NORTHWESTERN UNIVERSITY

TGF-beta and Cancer Development: The Role of TGF-beta in Converting $CD4^+CD25^-$ T Cells into $CD4^+CD25^+$ T Regulatory Cells and A TβRIIDN-tk/Ganciclovir Suicide System in Cancer Gene Therapy

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

for the degree

DOCTOR OF PHILOSOPHY

Field of Cancer Biology

By

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EVANSTON, ILLINOIS

June 2007

ABSTRACT

TGF-beta and cancer development: the role of TGF-beta in converting $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T regulatory cells and a TβRIIDN-tk/Ganciclovir suicide system in cancer gene therapy

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Initial studies described the ability of $CD4^+CD25^+$ T regulatory (T_{reg}) cells in suppressing autoimmune diseases in animal models. An emerging interest has focused on the potential role of T_{reg} cells in cancer development and progression as they have been shown to suppress anti-tumor immunity. In the present study, $CD4^+CD25^-$ T cells cultured in conditioned medium (CM) derived from tumor cells RENCA or TRAMP-C2, possess characteristics as those of naturally occurring T_{reg} cells. Further investigation revealed a critical role of tumor-derived TGF-β in converting $CD4^+CD25^-$ T cells into T_{reg} cells, as a neutralizing antibody against TGF-β, 1D11, completely abrogated this conversion. CM from a non-tumorigenic cell line, NRP-152, