomic DNA was extracted from TRAMPC1P3/TR/SLC clones 2-19 and 5-4 (Fig. 13) and transfected SLC gene was amplified by PCR. This analysis indicated that the transfected SLC gene was not detectable by PCR in either of the two previously positive sub-clones (Fig. 14).

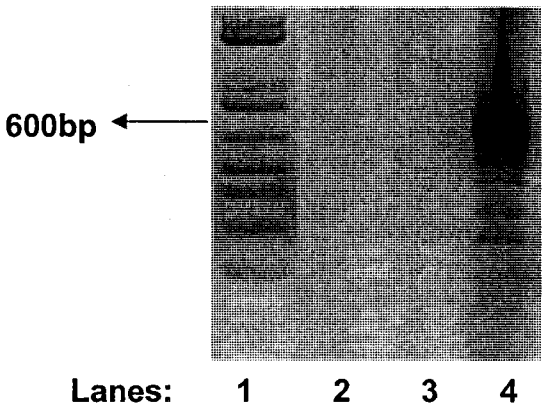


Figure 14. Loss of the SLC gene in transfected TRAMPC1P3 cells during expansion *in vitro*.
Genomic DNA was extracted from TRAMPC1P3/TR/SLC sub-clones (2-19 and 5-4) that originally had displayed tet-inducible SLC production. The transfected SLC gene was amplified by PCR using primers specific for the SLC gene and tetracycline operator sequences present in the vector. Using these primers, no product was detected using parental TRAMPC1P3 DNA (data not shown). Lane 1 contains molecular weight markers and lanes 2 and 3 were loaded with DNA from sub-clones 2-19 and 5-4. PcDNA4/TO/SLC expression vector was used as positive control for the PCR reaction (lane4).

Isolation and characterization of transfected tetracycline inducible TRAMPC2 clones with high SLC production *in vitro*

The difficulty in isolating stable transfectants of TRAMPC1P3 cells expressing inducible cytokines prompted us to evaluate TRAMPC2 tumor cells. The TRAMPC2 cell line was derived from the same TRAMP tumor as TRAMPC1 and is very similar to TRAMPC1 (153). There is one major difference between these two TRAMP lines; TRAMPC2 is metastatic when injected subcutaneously, whereas, TRAMPC1 is locally invasive but not metastatic (115, 174). We therefore transfected both TRAMPC2 and TRAMPC1P3 cell lines with TR and pcDNA4/TO/SLC expression vectors using Fugene 6 transfection reagent. Transfected cells then were selected in antibiotics (zeocin and blasticidine) for about 3 weeks. The antibiotic resistant clones were expanded and tested for inducible expression of SLC by ELISA. Six TRAMPC2/TR/SLC clones were isolated that possessed low constitutive expression of chemokine and an impressive 12- to 60-fold induction of SLC in the presence of tetracycline (Fig. 15, panel A). Two of the TRAMPC1P3/TR/SLC clones showed a 5-6-fold induction of SLC similar to what was observed in the previous experiments (data not presented). To test if TRAMPC2/TR/SLC lines would maintain tet-inducible expression, cells were re-tested after 3 and 6 additional passages. Three of clones still had a remarkable induction with tetracycline (Fig. 15 panel B). These clones also had a very low constitutive level of SLC expression (Fig. 15, note scale change). As shown in Figure 15 clone #6 had low background (100 pg/ml)

and 1800pg/ml tet induced level of SLC. After 3 and even 8 in vitro passages clone #6 still showed low level of background but the level of tet induced SLC decreased to about 700 pg/ml after 3 passages and to 500pg/ml after 8 passages. Clones #4 and 5 had high level of SLC induction at passage 1 (1900 and 1000 pg/ml, respectively). At passage 3 the level of inducible expression of clones #4 and 5 dropped to 1300 and 600 pg/ml. But after 8 passages the level of tet inducible expression of clones #4 and 5 increased to 3500 and 3200 pg/ml (panel C). These two clones maintained very low background through all the passages. Although these clones had variable levels of SLC induction they maintained the expression of SLC after *in vitro* passages.

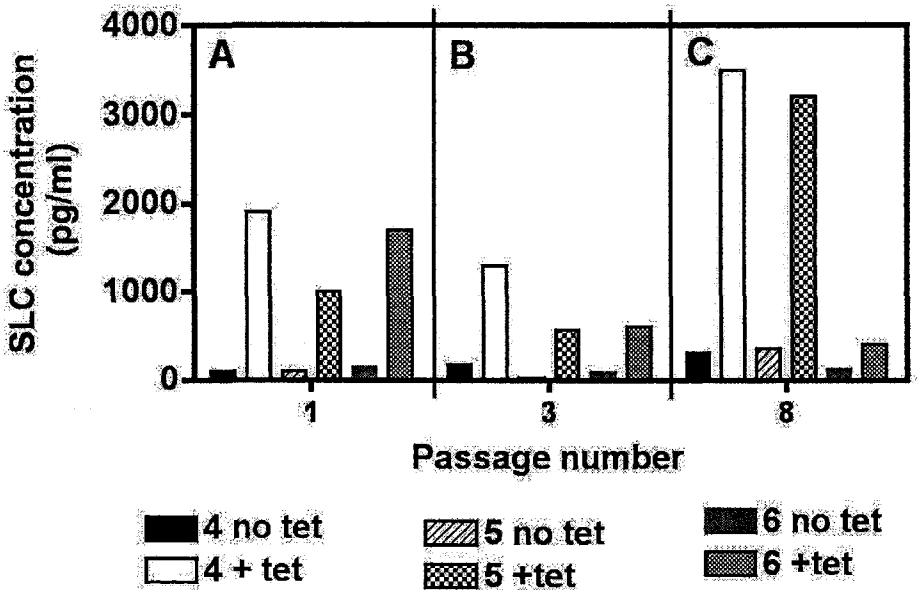


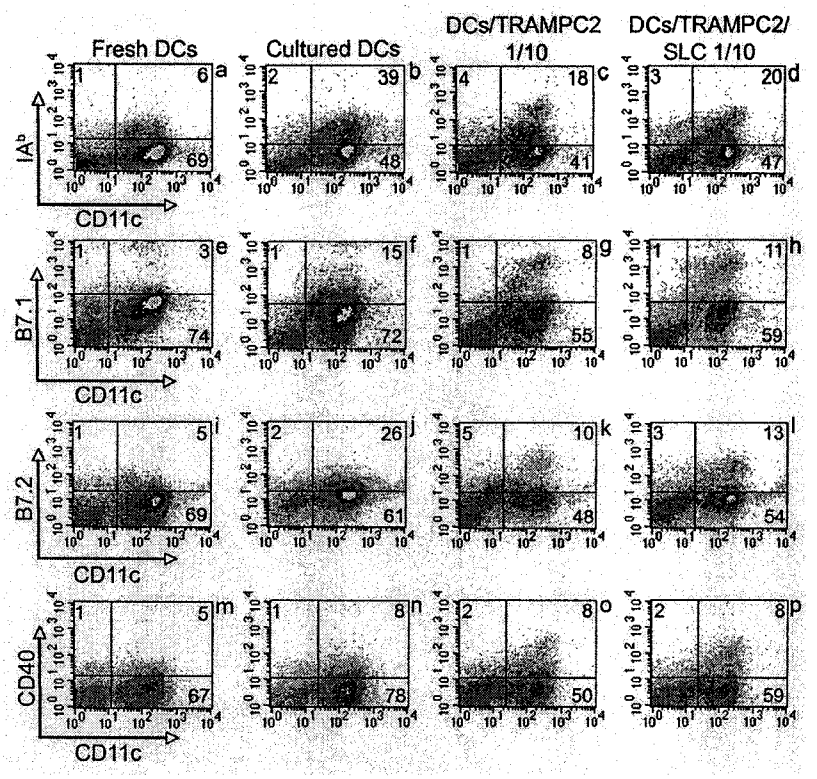
Figure 15. TRAMPC2/TR/SLC cells maintain regulated expression of SLC *in vitro*.
TRAMPC2 cells were transfected with the repressor and SLC expression vectors using Fugene 6 transfection reagent. Two days after transfection cells were passed into antibiotics containing media. A: After about three weeks of selection in antibiotics the antibiotics resistant clones (TRAMPC2/TR/SLC #4, 5 and 6) were tested for SLC expression with and without 2ug/ml of tetracycline by ELISA. ELISA was performed after 3 (B) and 8 (C) passages to test whether these clones maintained the inducible expression of the transgene.

Secondary lymphoid tissue chemokine secreted by TRAMPC2 cells does not induce DC maturation *in vitro*

Having established TRAMPC2/TR/SLC cell lines we next studied the impact of SLC on DC maturation when secreted by transfected TRAMPC2 cells *in vitro*. In this experiment DCs (X number) purified from the spleen of B16FL tumor bearing mice were cultured alone, with 10X TRAMPC2 and 10X TRAMPC2/TR/SLC (in the presence of 2ug/ml tetracycline) overnight and stained with the indicated antibodies the next day. Fresh DCs were also stained for comparison. The concentration of CCL21 in the lymph node has been estimated to be 11-12 ug/ml, and is likely considerably higher within the T cell zones (175). The concentration of SLC in the media of cultured DCs was about 2ug/ml. As shown in Figure 16 only 6% of fresh $CD11c^+$ cells expressed IA^b (panel a) whereas 39% of cultured $CD11c^+$ cells express IA^b (panel b). TRAMPC2 cells prevented IA^b expression by DCs to about 18% (panel c) that was only increased to 20% when TRAMPC2 cells expressed SLC (panel d). This was still lower than IA^b expression when DCs were cultured alone (39%). While only 3% of fresh DCs expressed B7.1 (panel e), 15% of cultured DCs expressed this molecule (panel f). When DCs were cultured with 10 times more TRAMPC2 cells the percentage of B7.1 positive DCs dropped to 8% (panel g), which increased slightly (11%) when SLC was expressed byTRAMPC2 cells (panel h). Fresh DCs were 5% B7.2 positive (panel i) and cultured DCs were 26% B7.2 positive (j). The percentage of B7.2 positive DCs decreased when DCs were cultured with

TRAMPC2 cells to 10% (k) and it increased very modestly 13% when TRAMPC2 cells expressed SLC (panel l). The co-expression of IA^b and co-stimulatory molecules increased after over night culture compared to fresh DCs and TRAMPC2 cells caused down-regulation of these molecules as shown in Figure 16B. Expression of SLC in the culture by TRAMPC2 cells caused a very moderate increase in the co-expression of IA^b/B7.1 and IA^b/B7.2 by DCs although this up-regulation did not overcome the inhibitory effect of TRAMPC2 cells on DCs. Although $H2D^b$/CD40 co-expression decreased when DCs were cultured with TRAMPC2 cells compared to DCs cultured alone, SLC did not cause an increase in the co-expression of these molecules. Although SLC has been shown to induce DC maturation but it is not as potent as GM-CSF and CD40L.These results show that SLC could not reverse the inhibitory effect of TRAMPC2 tumor cells on DC maturation and did not cause maturation of immature DCs in the presence of tumor cells.

A



B

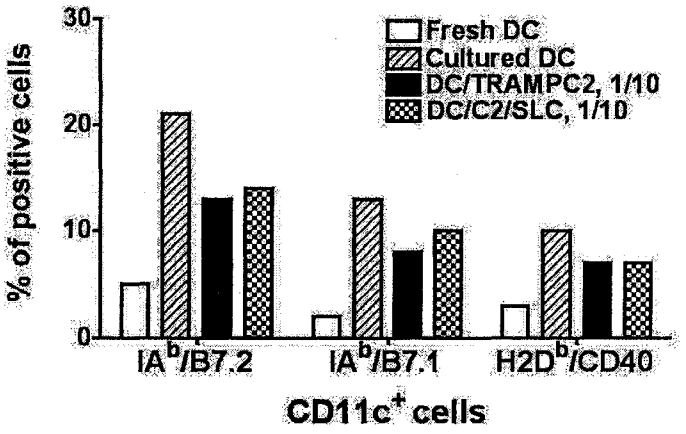


Figure 16. Secondary lymphoid tissue chemokine does not induce DC maturation.

Panel A: DCs were purified from spleen of B16fl tumor bearing mice using Nycodenz gradient centrifugation. Fresh DCs were stained for comparison (a, e, i and m). Dendritic cells were cultured alone (b, f, j and n), with 10X TRAMPC2 (c, g, k and o) or 10X TRAMPC2/SLC (d, h, I and p). Cultured DCs were harvested, stained with indicated antibodies the following day and analyzed with 4-color flow cytometer. Panel B: A CD11c gate was defined fro the data presented in panel A and the percentage of CD11c+ cells that co-express IA^b/B7.1; IA^b/B7.2 and $H2D^b$/CD40 in each population (Fresh DCs, cultured DCs, DCs/TRAMPC2 and DCs/TRAMPC2/SLC) has been plotted.

Isolation and characterization of transfected TRAMPC2 clones with high and stable production of CD40L inducible with tetracycline *in vitro*

In view of the fact that TRAMPC2 tumors are infiltrated with immature DCs we used the tetracycline inducible system to express CD40L in TRAMPC2 tumor microenvironment. It has been shown that CD40L in a potent inducer of DC maturation (65). We transfected TRAMPC2 cells with the repressor vector and the pcDNA/TO/CD40L expression vector. The transfected cells were selected with two antibiotics (zeocin and blasticidine). Antibiotic resistant clones were then isolated, expanded and tested for induction of CD40L expression after incubation with tetracycline. We isolated a single TRAMPC2/TR/CD40L clone that when induced with tetracycline more than 50% of the cells expressed CD40L compared to 11% background expression of CD40L without induction (Fig. 17).

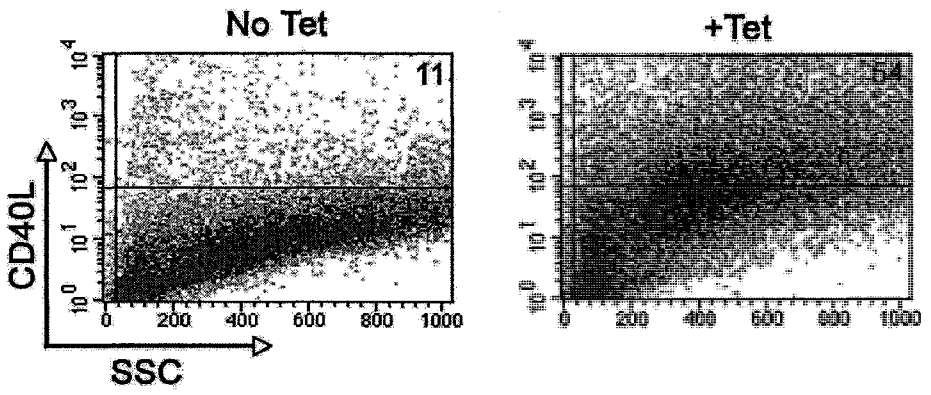


Figure 17. Tet-inducible regulation of CD40-ligand expression in transfected TRAMPC2 cells.
TRAMPC2 cells were transfected with repressor and pcDNA4/TO/CD40L expression vector. Transfected cells were selected in antibiotics (zeocin and blasticidine) containing media. Cells from an antibiotic resistant clone were seeded into 12 well plates (10^6/ml) in duplicate. The next day media was replaced with 1ml fresh media with or without 2ug/ml of tetracycline. Cells were harvested the following day, blocked with normal goat serum and then stained with a FITC-conjugated mouse anti-CD154 (CD40L) or FITC-conjugated hamster IgG (negative control). Following a final wash step, cells were analyzed by flow cytometry. Quadrants were drawn based on the isotype control. Plotted are CD40L staining versus side scatter (SSC) on cells incubated in the absence (left panel) or presence of tetracycline (right panel).

CD40L secreted by TRAMPC2 cells reversed prevention of dnedritic cell maturation induced by tumor cells *in vitro*

Activation of the CD40 receptors is one of the critical signals that allow the full maturation of DCs into potent APCs (165). Dendritic cell maturation signals may be delivered by an antigen-specific $CD4^+$ T cell when it recognizes antigen on the surface of the DC and signals the DC through CD40-CD40L interaction (176). Dendritic cell maturation may also be induced by exposure to cytokines (TNF-α, IL-1β) (177-179), bacterial components (LPS) (180), CpG containing DNA (181, 182) or double-stranded RNA (183). In addition, the T-cell-mediated maturation of DC may be mimicked by artificial CD40 triggering through anti-CD40 antibodies (184) or CD40L transfected cells (65). Therefore we studied whether CD40L expressed by TRAMPC2 cells can overcome the suppressor effect of TRAMPC2 cells on DCs *in vitro*. Purified immature DCs were cultured alone, with TRAMPC2 cells or stably transfected TRAMPC2/TR/CD40L cells (in the presence of tetracycline) and stained with the indicated mAbs the following day (Fig. 18). Fresh DCs were also stained for comparison. Fresh DCs were 8% IA^b positive (panel a) that increased to 29% when DCs were cultured (panel b). The presence of TRAMPC2 induced a reduction of IA^b positive DCs to 12% (panel c) and this was not considerably increased by CD40L expression on TRAMPC2 (panel d). TRAMPC2 cells reduced B7.1 and CD40 expression to 4 and 3%, respectively (panels g and o), whereas, 7 and 9% of DCs were positive for these molecules when cultured in media (panels f and n). The expression of

B7.1 and CD40 molecules by DCs was moderately up-regulated when DCs were cultured with TRAMPC2/TR/CD40L (10 and 12% respectively, panels h and p). While 2% of fresh DCs were B7.2 positive (panel i), cultured DCs were 13% B7.2 positive (panel j). TRAMPC2 cells decreased the number of B7.2 positive DCs to 8% (panel k) and expression of CD40L by TRAMPC2 caused up-regulation of this molecule to 14% (panel l). In Figure 18, panel B a CD11c gate was defined and the co-expression of IA^b/B7.1, IA^b/B7.2 and $H2D^b$/CD40 by DCs in each condition was studied. The co-expression of IA^b/B7.1 and IA^b/B7.2 increased after over night culture of DCs compared to fresh DCs. The co-expression of IA^b/B7.1 and IA^b/B7.2 was decreased by addition of TRAMPC2 cells to the culture. CD40L expression by TRAMPC2 cells caused an increase in the co-expression of IA^b/B7.2 and IA^b/B7.1. The level of IA^b/B7.1 expression by DCs in the presence of TRAMPC2/TR/CD40L was as high as the cultured DCs. TRAMPC2 cells caused down-regulation of $H2D^b$/CD40 co-expression compared to cultured DCs however, expression of CD40L by TRAMPC2 cells caused up-regulation of these molecules but not even to the same level as cultured DCs. Therefore CD40L that has been shown to induce DC maturation, when expressed by TRAMPC2 cells could reverse the inhibitory effect of TRAMPC2 cells. Although CD40L was not very potent inducer of DC maturation when secreted from TRAMPC2 cells but prevention of inhibitory effect of TRAMPC2 cells can be enough to induce sufficient immune response to inhibit tumor growth.

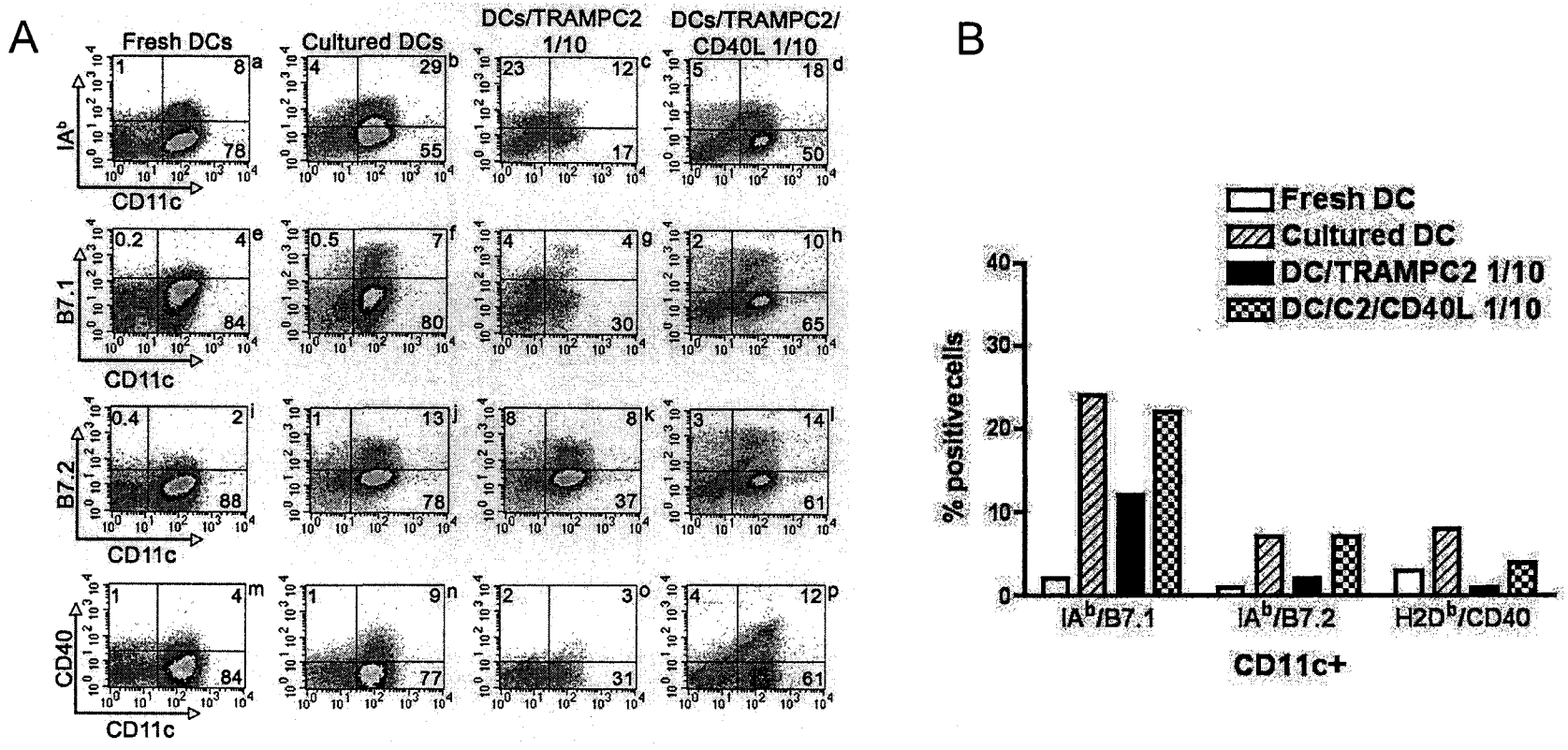


Figure 18. Inhibition of DC maturation by TRAMPC2 cells is reversed when CD40L expressed by TRAMPC2 cells *in vitro*. Panel A: DCs were purified from spleen of B16FL tumor bearing mice using Nycodenz gradient centrifugation. Fresh DCs were stained for comparison (a, e, i and m). DCs were cultured alone (b, f, j and n), with 10X TRAMPC2 (c, g, k and o) or 10X TRAMPC2/CD40L (d, h, l and p). Cultured DCs were harvested, stained with indicated antibodies the following day and analyzed with 4-color flow cytometer. Panel B: A CD11c gate was defined for data presented in panel A and the co-expression of IA^b/B7.1, IA^b/B7.2 and $H2D^b$/CD40 by DCs in each condition (Fresh DCs, cultured DCs, DCs/TRAMPC2 and DCs/TRAMPC2/CD40L) was plotted.

Isolation and characterization of transfected TRAMPC2 clones with high and stable and inducible production of GM-CSF *in vitro*

Expression of GM-CSF has been shown to stimulate the recruitment of antigen-presenting cells (DCs and macrophages) to the tumor site, suggesting the involvement of GM-CSF in the augmentation of tumor-antigen presentation (76). Moreover, GM-CSF induces the maturation of these DCs (128). In humans immature DCs require exposure to GM-CSF to undergo differentiation into mature antigen presenting cells (118). We speculated that murine DCs may also require exposure to this chemokine before maturation can be induced with CD40L and IFN-γ. Therefore, to examine the role of GM-CSF in DC maturation particularly within the TME, we co-transfected TRAMPC2 cells with the repressor and pcDNA4/TO/GM-CSF vectors. Antibiotic resistance clones were isolated and tested for induction of GM-CSF expression after incubation with tetracycline. Figure 19 illustrates a typical experiment where three isolated clones demonstrate tet-induced production of GM-CSF.

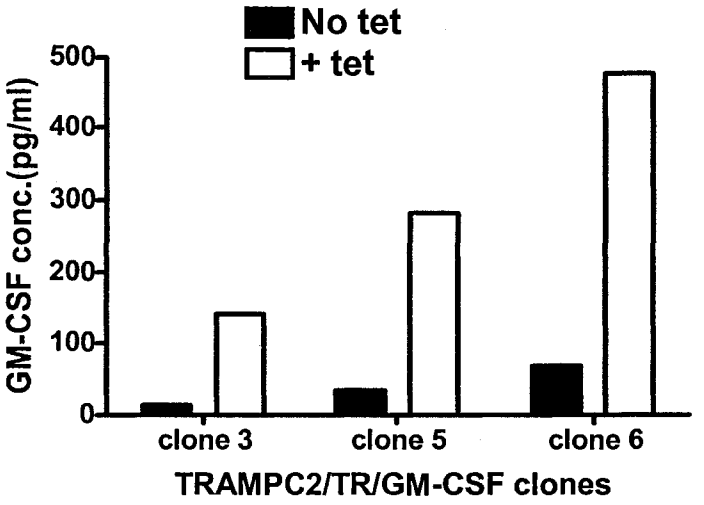


Figure 19. Tetracycline-regulated production of GM-CSF in TRAMPC2 tumor cells co-transfected with repressor and GM-CSF vectors.
TRAMPC2 lines were co-transfected with TR vector and pcDNA4/TO/GM-CSF expression vector. Antibiotic resistant clones were isolated and expanded *in vitro*. Cells from each clone were then seeded into 12 well plates in duplicate. The next day media was replaced with 1 ml fresh media with or without 2ug/ml of tetracycline. Supernatants were harvested the following day and tested by ELISA for GM-CSF expression.

Granulocyte macrophage-colony stimulating factor secreted by TRAMPC2 cells reversed down-regulation of dendritic cell maturation markers induced by tumor cells *in vitro*

Immature DCs enlarge, express more MHC, co-stimulatory and adhesion molecules when they are cultured with GM-CSF (185), characteristics of mature DCs. Therefore, we cultured purified immature DCs with TRAMPC2/TR/GM-CSF (in the presence of 2ug/ml of tetracycline) to study whether GM-CSF can induce DC maturation in the presence of TRAMPC2 (Fig. 20). As demonstrated in Figure 20, 10% of fresh DCs (panel a) and 25% of cultured DCs (panel b) expressed IA^b. When TRAMPC2 cells were added to the culture, the percentage of IA^b positive DCs decreased to 6% (panel c) that was partially reversed when cultured with transfected TRAMPC2 cells (panel d). Similar to class II antigens, the expression B7.1, B7.2 and CD40 increased when fresh DCs were cultured *in vitro* relative fresh DCs [compare fresh DCs (panels e, i and m) to cultured DCs (panels f, j and n)]. TRAMPC2 cells in culture caused a reduction of B7.1, B7.2 and CD40 expression by DCs (panels g, k and o). When GM-CSF was secreted from TRAMPC2/GM-CSF cells the percentage of B7.1, B7.2 or CD40 positive DCs increased almost to the same level as cultured DCs [compare cultured DCs (panels f, j and n) to DCs cultured with TRAMPC2/GM-CSF (panels h, l and p)]. Therefore, It seems that GM-CSF when expressed by TRAMPC2 cells can reverse the inhibitory effect of TRAMPC2 cells on DC maturation *in vitro* but it could not induce further maturation of DCs. Figure 20 panel B shows that when

gated on $CD11c^+$ cells the co-expression of IA^b/B7.1, IA^b/B7.2 and $H2D^b$/CD40 decreased when DCs were cultured with TRAMPC2 cells compared to cultured DCs. Although expression of GM-CSF by TRAMPC2 cells did not cause markedly high expression of the class II antigens and co-stimulatory molecules but at least in some cases it partially and in some other cases completely overcame the inhibitory effect of TRAMPC2 cells. Although the effect of GM-CSF was not very striking *in vitro* this could be very important in the initiation of a potent immune response *in vivo*.

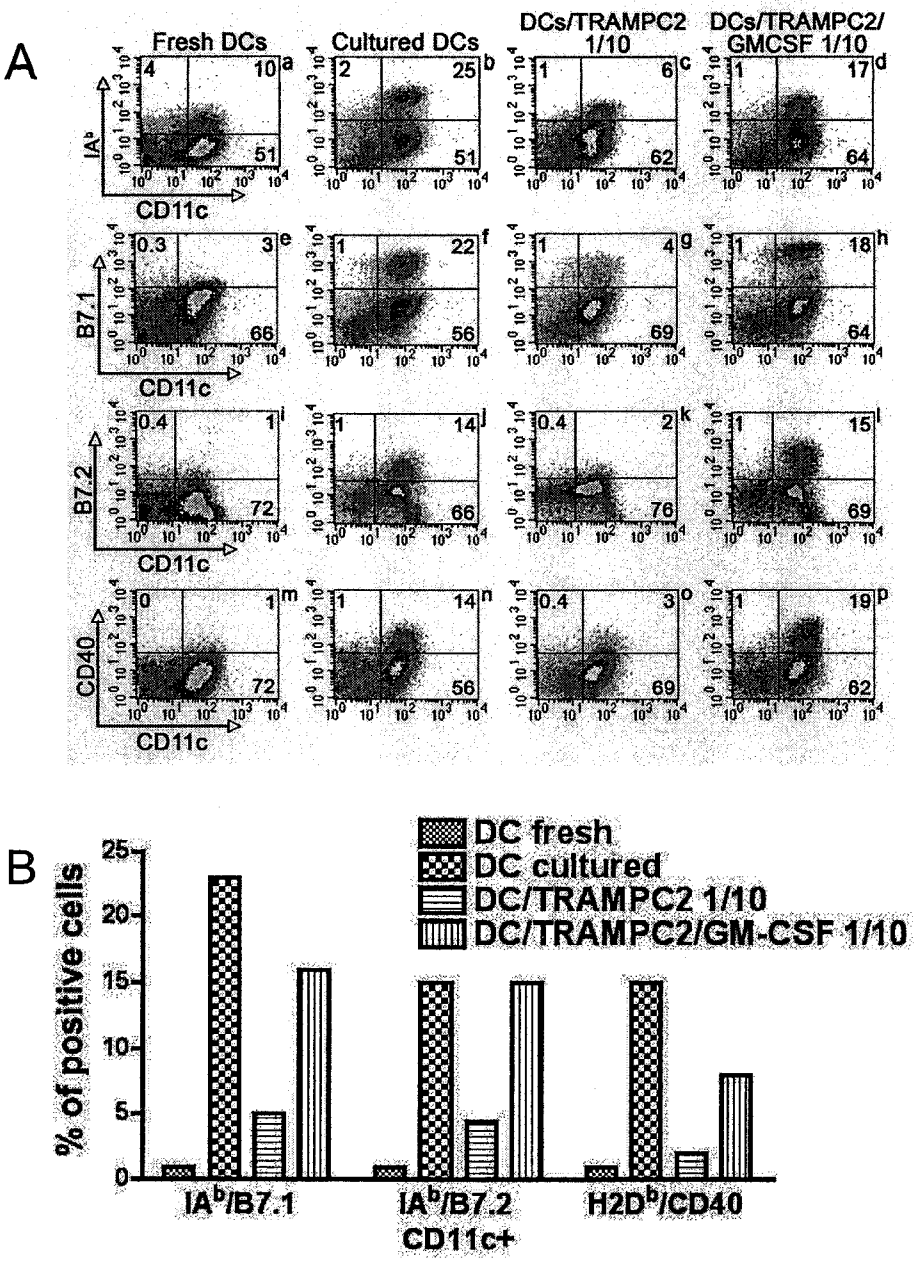


Figure 20. GM-CSF secreted by TRAMPC2 cells reverses down-regulation of DC maturation markers induced by tumor cells *in vitro*.
Panel A: DCs were purified from spleen of B16fl tumor bearing mice using Nycodenz gradient centrifugation. Fresh DCs were stained for comparison (a, e, i and m). DCs were cultured alone (b, f, j and n), with 10X TRAMPC2 (c, g, k and o) or 10X TRAMPC2/GM-CSF (d, h, l and p). Cultured DCs were harvested, stained with indicated antibodies the following day and analyzed with 4-color flow cytometer. Panel B: A CD11c gate was defined for data presented in panel A and the co-expression of IA^b/B7.1, IA^b/B7.2 and $H2D^b$/CD40 by DCs in each condition (Fresh DCs, cultured DCs, DCs/TRAMPC2 and DCs/TRAMPC2/GM-CSF) was plotted.

To characterize the tumor infiltrating leukocytes (TILs) in the TRAMPC tumor expressing SLC, CD40L or GMCSF *in vivo* and to evaluate the efficacy of expression of these genes in the tumor microenvironment to eradicate residual prostate

It is known that GM-CSF, CD40L and SLC induce DC maturation and SLC causes co-localization of DCs and T cells. We also showed that GM-CSF and CD40L when expressed from TRAMPC cells could reverse the inhibitory effect of tumor cells of DC maturation *in vitro*. These results prompted us to study the effect of expression of these cytokines on tumor growth *in vivo*. Even if the expression of these cytokines could cause maturation of a part of DCs infiltrating TRAMP tumor this can be enough to induce a potent immune response and prevent tumor growth. Although SLC could not induce DC maturation *in vitro* but it can still induce a potent immune response through co-localizing DCs and T cells. Since we used the tetracycline inducible expression system, first we wanted to make sure the repressor protein was not immunogenic and therefore we injected mice with the TRAMPC1P3 cells stably transfected with the repressor vector. Next, we designed experiments to inject TRAMPC2/TR/CD40L, TRAMPC2/TR/GM-CSF or TRAMPC2/TR/SLC cells into mice and study the effect of cytokine/chemokine expression on the tumor growth, metastasis and survival of the tumor bearing mice.

In vivo growth of TRAMPC1P3 cells expressing the repressor protein

The possibility exists that any host that has a competent immune system will reject tumor cells that express a foreign (bacterial) protein. This is a serious complication for a project evaluating novel immunotherapies where a competent immune system is a necessity. It has been shown that the expression of membrane-bound heat shock protein (mbHSP70) on the surface of the mouse mastocytoma cell line P815 enhanced immunogenicity of tumor cells (186). In this study the *in vivo* effect of mbHSP70 was evaluated by comparing the growth of mbHSP70 transfected cells to that of mock-transfected cells in DBA/2 mice. Fifty percent of mice rejected mbHSP70 transfected cells while 100% mice developed tumors in the control group (186). Furthermore, we had already experienced this problem when we injected mice with TRAMPC1 cells that were transfected with green fluorescent protein (TRAMPC1/GFP). TRAMPC1/GFP cells grew efficiently in athymic nude mice and remained GFP^+ even after several months of growth *in vivo*; however, these cells never produced tumors in immunocompetent C57BL6 mice (data not shown). They were also not transplantable into the prostate glands of syngeneic mice, a site considered immunologically "privileged". To address this issue, TRAMPC1P3/TR cells were transplanted subcutaneously into 4 mice. Control mice received the parental TRAMPC1P3 tumor. All mice that received TRAMPC1P3/TR tumor cells developed tumors at rates indistinguishable from mice that received parental

TRAMPC1P3 cells (data not shown). After several months of growth, tumors were excised, cut into small pieces and cultured in TRAMP media. Cells from the developed cell line were stained with an anti-TR monoclonal antibody and visualized by confocal microscopy. Three out of four tumors had sufficient TR protein levels in the cytosol and nucleus to be detected by this technique (Fig. 21). These data suggest that the TR protein is either not immunogenic or not sufficiently immunogenic to induce an immune response that alters the growth properties of TRAMPC1P3/TR tumor cells.

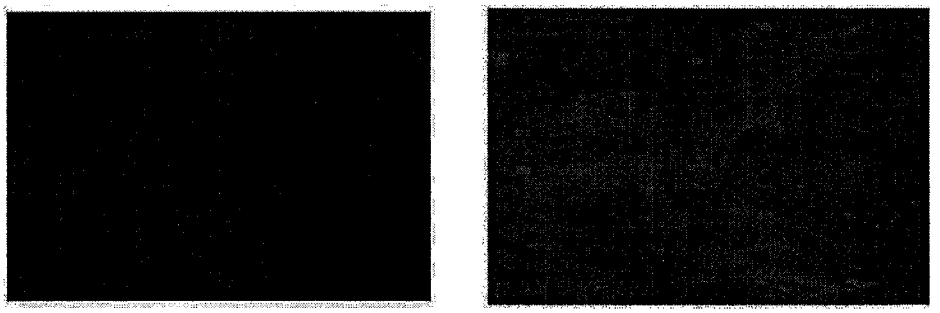


Figure 21. Detection of repressor protein in TRAMPC1P3/TR cells after long-term growth *in vivo.*
Mice received either 5 x 10^6 TRAMPC1P3 or TRAMPC1P3/TR cells subcutaneously and then were monitored for tumor growth twice weekly. Tumors were excised when they were $\geq$100 cm^2, grown in tissue culture chambers and then stained with either an isotype matched control or anti-TR protein antibody. TR protein was detected in TRAMPC1P3/TR cells incubated with the anti-TR antibody (B) but not with the isotype control antibody (A). Similarly, no staining was detected in TRAMPC1P3 cells stained with anti-TR protein antibody (data not shown). Fluorescence images were visualized by confocal microscopy and analyzed using Metamorph software.

In vivo growth of TRAMPC2/TR/GM-CSF clones

TRAMPC2/TR/GM-CSF cells clone 3, 5 and 6 (Fig. 19) were pooled and injected into the prostate of 7 mice, 6 of them grew tumors that were removed, diced and cultured without antibiotics. From 74 explants we developed cell lines that were tested by ELISA for inducible expression of GM-CSF. Explants from two tumors were not inducible by tetracycline and were not studied further. Cells derived from the four remaining tumors were inducible with tetracycline but manifested relatively low production of GM-CSF. A representative example is presented in Figure 22, panel A. Because cell lines from individual tumors were similar in terms of GM-CSF production, we pooled clones derived from each tumor and subjected them to antibiotic selection. After this selection step cell lines were expanded and tested for tet-induced production of GM-CSF. Although this process enhanced GM-CSF production, most lines had high constitutive GM-CSF production and displayed only a modest induction with tetracycline (panel B).

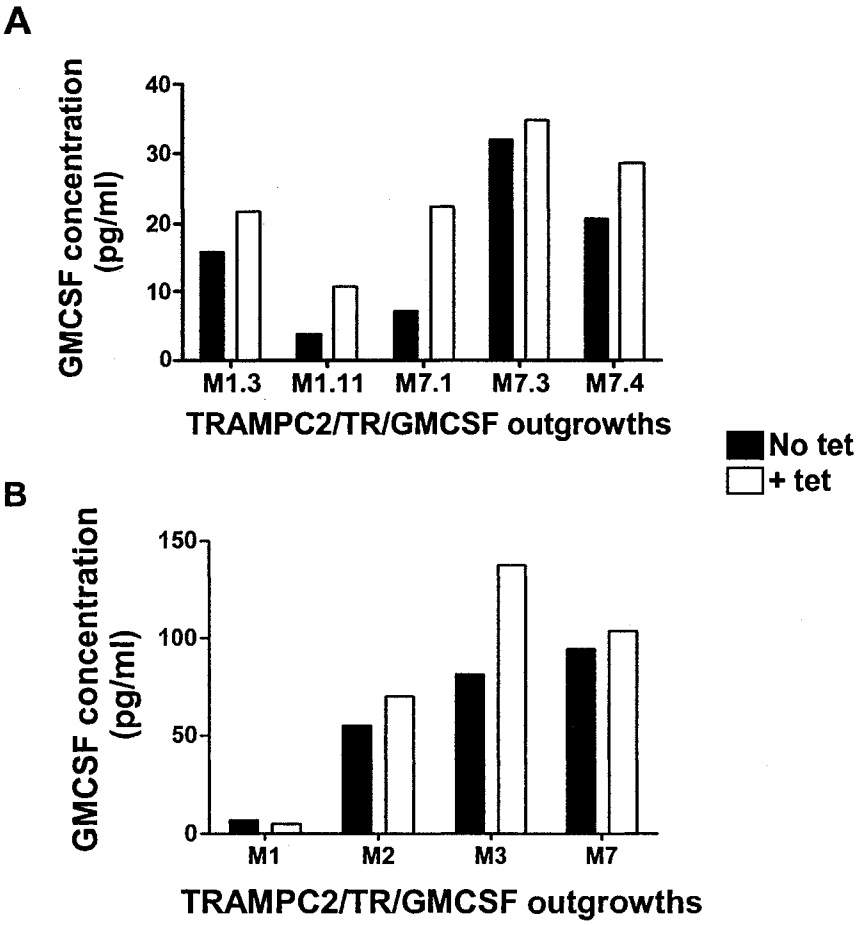


Figure 22. Diminished tet-regulated expression of GM-CSF following intraprostatic growth of TRAMPC2/TR/GM-CSF tumor cells.
Cells from TRAMPC2/TR/GM-CSF clones 3, 5 and 6 were pooled (Fig. 19) and injected into prostate glands of syngeneic mice. When tumors were palpable they were excised and explants incubated in vitro in tissue culture media in the absence of antibiotic selection. Panel A: Individual clones were isolated, expanded and then induced with tetracycline. The following day supernatants were tested by ELISA for GM-CSF expression. Shown are representative data from clones derived from two tumors from mouse #1 and #7 (M1, M7). Panel B: Clones derived from individual tumors were pooled, subjected to antibiotic selection and then expanded in vitro. Aliquots were incubated with tetracycline and the following day supernatants evaluated for GM-CSF production. Shown are data from tumor cells obtained from four individual mice (M1, M2, M3, M7).

To determine the cellular basis for low GM-CSF production, pooled TRAMPC2/TR/GM-CSF cells (clones 3, 5, 6) were grown under limiting dilution conditions. We identified clones producing GM-CSF by ELISA and then performed an ELISPOT assay (R&D, Minneapolis, MN) to estimate the number of cells producing GM-CSF. This study revealed that few cells produced GM-CSF in the absence of tet (Fig. 23, left panel). However <10% of TRAMPC2/TR/GM-CSF tumor cells secreted GM-CSF when induced in the presence of tet (right panel). Since these cells represent the progeny of a single cell, these data demonstrate that TRAMPC2/TR/GM-CSF tumor cells either lose or silence the transgene during clonal expansion.

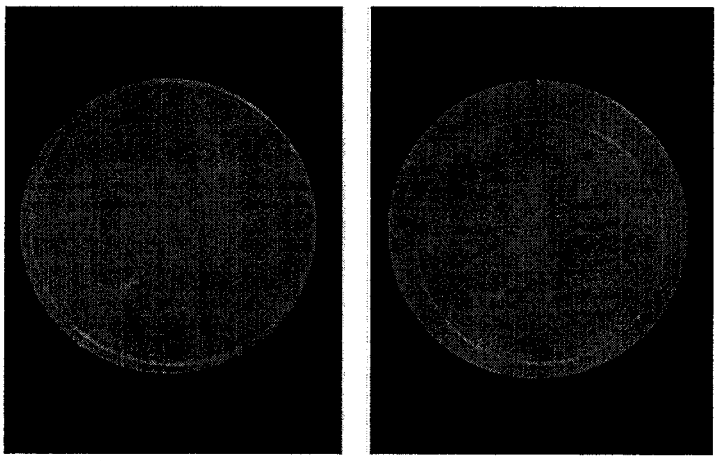


Figure 23. Evidence that TRAMPC2/TR/GM-CSF tumor cells lose or silence the GM-CSF gene during clonal expansion.
TRAMPC2/TR/GM-CSF cells were grown under limiting dilution conditions and clones producing SLC identified by ELISA. Cells producing this chemokine were expanded and then subsequently incubated overnight in the absence (left panel) or presence of tetracycline (right panel). The following day the percentage of cells producing GM-CSF was estimated by ELISPOT assay. Shown are individual wells from an ELISPOT plate containing 1000 input TRAMPC2/TR/GM-CSF cells.

Growth characteristics of a TRAMPC2/TR/CD40L clone *in vivo*

A TRAMPC2/TR/CD40L clone (from over 50 clones tested) that expressed CD40L after induction with tetracycline (Fig. 17) was tested for *in vivo* growth. To assess whether this cell line was still tumorigenic *in vivo* and maintained inducible CD40L expression, five mice were implanted ectopically with TRAMPC2/TR/CD40L cells. None of these mice developed ectopic (subcutaneous) tumors three months post implantation.

Next we tested whether TRAMPC2/TR/CD40L cells would grow in the prostate gland. We orthotopically injected five mice with TRAMPC2/TR/CD40L tumor cells and monitored these mice for tumor growth. None of these mice grew tumors during 3 months that they were monitored. This can be due to the leakiness and therefore the background CD40L expression of these cell lines.

Growth characteristics of SLC-transfected TRAMPC2 tumor cells injected subcutaneously

In order to develop a TRAMPC2/TR/SLC cell line that grows *in vivo* and maintains the regulated expression of SLC 20 mice were injected subcutaneously with 5 different TRAMPC2/TR/SLC lines (table 2; cell lines #4, 5, 8, 12 and 31). Seventeen of 20 mice grew tumors (table 2). These tumors were removed, diced into small pieces and cultured in TRAMP media without antibiotics. 180 clonal cell lines were isolated and tested by ELISA for inducible

expression of SLC individually as explained in Material and Methods section. Five outgrowth cell lines (A18, B13 and B3, M9 and R3) from subcutaneous tumors had low induction level of SLC after exposure to tetracycline (Fig. 24) relative to the parental cell line. For example A18 (Fig. 24) had about 220pg/ml of SLC expression with no background expression. TRAMPC2/TR/SLC 4 parental cell line that A18 was generated from had an average induction level of 3200 pg/ml with background level below 400 pg/ml (Fig. 15).

Table 2. 5 cell lines developed from 20 TRAMPC2/TR/SLC subcutaneous tumors that have small SLC induction after passage *in vivo*.

Mice	Clone	Tumor incidence	# of outgrowth cell lines *	# of inducible cell lines †
A-F	4	5/6	63	3 (A18, B3, B13)
G-H	5	2/2	25	0
I-L	8	3/4	26	0
M-P	12	1/4	15	1 (M9)
Q-T	31	4/4	51	1 (R3)
Total		17/20	180	5

Mice A through T were injected with different TRAMPC2/TR/SLC clones as indicated in the second column. Tumors were removed, diced and cultured in TRAMP media without antibiotics (zeocin and blasticidine). Different numbers of outgrowth cell lines were generated from each tumor (*). All these cell lines were tested for tet-inducible expression of SLC. $1X10^5$ cells from each cell line were seeded in duplicates (with or without 2ug/ml tetracycline) and the next day the supernatants were tested by ELISA. Only five cell lines were identified with very low induction levels (†).

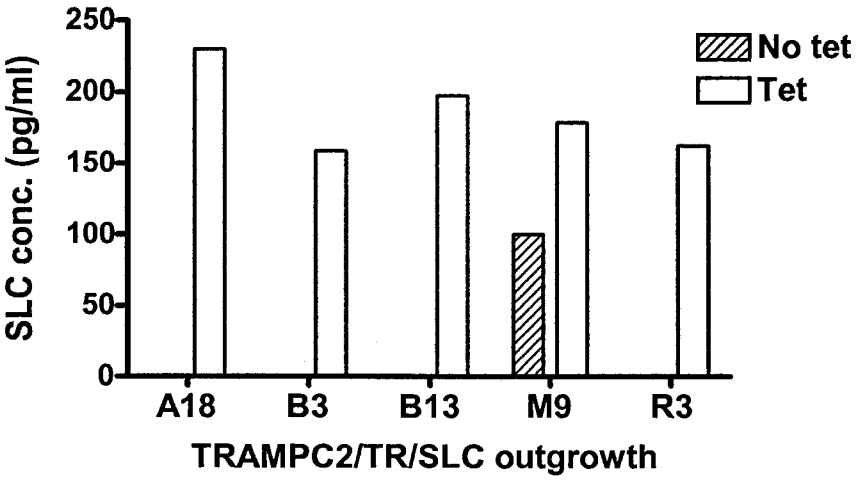


Figure 24. *In vivo* growth of TRAMPC2/TR/SLC clones is associated with reduced induction of SLC.
Different TRAMPC2/TR/SLC clones were injected subcutaneously in mice. Some mice grew tumors that were removed, diced and cultured in TRAMP media without selection in antibiotics. The developed cell lines were then tested for regulated expression of SLC. Only cell lines with regulated SLC induction are presented.

TRAMPC2/TR/SLC outgrowths shown in Figure 20 that had some SLC induction were expanded and subjected into antibiotic selection for about three weeks. All the cells of the first three cell lines (A18, B3 and B13) died. The other two lines (M9 and R3) were expanded and tested again for SLC induction (Fig. 25).

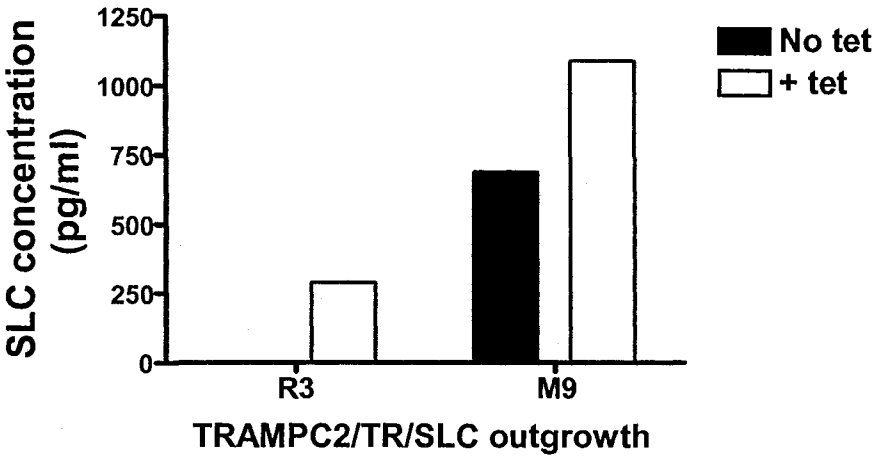


Figure 25. Antibiotics selection increased SLC induction level of TRAMPC2/TR/SLC outgrowth cell lines.
Different TRAMPC2/TR/SLC clones were injected subcutaneously in mice. Some mice grew tumors that were removed, diced and cultured in TRAMP media without selection in antibiotics. The developed cell lines were then tested for regulated expression of SLC and had low level of SLC induction compared to the parental cell line. These cell lines then were selected in antibiotics (zeocin and blasticidine). All the cells from three of these cell lines died during the selection process. R3 and M9 outgrowths were tested again for inducible SLC expression after they were expanded in the selection media. These two cell lines showed improved SLC induction after they were expanded in the presence of antibiotics.

Even after selection in antibiotics, SLC induction levels of TRAMPC2/TR/SLC lines was much lower than the parental cell lines that were injected into the mice (Fig. 25, R3) or they had much higher background (Fig 25, M9). For example, the parental cell line of R3 had induction levels between 1800 and 3500pg/ml (Fig. 15, clone #4), whereas, R3 produced 300pg/ml SLC after selection in antibiotics (Fig. 25). Similarly, the parental cell line from which the M9 clone was isolated constitutively produced low levels of SLC (10-50pg/ml, data not shown) and approximately 2000 pg/ml SLC in the presence of the inducer. In contrast, M9 showed high background level of SLC expression (Fig. 25, 700pg/ml) even after antibiotics selection. Although these cell lines (R3 and M9) maintained SLC expression after *in vivo* passage they did not survive antibiotics selection for more than 5 weeks. It seems that these cell lines were not genetically stable and as they were passaged *in vitro* they lost the antibiotic resistant gene or turn it off and therefore did not survive the antibiotic selection.

Growth characteristics of TRAMPC2/TR/SLC tumor cells following orthotopic implantation into the mouse prostate gland

The observation that most clonal outgrowths did not secrete SLC may indicate that cells secreting this chemokine were eliminated by an immunological mechanism(s). We therefore tested the notion that tumor growth in the "immunologically privileged" prostate gland may allow for the growth of cells with inducible expression of SLC. To that end, TRAMPC2/TR/SLC tumor cells (clone

#4, Fig. 18) were implanted into the prostate gland of nine mice. One mouse died without evidence of a palpable prostate tumor. Six mice developed palpable tumors approximately 2 months after implantation. These six tumors were excised and clonal outgrowths were obtained in TRAMP media without selection antibiotics. Outgrowths from two tumors were no longer tet-inducible and were not further studied (data not shown). Seventy clonal outgrowths were obtained from the remaining four tumors of which ten were inducible for SLC expression (Fig. 26). Clonal outgrowths derived from mouse 1 (M1) generally had low constitutive SLC levels but relatively weak induction for SLC. The remaining outgrowths demonstrated higher tet-induced SLC secretion but were "leaky" (high constitutive levels).

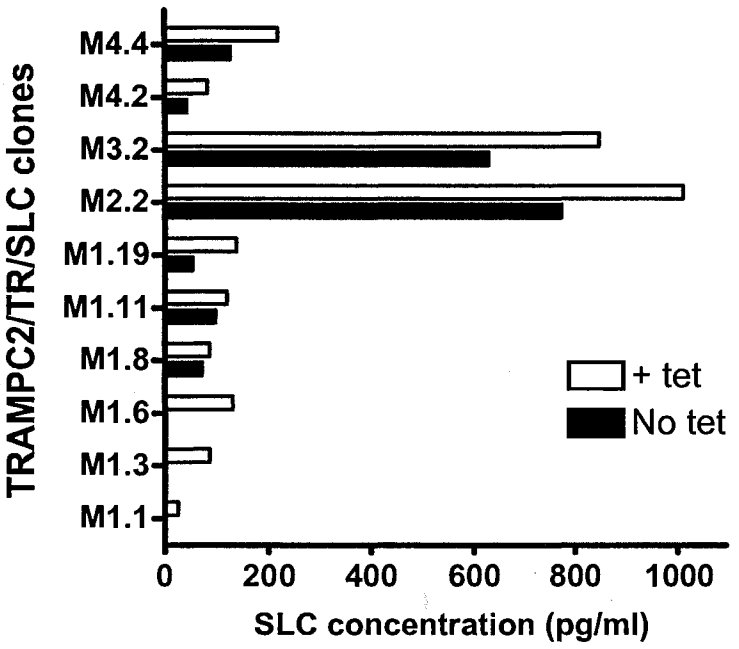


Figure 26. Tet-induced regulation of SLC in TRAMP/TR/SLC tumor cells following orthotopic implantation and growth.
Syngeneic mice were implanted orthotopically with TRAMP/TR/SLC tumor cells (5×10^5). After several months following implantation, palpable tumors were excised, diced and explants cultured in vitro. Clonal outgrowths were then isolated, expanded and tested for tet-induced secretion of SLC by ELISA. Four tumors were evaluated from individual mice (M1-4).

Impact of antibiotic selection on tet-induced production of SLC

Because the clonal outgrowths from intraprostatic tumors were isolated and grown in the absence of selection media, the relatively modest of SLC production or high background may indicate that TRAMPC2/TR/SLC tumor cells lost or silenced the SLC or the repressor gene during *in vivo* growth. To study whether the SLC gene was lost and to enrich for tumor cells with stable tet-inducible expression of SLC, clonal outgrowths from mouse 1 (M1.1-1.19) were pooled to generate TRAMPC2/TR/SLC-L1. The remaining lines were also pooled to produce TRAMPC2/TR/SLC-L2. Both transfected populations were then subjected to antibiotic selection. Figure 27 demonstrates that growth in selection media enhanced SLC production by TRAMPC2/TR/SLC-L1 cells (Line 1) approximately 5-fold without increasing constitutive SLC levels. Although TRAMPC2/TR/SLC-L2 (Line 2) cells had higher background expression relative to Line 1, tetracycline induced much higher levels of SLC production. These data indicate that both these lines have the capacity to grow both *in vitro* and *in vivo* in an orthotopic location. Therefore these cell lines that maintain stable and tet-inducible expression of SLC will be used for *in vivo* studies to investigate the effect of expression of SLC in TRAMP TME on tumor growth, survival and metastasis.

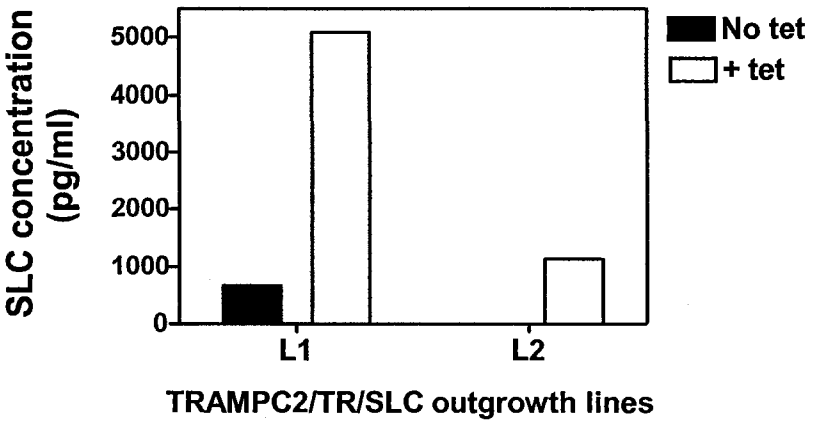


Figure 27. Enhanced tet-induced expression following intraprostatic outgrowth TRAMPC2/TR/SLC lines following antibiotic selection.
TRAMPC2/TR/SLC lines (L1 and L2) were expanded in vitro in the presence of zeocin and blasticidin. Samples from antibiotic resistant lines were then incubated in the absence (no tet) or presence (+tet) of tetracycline. The following day supernatants were evaluated for SLC levels by ELISA.

Impact of SLC production on the establishment of orthotopic and metastatic prostate tumors

TRAMPC2/TR/SLC clones (L1 and L2) displayed high levels of tet-inducible expression of SLC and had demonstrated the ability to grow *in vivo*. We therefore wanted to test the extent to which SLC expression in the TME influences tumor growth and metastatic disease. To that end, we implanted TRAMPC2/TR/SLC-L2 orthotopically for the second time into a total of 18 animals in two separate experiments (8 mice in experiment 1 and 10 mice in experiment 2, Fig. 28). In each experiment the mice were divided into two groups and one group were given doxycycline (Dox), a more stable derivative of tetracycline, in their drinking water one day after implantation. All the mice (control and treated) were sacrificed when the mice in the control group had ruffled fur, hunched up back and therefore were considered sick. The tumor weight and volumes were measured to determine if expression of SLC in the prostate TME alters these parameters. One of the mice given Dox 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 Dox in their drinking water. However, tumors excised from mice that received Dox in their drinking water were smaller in size and weighed less than TRAMPC2/TR/SLC-L2 tumors removed from control mice (Fig. 29). Therefore expression of SLC in the TRAMP TME inhibited tumor growth and treated mice lived longer.

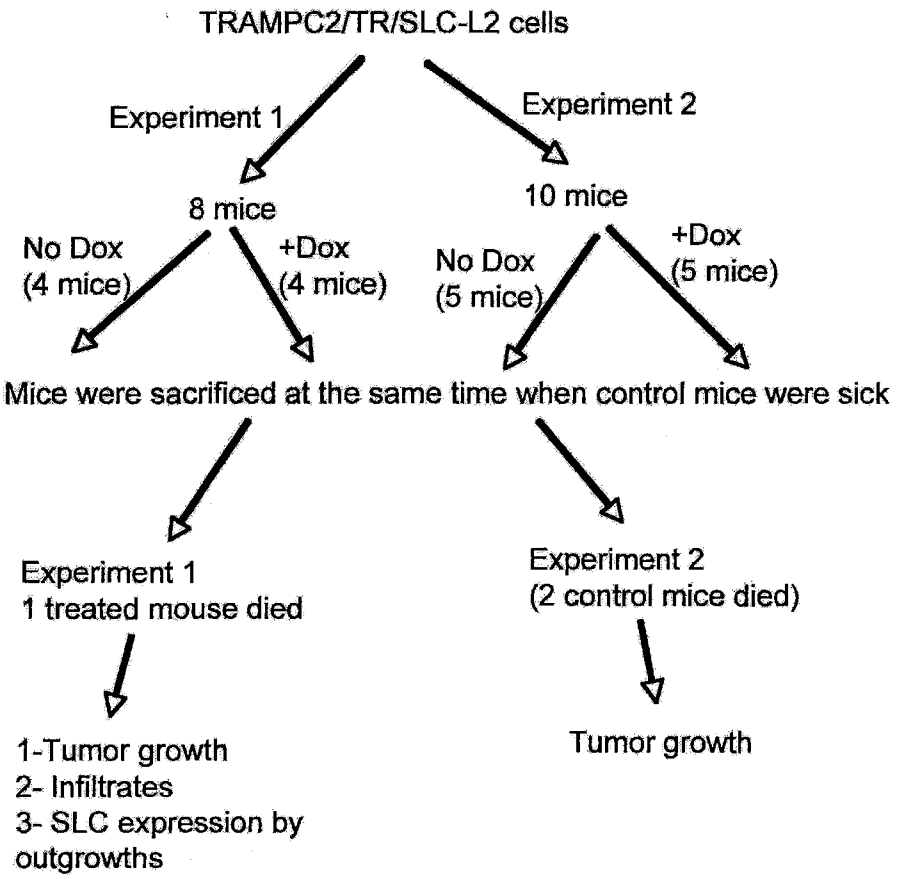


Figure 28. Schematic presentation of the *in vivo* experiments performed using TRAMPC2/TR/SLC-L2.
TRAMPC2/TR/SLC-L2 cells were injected into 18 mice in two independent experiments. 8 mice in experiment 1 and 10 mice in experiment 2 were divided into two groups and one cohort in each experiment received doxycycline in their drinking water. Mice in both experiments were sacrificed when the control group mice were sick. In experiment 1, tumor weights and volumes were measured, TILs of treated and control groups were phenotyped and SLC expression by tumor outgrowth cell lines was determined. In experiment 2, tumor weights and volumes were determined and metastasis to lymph nodes, lungs and pancreas was studied.

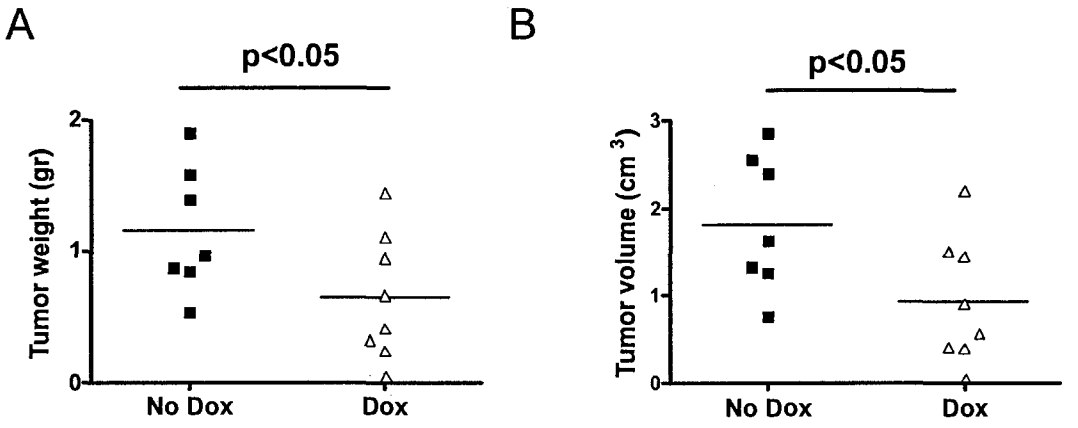


Figure 29. Expression of SLC in the prostate tumor microenvironment inhibits tumor growth.
Mice (total of 18, experiment 1 and 2) were given an orthotopic injection of $5x10^5$ TRAMPC2/TR/SLC-L2 cells. One cohort was given doxycycline in their drinking water after surgery and one group served as control. One mouse from doxycycline treated group and two from control group died a week after surgery without any tumor. Tumor growth was monitored by palpation and approximately two months after implantation, tumors were excised, weighed (panel A) and tumor volumes (Panel B) were measured.

A small piece of each tumor from experiment 1 was diced, cultured in TRAMP media and the derived outgrowth cell lines selected in antibiotics (zeocin and blasticidine) were tested for tetracycline-induced SLC secretion. Figure 30 illustrates that even the best clonal derivatives had very modest induction of SLC (≤10% of parental line 2, see Fig. 27) following exposure to tetracycline. TRAMPC2/TR/SLC-L2 cells that were injected orthotopically into mice had about 1200pg/ml of induced SLC expression (Fig. 27) with no background expression. However, after *in vivo* passage the tetracycline-induced level of SLC expression of M5.4 line that had the highest inducible level of expression among the generated cell lines was only 450pg/ml (Fig. 30). All these cell lines eventually lost inducible expression after a few *in vitro* passages. This experiment showed that even after two *in vivo* passages the generated cell lines were genetically unstable and lost the regulated expression of SLC.

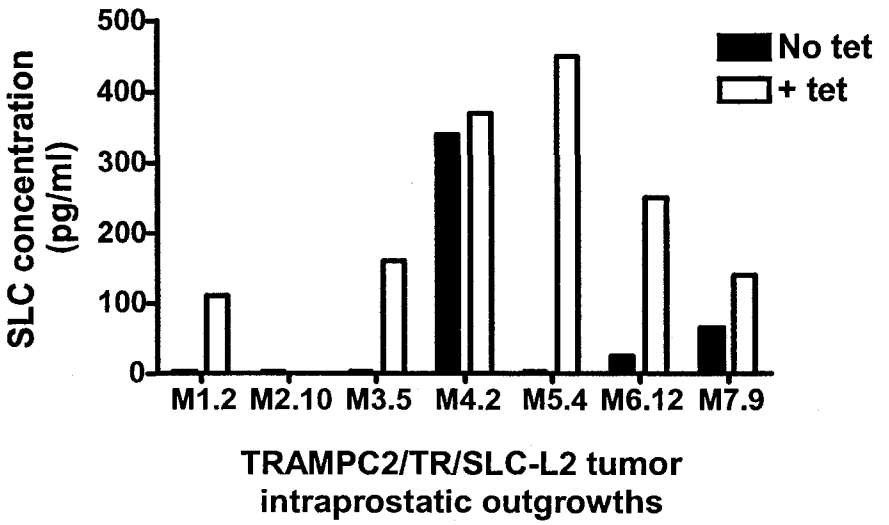


Figure 30. Representative tet-inducible secretion of SLC in TRAMPC2/TR/SLC clonal lines derived from tumors exposed to doxycycline *in vivo*. TRAMPC2/TR/SLC-L2 cells were implanted orthotopically into 8 mice (experiment 1). One cohort received doxycycline in their drinking water after surgery. Two months later, tumors were excised, diced and cultured in tissue culture media containing antibiotics. An aliquot (10^6 cells) of each clonal outgrowth was incubated in the absence or presence of tet (2ug/ml) overnight and the following day evaluated for SLC expression by ELISA.

Characterization of the cellular infiltrate in tumors that expressed SLC

The data above indicated that expression of SLC inhibited primary prostate tumor growth. This may have resulted from changes in the inflammatory response as a result of expression of SLC in the TME because SLC is an attractant primarily for DCs and T cells. Therefore we also pooled cells from the spleen and tumors from both untreated and doxycycline treated groups of mice (±Tet) in experiment 1 and stained them with the indicated monoclonal antibodies (mAbs) (Fig. 31). Viable cells were gated and then phenotyped by multi-parameter flow cytometry. The panel of mAbs used for this study detected the presence of DCs (CD11c), macrophages (F4/80), B cells (B220), T cells (CD3e) and granulocytes (Gr-1). Spleens from normal or tumor bearing mice were used as controls. Since SLC is known to attract DCs and T cells, we expected to detect higher percentages of these cells in tumors from mice treated with doxycycline. However, we did not detect any major difference between the doxycycline treated and untreated groups (Fig. 31, panel A). There was an increase in the number of B cells ($B220^{+}$) in the tumor of treated mice, but the role played by B cells in cancer biology is complex and somewhat controversial. Previous studies using genetically engineered mice suggest that B cells may be immunosuppressive and inhibit tumor rejection. However, the effects of B-cell depletion employing an antibody in mice bearing solid tumors has not been tested owing to difficulties in making an effective antimouse CD20 antibody (187). We also analyzed DCs for the expression of MHC class I and II antigens

and the co-stimulatory molecules CD80 and CD86 (Fig. 31, panel B). These molecules are indicative of DC maturation and are important for optimal antigen presenting function. Expression of CD40 on DCs is an important receptor for maturation signals delivered by CD40-ligand usually expressed on activated $CD4^+$ T cells. However, doxycycline treatment did not influence the maturational state of intratumoral DCs at least based on the cell surface expression of these molecules (panel B).

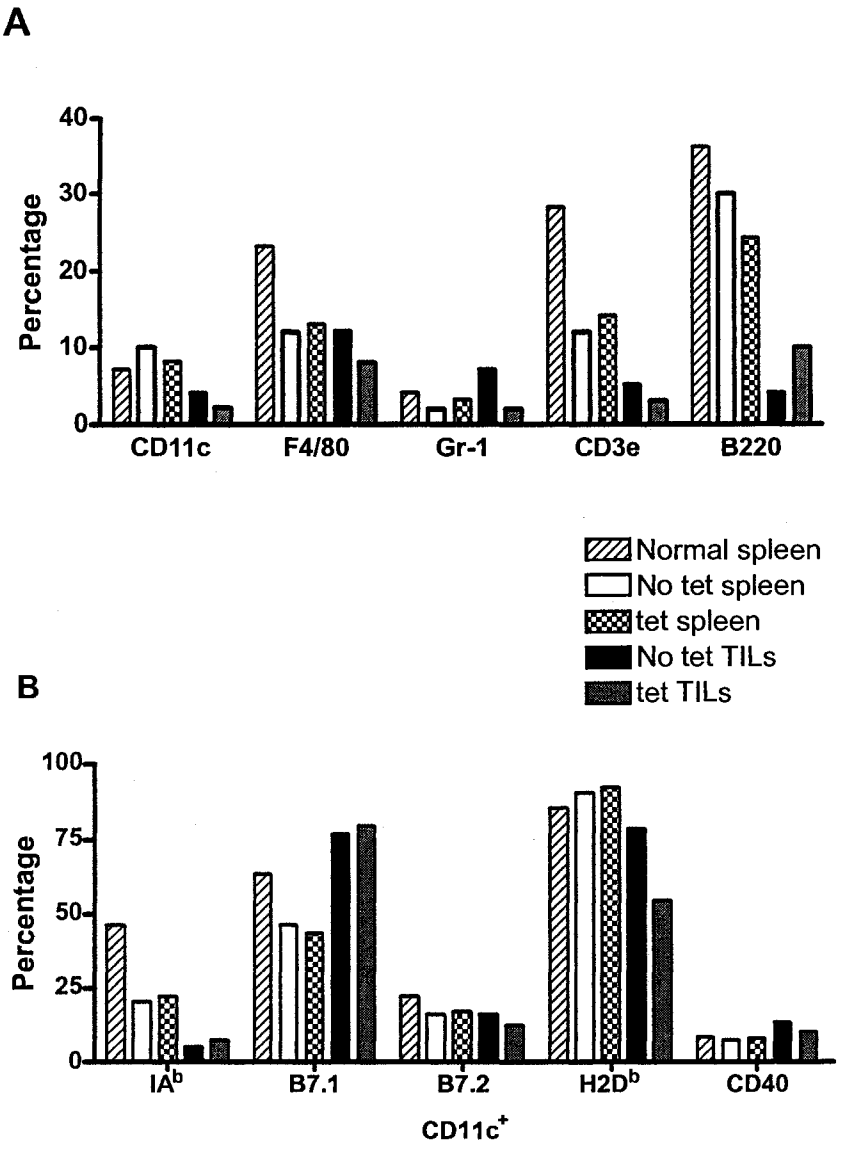


Figure 31. Characterization of host cells infiltrating TRAMPC2/TR/SLC tumors treated with doxycycline.
TRAMPC2/TR/SLC-L2 was injected into the prostate of 8 mice (experiment 1). On the day after the surgery, half the mice received water that contained doxycycline (20 ug/ml). Tumors were removed 60 days post implantation and single cell suspensions from each group (treated or not treated) were pooled for flow cytometric analysis. Tumor bearing spleens (TBS) and normal spleen (NS) were used as control. Panel A. Phenotype of tumor infiltrating leukocytes (TILs). Panel B. A $CD11c^+$ gate was defined and expression of the indicated molecules was determined on $CD11c^+$ cells.

Calculating the absolute number of each cell type per spleen or tumor showed that doxycycline treatment did not have any affect on the number of any cell type in the spleen (Fig. 32A) other than B cells ($B220^+$ cells) compared to spleen of untreated mice. The number of B cells was lower in the spleen of doxycycline treated mice ($1.3x10^7$ cells/spleen) than in untreated mice ($1.9x10^7$ cells/spleen). As shown in Figure 32B there was actually more B cells in the tumor of treated mice ($4x10^5$ cells/tumor) than untreated mice ($1x10^5$ cells/tumor). The number of all other cell types was actually lower in the tumor of treated mice than untreated mice, particularly T cells and Gr-1 cells. We also compared the number of DCs from treated and untreated mice that express IA^b, B7.1, B7.2 and CD40 molecules (Fig. 32 C and D). The number of splenic DCs that expressed IA^b, B7.2, CD40 was slightly higher when mice were treated with doxycycline. In the tumor the number of $CD11c^+$ $B7.1^+$ cells was higher in the treated mice than untreated mice (panel D). Although treated mice had smaller tumors there was not any major difference between the number or the percentage of the cells infiltrating tumors. This can be due to the fact that these mice were sacrificed when they had progressed disease and although treatment slowed down the progression of the disease it did not prevent it. It is possible that major differences in the TILs could be detected at earlier time points of the tumor progression.

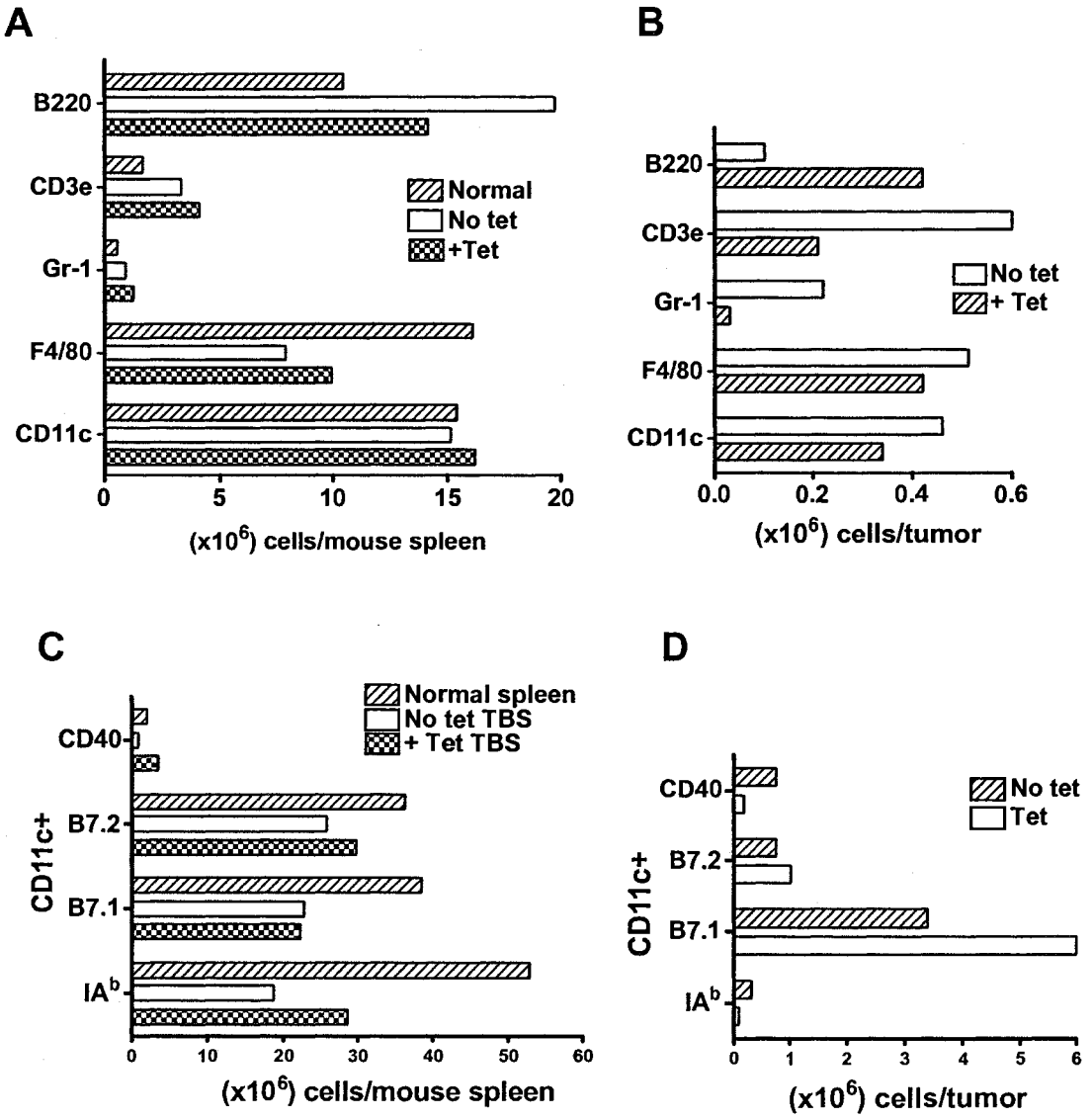


Figure 32. Characterization of host cells infiltrating TRAMPC2/TR/SLC tumors treated with doxycycline.
TRAMPC2/TR/SLC-L2 was injected into the prostate of 8 mice (experiment 1). On the day after the surgery, half the mice received water that contained Dox (20 ug/ml). Tumors were removed 60 days post implantation and single cell suspensions from each group (treated or not treated) were pooled for flow cytometric analysis. Tumor bearing spleens (TBS) and normal spleen (NS) were used as control. Panels A and B: Phenotypic analysis of spleen cells from normal mice and mice treated or not with Dox (A) and of TRAMPC2/TR/SLC-L2 TILs of mice treated or not with Dox (B). Panels C and D: A $CD11c^+$ gate was defined and expression of the indicated molecules was determined on DCs from normal spleen and spleens of mice treated or not with Dox (C) and DCs infiltrating TRAMP/TR/SLC-L2 tumor treated or not with Dox (D).

Functional assessment of the promoter (CMV) of the exogenous SLC gene

It is apparent from the data presented above that TRAMPC2/TR/SLC tumor cells displayed very weak induction of SLC following ectopic and orthotopic implantation. Two possibilities were considered: loss of the transgene or alternatively, silencing of the promoter. We therefore next tested whether the poor induction with tetracycline reflected loss of the transgene. DNA was extracted from the outgrowth cell lines derived from TRAMPC2/TR/SLC line 2 tumors and PCR was performed using specific primers to amplify SLC transgene. It is apparent from Figure 33 panel A that outgrowths obtained from orthotopic TRAMPC2/TR/SLC tumors still contained the SLC transgene. The absence of a product in the control mouse DNA confirmed that the primers did not amplify endogenous SLC (lane 9). To test the possibility that the promoter was silenced by methylation, we evaluated the methylation pattern of the CMV promoter. To perform this assay, DNA isolated from tumor pieces or outgrowth cell lines were bisulfite treated. 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 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 (188). Figure 33B demonstrates that when the pcDNA4/TO/SLC plasmid was tested following bisulfite conversion, PCR reactions with both primers produced a product indicating that the original plasmid DNA was not

methylated (TO/SLC, oligos 1 and 2). In contrast, when DNA was extracted from two excised TRAMPC2/SLC-L2 tumors (prior to *in vitro* expansion), promoters appeared to be methylated because there were no signals when oligos 2 were used, whereas a PCR product was formed with oligos 1 (tumor M1 and M2, oligos 1 snd 2). When a clonal outgrowth derived from one of these tumors (line M1, oligos 1 and 2) 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 SLC in some clonal outgrowth *in vitro* of explants of TRAMPC2/TR/SLC tumor (Fig. 30). We did not investigate the expression of repressor protein but the repressor protein is also expressed from a CMV promoter and therefore in the cell lines that with methylated promoter the repressor gene CMV promoter was also silenced.

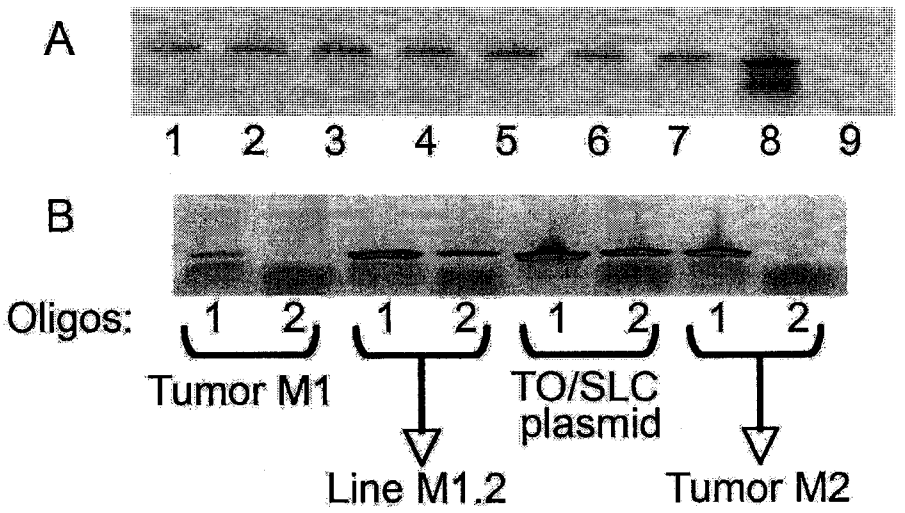


Figure 33. Detection of SLC gene in TRAMPC2/TR/SLC tumors and methylation status of CMV promoter following growth *in vivo*.
A: DNA extracted from outgrowth cell lines derived from TRAMPC2/TR/SLC line 2 tumors were tested for the presence of transfected SLC gene by PCR reactions using primers specific for the transfected SLC gene. PcDNA4/TO/SLC plasmid used for transfection was included as a positive control and mouse DNA was used as negative control. Lanes: 1-7 represent PCR products obtained when DNA from two TRAMPC2/TR/SLC Line 2 tumors were amplified by PCR. Lanes 8 and 9 represent the PCR products obtained when pcDNA4/TO/SLC plasmid 9 and mouse DNA were used, respectively. B: DNA extracted from TRAMPC2/TR/SLC line 2 tumors (M1 and M2) and a cell line derived from tumor M1 were tested for methylation status of SLC gene promoter. PcDNA4/TO/SLC plasmid used for transfection was used as negative control. Lane 1- tumor M1, oligo 1; lane 2- tumor M1, oligo 2; lane 3- M1.2 line, oligo 1; lane 4- M1.2 line, oligo 2; lane 5- pcDNA4/TO/SLC plasmid, oligo 1; lane 6- pcDNA4/TO/SLC plasmid, oligo 2; lane 7- tumor M2, oligo 1; lane 8- tumor M2, oligo 2.

Expression of SLC in the TRAMPC2 TME prolonged survival of tumor bearing mice

In order to establish that SLC expression delays tumor growth in a different experiment, 8 mice were injected with TRAMPC2/TR/SLC-L2 cells orthotopically and randomly divided into two groups. One group received doxycycline in their drinking water starting the day after the surgery. The mice were sacrificed when they showed signs of morbidity (they were not sacrificed all on the same day). Although tumors of doxycycline treated mice were bigger in size and weighed more when the mice were sacrificed, it appeared that SLC prolonged the survival of treated mice. As shown in Figure 34 the non-treated mice all died by day 32 after implantation, however, doxycycline treated mice lived up to 50 days. Therefore, expression of SLC in TRAMPC2 TME prolongs the survival of tumor bearing mice.

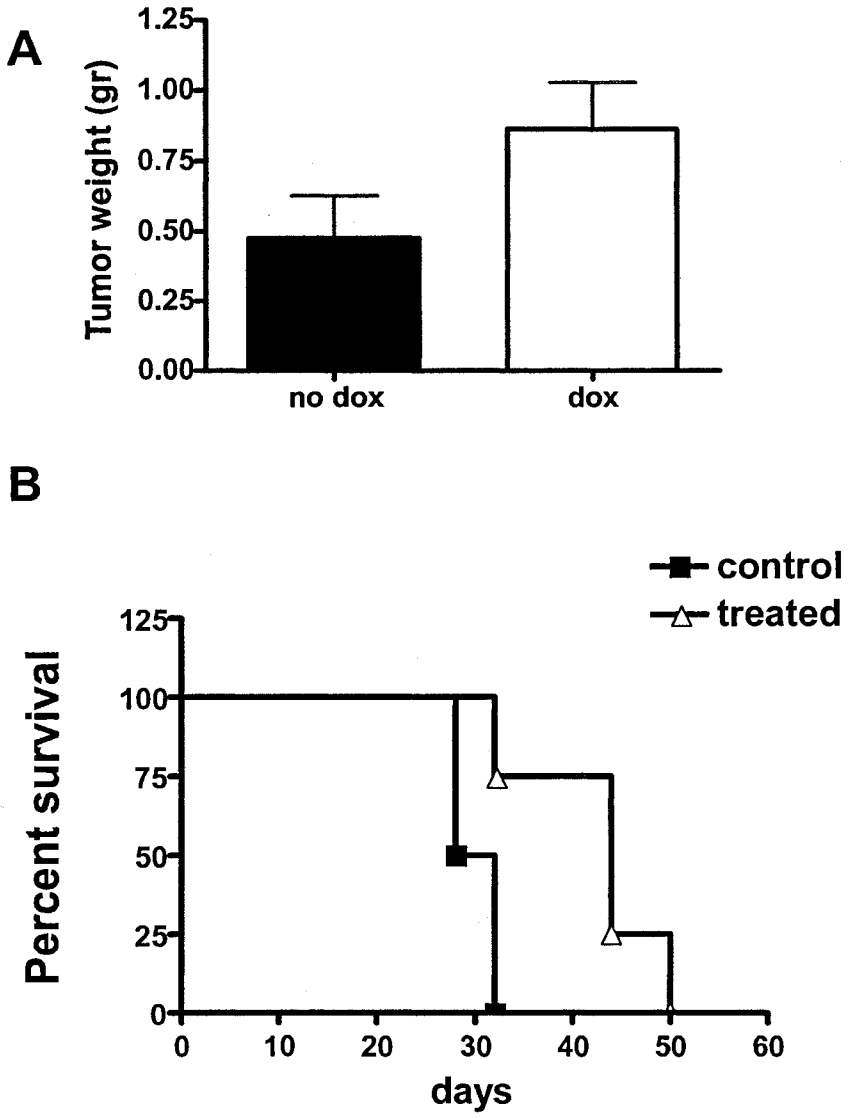


Figure 34. Inducible expression of SLC in the tumor microenvironment enhances survival in mice bearing orthotopic prostate tumors.
8 Mice were transplanted orthotopically with TRAMPC2/TR/SLC-L2 cells. 4 mice were given Dox in their drinking water the day after implantation. Tumor growth was monitored by palpation and mice were euthanized when tumors were palpable and mice showed signs of morbidity. Panel A: The average of tumor weights of the tumors from mice treated or not with doxycycline. Panel B: Survival curve of TRAMPC2/TR/SLC-L2 tumor bearing mice treated or not with Dox.

We also evaluated the metastasis to different organs like lymph nodes, lungs, pancreas and bone marrow (one mouse from the untreated group was found dead before we could access metatstatic disease). All draining cervical lymph nodes from untreated mice had metastatsis, whereas, only 2 lymph nodes from treated mice yielded tumor cell outgrowth. 50% (2/4) of the untreated control mice also had lung metastasis with one animal with both lung and pancreatic metastases. Only 1/4 of the treated mice had metastasis to lung and none of the treated mice had metastatis to the pancreas (table 3).

Table 3. Frequency of lymph node, lung and pancreas metastasis in TRAMPC2/TR/SLC-L2 orthotopic tumor bearing mice treated with doxycycline.

metastasis location	No. of mice with metastasis/group	
	Carrier treated	Dox treated
Lymph node	3/3	2/4
Lung	2/3	1/4
Pancreas	1/3	0/4

8 Mice were transplanted orthotopically with TRAMPC2/TR/SLC-L2 cells. 4 mice were given doxycycline in their drinking water the day after implantation. Tumor growth was monitored by palpation and mice were euthanized when tumors were palpable and mice showed signs of morbidity. Lymph nodes, lungs and pancreases were removed, diced and cultured as described previously. Organs with tumor metastases grew TRAMPC2 cells out of the organ piece and were harvested as a cell line. Demonstrating that these cell lines contained aneuploid DNA validated that the outgrowths represented tumor cells.

Next we wanted to determine whether SLC expression could induce tumor regression after the tumor became palpable. Therefore we injected 9 mice with TRAMPC2/TR/SLC-L2 orthotopically and divided them into three groups. One group received doxycycline from day 1, the second group from day 20 (after one of the mice had a palpable tumor) and the third group served as control. The survival curve of these mice is presented in Figure 35 panel A. One mouse from the control group did not grow tumor at all and was excluded from this graph. As shown in this Figure, control mice died by day 50. However, mice from doxycycline treated (both day 1 and day 20) lived only 4 days longer. Although the average tumor weight of the mice treated with doxycycline from day 1 was lower than both control group and mice treated with doxycycine from day 20, this difference was not significant (Fig. 35, panel B).

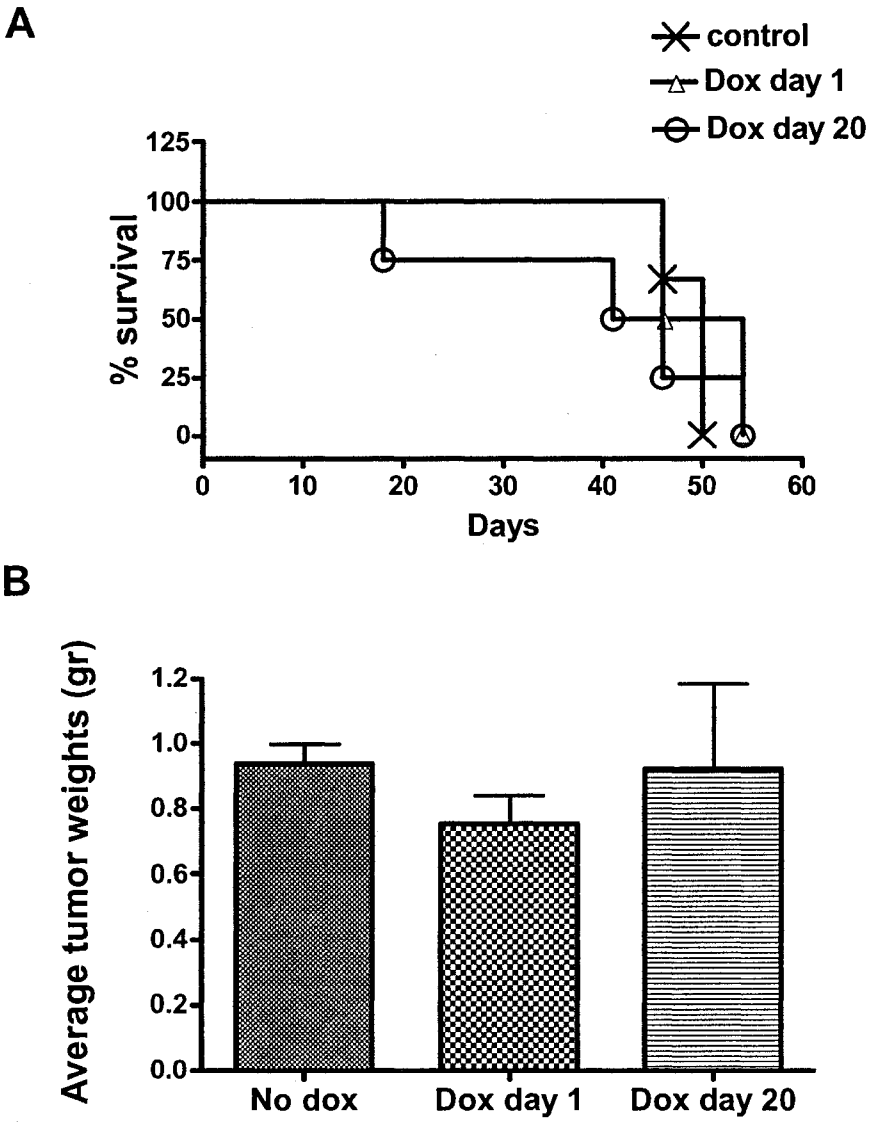


Figure 35. SLC does not inhibit growth of established TRAMPC2 tumor. 9 mice were injected with TRAMPC2/TR/SLC-L2 and divided in 3 groups. One group (2 mice) received doxycycline in their drinking water 1 day after surgery and the second group (4 mice) 20 days after surgery when one mouse in the group had a palpable tumor. The third group (3 mice) did not receive doxycycline and served as control (one mouse from this group did not grow a tumor). Panel A: The survival curve of TRAMPC2/TR/SLC-L2 tumor bearing mice treated or not with doxycycline. Panel B: The average tumor weight of TRAMPC2/TR/SLC-L2 tumor bearing mice treated or not with doxycycline.

CHAPTER V

DISCUSSION

Maturation of dendritic cells is inhibited by TRAMPC2 cells but not Gr-1$^+$ cells

Dendritic cells play a major role in orchestrating immune responses. Immature DC are characterized by their ability to capture, process and load antigens to MHC class I- or class II- molecules (165). Following antigen uptake, DCs migrate to the secondary lymphoid organs where they mature, express markers like B7.1, B7.2 and CD40 molecules and become APCs that are able to select and activate naive antigen-specific T cells (183). The presence of DCs has been reported in numerous tumors (137). Despite the presence of such tumor-infiltrating DCs, tumor cell outgrowth often occurs indicating that immunity against tumor cells is either improperly induced or evaded by the tumor. This raises questions regarding the status of TIDCs. Therefore we decided to use TRAMP transplantable murine prostate model to characterize DCs infiltrating these tumors. Dendritic cells infiltrating TRAMP tumors had lower expression of MHCII, B7.2 and CD40 molecules compared to splenic DCs. Furthermore CCR7, CD8 and PDL2 molecules were down-regulated in TIDCs compared to normal spleen DCs. Therefore, TRAMP tumor infiltrating DCs are considered phenotypically immature. Although a small percentage of TRAMP TIDCs express MHCII and co-stimulatory molecules, it may not be sufficient for T cell activation. Determining their level of functional activity requires isolation and purification of these cells. In

this study we tried different yet common methods to purify TIDCs. These methods included selective adherence, Nycodenz gradient centrifugation, discontinuous Percoll gradient centrifugation and finally anti-CD11c magnetic beads. However, all of these methods failed to enrich TRAMP TIDCs. Enrichment of TIDCs by percoll gradient centrifugation showed that splenic DCs were denser than TIDCs as they appeared in the interface of 70 and 40% percoll, whereas, TIDCs were localized primarily at he 20-40% interface (Fig. 5). Therefore, TRAMPC2 TIDCs do not only differ from splenic DCs in the expression of cell surface molecules but also they behave differently as shown by performing discontinuous percoll gradient centrifugation. Tumor cells are known to activate granulocytes by producing different cytokine such as TNF-α and IL-8. These cytokines are known to be elevated in the serum of cancer patients (189) and stimulate the oxidative burst (190). Although it has not been reported it is highly probable that other cells of the immune system could be equally affected by the same oxidative stress induced by tumor cells.

Numerous findings indicate that tumor-derived factors promote the immunosuppressive phenotype of DCs. Indeed, conditioned media from tumor cells lines can inhibit the *in vitro* differentiation of DCs from their precursors (170). After trying different techniques to enrich TIDCs we decided to determine the cell type responsible for immature phenotype of DCs in the TRAMP TME. When immature DCs were cultured alone the expression of MHCII and co-stimulatory and CD40 molecules were up-regulated compared to fresh DCs (Fig. 6). However, the presence of TRAMPC2 cells in the culture inhibited the

expression of these molecules. We also showed that TRAMPC2 cells inhibited DC maturation of DCs through direct contact. When contact of DCs with TRAMPC2 cells was prevented using transwells, TRAMPC2 cells failed to inhibit the expression of IA^b, B7.1 and B7.2 (Fig. 7). Although there are no reports about molecules involved in the prevention of DC maturation by tumor cells through direct contact we reported that TRAMPC2 cells inhibit up-regulation of class II antigens and co-stimulatory molecules by immature DCs through direct contact. Tumor cells have developed strategies to down-regulate immune responses. Previous studies have shown that tumor cells produce molecules that inhibit DC maturation. Some of these molecules include IL-10 (191, 192), vascular endothelial growth factor (170), PGE_2 (192), and TGF-β (171). Furthermore, purified TIDCs were shown to be refractory to *ex-vivo* maturation stimuli because of autocrine production of IL-10 (137). This observation is consistent with their inability to induce appropriate allogeneic T cell activation (100). Taken together, our results and the above reports suggest that the TME is immunosuppressive for DC and has the ability to selectively modulate TIDCs.

We have previously shown that TRAMP tumors are infiltrated with immature myeloid cells (84) that are known to be immunosuppressive through different mechanisms (172). Immature myeloid cells (iMCs) are present in the bone marrow and spleen of healthy mice and differentiate into mature myeloid cells including granulocytes, macrophages and DCs in the presence of GM-CSF *in vitro* or after adoptive transfer to healthy, naive recipients *in vivo* (193). However, they accumulate in the spleen and, to some extent, in the lymph nodes

of mice bearing many different tumors (194, 195). The functional activity of iMCs involves the inhibition of IFN-γ production by $CD8^+$ T cells both *in vitro* and *in vivo* in response to peptide epitopes presented by MHC class I molecules at the cell surface of iMCs (196). We (unpublished data) and others (197) found that oxidative stress, caused by the production of reactive oxygen species by iMCs, suppressed CD3ζ expression by T cells. This effect was mediated by molecules such as hydrogen peroxide (198) that are involved in many signaling pathways and can also be responsible for immature phenotype of DCs in TME. Therefore we investigated the possibility of a contribution of myeloid cells to the immature phenotype in the TRAMP TME. Purified $Gr\text{-}1^+$ granulocytes obtained from flt3-ligand treated mice did not prevent maturation of DCs in vitro. But the combination of $Gr\text{-}1^+$ and TRAMPC2 cells caused reduction in the expression of IA^b and B7.1 molecules that was not greater than reduction induced by only TRAMPC2 cells. Thus, TRAMPC2 cells seemed to be the major cell type that prevented TIDCs maturation and this was mediated through direct contact.

It has been reported by others that TIDCs in several transplantable and transgenic mouse tumor models had an immature phenotype and were refractory to activation with microbial or T cell-derived stimuli like LPS, IFN-γ, and anti-CD40 agonist antibody (137, 199). We showed that exposure to TRAMPC2 did not prevent immature DCs undergoing maturation when stimulated with LPS *in vitro*. Even though other factors may be involved in TRAMP TME, this indicates that TIDCs did not lose their capacity to mature when stimulated with a potent TLR4 ligand.

As discussed previously GM-CSF, SLC and CD40L can induce maturation of DCs. SLC secreted from TRAMPC2 cells partially reversed the down-regulation of maturation markers induced by TRAMPC2 cells, but the level of maturation markers expression was still lower than the level of expression of these molecules when DCs were cultured alone. GM-CSF and CD40L were more potent in induction of DC maturation when secreted from TRAMPC2 cells as they completely reversed the down-regulation of maturation markers induced by TRAMPC2 cells. However, the effect of GM-CSF and CD40L did not exceed the maturation level of DCs cultured alone. Although this maturation level seems to be low compared to LPS induced maturation, this may be physiologically relevant *in vivo*. It has been shown ratio of DCs:T cells required to induce T cells proliferation was 1:40 using tumor RNA-pulsed DCs in a pancreatic cancer model (200). Therefore, even a low level of DCs maturation may be sufficient to induce an anti-tumor immune response.

Gene silencing through promoter methylation in the loss of transgene expression in TRAMPC2 transfected cells

Regulated expression of specific cytokines and chemokines was a crucial component of designing these studies that made our model more clinically relevant. The repressor protein of tetracycline regulated expression system was not detectable after TRAMPC1P3 cells that were transfected with the repressor protein were passed *in vitro*. Furthermore, when TRAMPC1P3 cells were transfected with both TR and the TO/SLC expression vectors, the inducible level

of expression was very low. These data led us to try TRAMPC2 cells that turned out to have higher transfection efficiency. Furthermore, TRAMPC2 cells transfected with TR and TO/SLC vectors had higher level of inducible expression. Other studies have also reported the difficulties with specific cell lines to generate stable inducible clones (201).

Although TRAMPC2 cells produced more SLC when induced with tetracycline compared to TRAMPC1P3 cells, the amount of protein produced varied from one passage to another and some lost the expression of the transgene altogether. Chromosome instability that is a common feature of human cancer and results in aneuploidy could be one explanation for different amount of protein produced by transfected TRAMPC2 cells (202). Another explanation that was addressed in this study was gene silencing through promoter methylation (188). After transfected TRAMPC2/TR/SLC cells with low background and high inducible expression were passaged *in vivo*, some of the cell lines developed from tumors did not express SLC at all although they possessed the transgene as determined by PCR. Methylation of the CMV promoter was detected in these cell lines as well as in some of the tumor pieces. This assay could not detect methylation of CMV promoter if there were mixed population of cells with methylated and un-methylated promoter. Therefore it was not possible to detect methylation of promoter in cell lines that had low expression of SLC. Furthermore, the repressor protein is also expressed under the control of CMV promoter in the T-Rex system. Therefore high background expression of SLC in some of the cell lines could be due to methylation and silencing of the repressor

protein promoter. In the cell lines with no SLC expression where we detected CMV promoter methylation the repressor protein promoter was also methylated since this assay could not detect methylation of CMV promoter if there were mixed population of cells with methylated and un-methylated promoter. It has been demonstrated by others that the CMV promoter, a promoter most widely used for transfection, may be silenced in mouse (203) and human (204) cells after transfection.

Regulated expression of GM-CSF by TRAMPC2 cells *in vivo* and *in vitro*

Cytokine-modified cell-based vaccines are currently the major form of cancer vaccine tested clinically (205). Among the different cytokine genes used to modify tumor immunogenicity, GM-CSF is the most potent stimulator of systemic anti-tumor immunity when transduced into autologous tumors (55). The potency of GM-CSF in modulating an anti-tumor effect has been attributed to its role as an important growth and differentiation factor for DCs, at the vaccination site (59, 76). This made GM-CSF a good candidate to be tested for immunotherapy of TRAMPC2 prostate cancer model since TRAMPC2 tumors are infiltrated with a small number of DCs that are phenotypically immature. Therefore GM-CSF could both expand the number of DCs and induce their maturation. Furthermore, it has been shown that the introduction of GM-CSF into tumor cells produced the most active vaccine compared to cytokines such as IL-2 and IFN-γ or the co-stimulatory molecules like B7.1 that provides signal for T cell

activation in the absence of APCs (206, 207). Using different murine tumor models including B16 melanoma, CT-26 colon carcinoma, CMS-5 fibrosarcoma, RENCA renal cell carcinoma and WP-4 fibrosarcoma, Dranoff et al, showed that irradiated GM-CSF expressing tumor cells were more effective than irradiated cells alone in eliciting systemic immunity (55). TRAMPC2 cells transfected with GM-CSF could be induced to express GM-CSF in the presence of tetracycline. However the amount of GM-CSF produced by these cell lines never exceeded 500pg/ml/million cells that was low compared to levels of SLC produced by TRAMPC2/TR/SLC cells that produced 5000pg/ml/million cells. ELISPOT assays on cloned TRAMPC2/TR/GM-CSF cells revealed that <10% of the cells produced GM-CSF. This indicates that most of the cells lost inducible expression of GM-CSF but retained antibiotic resistant genes. Furthermore, the cell lines that were derived from TRAMPC2/TR/GM-CSF (clone 6) prostate tumors had very low or no expression of GM-CSF and eventually died when we tried to select the antibiotic resistant cells to expand for repeated *in vivo* growth. This behavior may reflect loss of the transgene or silencing of the promoter as discussed before. We could not establish a TRAMPC2/TR/GM-CSF that maintained regulated expression of GM-CSF after *in vivo* passage. Therefore, we decided to use TRAMPC2/TR/GM-CSF (clone 6) and study the effect of GM-CSF expression in the TME on tumor growth and metastasis. However, this cell line failed to grow *in vivo* although it grew before that be due to the fact that these cell lines are genetically very unstable and they change dramatically after *in vitro* or *in vivo* passages.

CD40L expressing TRAMPC2 cells were not tumorigenic *in vivo*

CD40L is expressed mainly by activated CD4+ T lymphocytes in highly regulated manner. At any one time a very limited number of circulating T cells are positive for CD40L expression (208). To date, the physiologic role(s) of CD40 on T cells has not been characterized, nor have the mechanisms by which CD40 affects T cell function been defined. However, after engagement of TCR with MHC-peptide complexes on the surface of APCs, T cells rapidly express CD40L. Then, CD40L binding to CD40 causes up-regulation of co-stimulatory/adhesion molecules [B7.1, B7.2, leukocyte function-associated molecule 3 (LFA-3) and intercellular adhesion molecule 1 (ICAM-1)] on the surface of DCs, which provides the second signal required to activate naive T cells, amplify the immune response and prevent anergy or tolerance induction (209). While CD40 itself cannot induce IL-2 production, it augments the CD3 signal and gives maximal stimulation together with CD3 and CD28 signals (210).

We developed an inducible TRAMPC2 cell line that expressed CD40L with addition of tetracycline. To study the extent to which CD40L expression modulates tumor growth, TRAMPC2/TR/CD40L cells was implanted orthotopically into mice. However, after 3 months of observation, no tumors were detected in the prostate gland. This cell line had a low basal level of CD40L expression (11%). It is possible that this low background level of expression was sufficient to induce an immune response that caused the rejection of TRAMPC2 tumor. Grossman *et al.* showed that murine neuroblastoma cells that expressed

CD40L grew slower than the parental cell line. They also showed that not all tumor cells needed to express CD40L to observe the growth inhibitory effect. In fact when only 1.4% of the injected cells were CD40L transfected the tumors were smaller than control (211). Expression of CD40L by tumor cells induced systemic immune response as the number of CD4+ and CD8+ T cells increased significantly in the spleen of CD40L treated mice compared to control (211). Therefore, limited background expression of CD40L by TRAMP tumor cells may have been sufficient to induce an anti-tumor immune response that prevented tumor growth.

A recent study by Dzojic *et al.,* showed that TRAMP-C2 cells that were transduced with an adenoviral vector encoding CD40L were not tumorigenic *in vivo*. They also showed that phenotype of CD40L expressing TRAMPC2 cells compared to the parental cells and they expressed more CD40. CD40L expression by TRAMPC2 cells reduced cell viability and induced apoptosis through activation of caspases. These effects were CD40-dependent since a neutralizing anti-CD40L antibody blocked the reduction in cell viability in a concentration-dependent manner (212). This could be another possible mechanism for the inability of TRAMPC2/TR/CD40L to grow *in vivo* although CD40 was not detectable on TRAMPC2 or TRAMPC2/TR/CD40L cell lines (data not shown).

In summary our studies demonstrated that TRAMP tumor infiltrating DCs are not only phenotypically immature but also fractionate differently than splenic DCs. We also showed that SLC, GM-CSF and CD40L could reverse the

immunosuppressive effect of TRAMPC2 cells on DCs *in vitro* that made them good candidates for *in vivo* studies. Transplantation studies *in vivo* showed that SLC inhibited tumor growth and metastasis in an orthotopic TRAMP prostate cancer model. *In vivo* studies with GM-CSF or CD40L transfected TRAMPC2 cells were not successful due to either the genetic instability of TRAMPC2 cells that resulted in unstable expression of chemokines or the "leakiness" of the system that induced a tumor rejection response.

Expression of SLC in TME inhibits tumor growth and metastasis in the murine prostate cancer model

Dendritic cells, the most potent APCs, play a critical role in the induction of anti-tumor response through a series of functions including antigen capture and processing, up-regulation of co-stimulatory signals, and finally antigen presentation and activation of T cells (165). Efficient transport of DCs into T-cell-rich areas of lymphoid organs is primarily mediated by SLC (213) through CCR7 receptor. Previous works demonstrated that the chemotactic activity of SLC for DCs and T cells could be used to generate anti-tumor immune responses (214-216) and all of these reports indicated that the anti-tumor effect of SLC 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 reports we found that the expression of SLC in TRAMPC2 TME inhibited tumor

growth (Fig. 29). The mice were sacrificed when they all had tumors and the majority of the non-treated mice were sick. This could be the reason that there was not any significant difference in the percentage or composition of TILs from treated and non-treated group, and the infiltrate could be different at earlier time points (Fig. 31 and 32). TRAMPC2/TR/SLC cell lines had high inducible expression of SLC at the time of injection and the mice received tetracycline in their drinking water right after the surgery. Therefore expression of SLC in the TME could change the composition of TILs as the tumor began to establish and grow. However, by the time we sacrificed the mice and studied the infiltrates the cells lost the expression of SLC due to promoter methylation and silencing. Theresore there was not any difference between TILs of treated and control groups. It has been shown that when mice with 5-day-old lung cancer were administered with SLC for two weeks, the frequency of $CD4^+$ and $CD8^+$ T cells and $CD11c^+$ DCs that infiltrated both the tumor and lymph nodes increased (136). Although SLC seemed to inhibit tumor growth when its expression started soon after tumor injection, it did not delay tumor growth when expression was induced after the appearance of palpable tumors (Fig. 35). Ochsenbein *et al.* showed that sarcoma cells expressing a strong viral tumor antigen were capable of inducing a protective cytotoxic T cell response if transferred as a single cell suspension. However, if they were transplanted as small tumor pieces, they did not induce a CTL response and tumors readily grew (217). Therefore, it seems that tumors with established microenvironment prevent presentation of tumor antigens in the draining lymph nodes and consequently, tumor specific T cells

can remain ignorant. Liang *et al.* showed that intra-tumoral injection of recombinant adeno-associated viruses vector (rAAV)-SLC into the established tumor also inhibited tumor growth, but the pre-transduced tumor cells, which expressed SLC from the time of administration, displayed a stronger anti-tumor response (218).

In the next study we asked the question whether SLC expression in TME would increase survival and decrease metastasis of TRAMPC2/TR/SLC tumors. Therefore, mice were sacrificed when they became morbid at different time points. Treated mice lived longer and had less metastatic disease compared to non-treated mice. Consistent with our data, Liang et al showed that long-term and local expression of SLC in the TME mediated by rAAV-SLC caused delay in progression of ectopic Hepal-6 liver tumors and also reduced tumor weight in these mice compared to tumors that expressed GFP (218). They also showed that SLC generated systemic anti-tumor responses, accompanied by extensive infiltration of $CD11c^+$ DCs and $CD4^+$ and $CD8^+$ T cells into the tumor site, especially activated $CD3^+$ $CD69^+$ T cells (218). As mentioned before SLC is expressed in high endothelial venules (HEVs), the main entrance of lymphocyte into peripheral lymph nodes and spleen (133) that may help organize T cells/DCs co-localization and interaction. Immunohistochemical staining of tumor sections showed that infiltration of T cells and DCs into the tumor formed a new lymphoid-like tissue within the established tumor (218). Furthermore, expression of SLC in transgenic mice with islet β-cell-specific expression of SLC has been shown to trigger formation of lymphoid-like tissue in the pancreatic islets by recruiting the

lymphocytes and DCs to pancreatic islets (219). Thus, these results coincident with previous studies showed that local expression of SLC in the TME elicited anti-tumor effects by induction of lymphocyte infiltration as well as facilitating their interaction.

Although SLC is important in recruiting DCs and T cells and is classified as a CC chemokine (binds to CCR7 receptor), murine SLC has been shown to bind to CXC chemokine receptor CXCR3 (220). This is a property that SLC shares with two other angiostatic chemokines, interferon-inducible protein 10 (IP-10) and monokine induced by interferon-γ (MIG) (221). Therefore anti-tumor activity of SLC can also be associated with its angiostatic activity through binding to CXCR3 receptor. Arenberg *et al.*, showed that injection of SLC 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 (222).

Many tumors produce chemokines, which may explain the presence of tumor-associated leukocytes. These chemokines may assist in immuno-surveillance and help to eliminate the tumor (223, 224). However, they may also promote tumor growth and invasion. Chemo-attracted host-derived inflammatory cells that infiltrate tumor tissues can create an environment that favors tumor progression. They promote tumor angiogenesis and tumor progression by producing angiogenic factors and matrix-degrading enzymes, respectively (198, 225). Yang *et al.* demonstrated that tumors could use different mechanisms to promote tumor development and growth. They demonstrated that immature

granulocytes consist about 5-6% of the leukocytes infiltrating colorectal cancer (MC26) and Lewis lung carcinoma (3LL) tumors. The percentage of these cells also increased in tumor bearing spleen compared to normal spleen. Tumor growth was increased when tumor cells were co-injected with Gr-1^{+}CD11b^{+} cells purified from the spleen of tumor bearing mice suggesting that these cells promoted tumor growth. Furthermore, increased tumor angiogenesis, vascular maturation, and decreased tumor cell apoptosis and tumor necrosis were observed in tumors co-injected with Gr-1^{+}CD11b^{+} cells that was mainly related to production of matrix metalloproteinase 9 (MMP9) by these cells (226). Studies have shown that MMP9 functions as an angiogenic switch during tumorigenesis by releasing vascular endothelial growth factor (VEGF) from the matrix (227). Importantly, deletion of MMP9 abolished the angiogenesis-promoting activity (226). Tumor associated macrophages can also release a number of potent growth and angiogenic factors such as platelet-derived growth factor, epidermal growth factor, fibroblast growth factor, and IL-8 (228-230). Furthermore, Lin *et al.*, showed that the absence of macrophages from the TME in the mammary-tumor prone mouse model markedly delayed benign to malignant transition and pulmonary metastasis (231). We could not detect any major phenotypic differences between the infiltrates of tumors that expressed SLC and control tumors when morbid animals were evaluated. However, we cannot exclude the possibility that differences in the cellular composition of the infiltrate existed during early tumor growth of TRAMPC2 and TRAMPC2/SLC.

CHAPTER VI

CONCLUSION AND FUTURE DIRECTIONS

Dendritic cells are known as the most efficient antigen-presenting cell type with the ability to interact with T cells and initiate an immune response. Dendritic cells are receiving increasing scientific and clinical interest due to their key role in the immune response (71). However tumors have been shown to be immunosuppressive and prevent initiation of the immune response by different mechanisms. We showed that at least one possible mechanism for TRAMP tumor cells to escape immune recognition could be the immature phenotype of DCs in the TME. Maturation of DCs is a required step for T cell activation and induction of immune response. In this study we showed that although TRAMP TME is immunosuppressive and prevents maturation of tumor infiltrating DCs but these DCs are not refractory to activation by bacterial stimuli. This is important since the major goal of this study was to generate vaccines to induce an immune response by co-localizing DCs and T cells and/or inducing DC maturation. Although expression of CD40L and GM-CSF by TRAMPC2 cells caused DC maturation to some extent *in vitro* but these cell lines did not grow *in vivo* that could be due to the fact that these cell line had some background expression of the cytokine that could be enough to generate sufficient immune response to reject the tumor.

Expression of SLC *in vitro* did not induce DC maturation when it was secreted from TRAMPC2 tumor cells, however when it was expressed *in vivo* it delayed tumor growth and prolonged survival of tumor bearing mice. The

mechanism of SLC function could be co-localization of DCs and T cells rather than induction of DC maturation. Furthermore, SLC reduced the frequency of metastasis. Expression of SLC in TRAMP TME did not have any effect on the growth of established tumors however this could be due to loss of SLC transgene expression through methylation of its promoter. It seemed that gene silencing through promoter methylation could be a possible mechanism for loss of transgene expression by TRAMPC2 cells therefore replacing the CMV promoter with a promoter that is less susceptible to methylation could actually prevent the loss of transgene expression by TRAMPC2 cells. The replacement of the promoter can help with the leakiness of these cell lines and lower the background expression of the cytokines. This is important specifically in case of CD40L and GM-CSF as the inability of these cell lines to grow in vivo could be due to immune response induction through the background expression.

We did not detect any difference between the infiltrate compositions of TRAMP tumors that expressed SLC or control group. We phenotyped the infiltrates when the mice had progressed disease and it seemed that the expression of SLC had role in preventing the establishment of tumors and did not affect the growth of established tumors. This could also be the result of promoter methylation that seemed to cause silencing of the SLC gene throughout the tumor growth. Therefore the infiltrate composition of treated and not treated mice could be different at earlier time points when the tumor is just established and tumor cells still secret SLC in the tetracycline treated mice and SLC gene is not silenced due to promoter methylation.

Flt3-L is known to be a haematopoetic growth factor for both myeloid and lymphoid precursors (108). Systemic injection of flt3-L into the mice with established TRAMP tumors induced growth inhibition and prolonged their survival (115). The tumor growth inhibition was correlated with the infiltration of mainly myeloid cells but not T cells (115). This therapy alone was not successful and did not induce long-term immunity because disease relapse occurred after the termination of treatment. Furthermore myeloid cells are known to be immunosuppressive and it has been shown that elimination of these cells improved CD4 and CD8 mediated tumor-specific immune response (232). On the other hand we showed that expression of SLC in TRAMP TME also enhanced the survival of tumor bearing mice and inhibited tumor growth. The major function of SLC is known to be attraction of DCs and T cells. Therefore combination of these therapies can induce a more potent immune response. To avoid consecutive daily injections transgenic mice that conditionally express murine flt3-L in the presence of tetracycline could be used (110). Expression of flt3-L in these transgenic mice led to dramatic increases in the number of DC in multiple tissues and expansion of different subsets of DCs including $CD8^+$ DCs (110). Treatment of flt3-L transgenic mice implanted with TRAMPC2/TR/SLC tumor cells with tetracycline would cause expression of both flt3-L and SLC. Flt3-L would expand the number of DCs and SLC that is expressed by the tumor cells would attract DCs and T cell in the TME. The interaction of these cells can induce a potent immune response to prevent tumor growth.

Delivering a therapeutic gene that encodes a molecule that directly or

indirectly kills tumor cells is an attractive approach in cancer gene therapy. Gene modified cancer vaccines are usually composed of autologous tumor cells stably transfected with an immunostimulatory gene (207). The original hypothesis was that the expression of cytokines or the co-stimulatory molecules would allow the tumor cell to provide all of the signals for activation of T cells, bypassing the need for host APCs (76, 206). However, preclinical models showed that introduction of GM-CSF that expands the number of DCs into tumor cells produced the most active vaccine (55). These cytokine-modified autologous tumor cell vaccines have been tested in clinical trials for several years (129). The manufacture of autologous tumor cell vaccines requires tumor cell cultures from each patient, transfection of these cells and selection of cells that express the transgene. To avoid this long process, other methods have been explored, including the use of allogeneic gene-modified tumor cell vaccines, transfection of autologous non-cancerous cells that are easier to obtain and gene-modify (usually fibroblasts) or the use of other bystander cells coinjected with autologous tumor cells (233). These strategies that decrease vaccine production time could be applied to generate prostate cancer vaccines using flt3-L and/or SLC.

In summary our studies demonstrated that TRAMP tumor infiltrating DCs are not only phenotypically immature and this immature phenotype was induced by TRAMP tumor cells but not $GR1^+$ cells infiltrating the tumor. We also showed that GM-CSF and CD40L could reverse the immunosuppressive effect of TRAMPC2 cells on DCs *in vitro* that made them good candidates for *in vivo* studies. *In vivo* studies with GM-CSF or CD40L transfected TRAMPC2 cells were

not successful due to either the genetic instability of TRAMPC2 cells that resulted in unstable expression of chemokines or the "leakiness" of the system that induced a tumor rejection response. SLC was not as effective as CD40L and GM-CSF to induce DC maturation *in vitro* however, transplantation studies *in vivo* showed that SLC inhibited tumor growth and metastasis in an orthotopic TRAMP prostate cancer model.

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APPENDICES

A. ADDITIONAL REAGENTS:

Tumor digestion buffer (in 20ml 1X PBS):

20mg/20ml Collagenase Type I (Sigma)

0.20mg/20ml DNase I (Sigma)

50U/20ml Hylauronidase (Sigma)

Red blood cell lysis buffer:

Reagent 1: 15gr NH_4Cl added to 1L of sterile, distilled water.

Reagent 2: 12.5ml Na_2HPO_4 (0.5M)

3.9ml sterile NaH_2PO_4 (0.5M)

The solution was brought up to 240ml with sterile water. The pH was adjusted to 7.2 using concentrated HCl and filter sterilized.

To prepare the red blood lysis buffer:

1 part reagent1 was mixed with 1 part reagent 2 and stored at 4°C.

Wash buffer for flow cytometery:

0.1% Goat serum

0.01% sodium azide in 1X PBS

B: ANTIBODIES

Optimal concentrations of antibodies used for flow cytometry:

Antibody	*Concentration/10^6 cells*	*Source*
APC-GR1	0.1µg	eBioscience
FITC-CD8a	0.13µg	eBioscience
PE-F4/80	0.13µg	eBioscience
APC-CD11c	0.1µg	eBioscience
Cy7-B220 (CD45R)	0.13µg	eBioscience
Biotin-anti-GR1	0.1µg	eBioscience
FITC-MHC class II	0.5ug	eBioscience
FITC-MHC class I	0.5ug	eBioscience
Cy7-CD86	0.1ug	Biolegend
PE-CD80	0.1ug	eBioscience
PE-CD40	0.1ug	eBioscience
PE-B7-DC (PD-L2)	0.1ug	eBioscience
PE-CCR7	0.1ug	eBioscience
PE-CD154 (CD40L)	0.1ug	eBioscience

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Title of thesis: Gene therapy to modulate prostate tumor microenvironment using tet-repressor system.
09.1996 - 02.1999: M.S. Degree in Biochemistry, Middle East Technical University (METU), Faculty of Arts & Science, Department of Biochemistry, ANKARA-TURKEY (CGPA 3.64).
Title of thesis: Synthesis and Characterization of a Cyclodextrin based artificial Enzyme.
09.1989 - 09.1993: B.S. Degree in Chemistry, Shahid Beheshti University, Department of Chemistry, TEHRAN- IRAN (CGPA 2.99).
09. 1984 - 07. 1988: High School Degree from Bu Ali Sina High School, TEHRAN- IRAN.

Publications:

1- Somers KD, Brown RR, Holterman DA, Yousefieh N, Glass WF, Wright GL Jr, Schellhammer PF, Qian J, Ciavarra RP. Orthotopic treatment model of prostate cancer and metastasis in the immunocompetent mouse: efficacy of flt3 ligand immunotherapy. **Int J Cancer. 2003 Dec 10;107(5):773-80.**
4- Yousefieh N, Jones C, Ciavarra RP. Impact of the prostate tumor microenvironment on host cell ROS production: identification and molecular characterization of effector molecules. *(manuscript in preparation)*
5- Yousefieh N, Ciavarra RP. Secondary lymphoid tissue chemokine inhibited tumor growth and metastasis of prostate tumor when expressed in the tumor microenvironment. *(manuscript in preparation)*

Presentations:

1- Poster presentation, Experimental Biology 2008, San Diego
Yousefieh N, Ciavarra R.P.
Title: CCL21 (SLC) inhibits primary prostate tumor growth and metastases
2- Oral presentation, The American Association of Immunologists meeting 2008,
Yousefieh N, Ciavarra R.P.
Title: CCL21 (SLC) inhibits primary prostate tumor growth and metastases

Award:

The American Association of Immunologists trainee abstract award, 2008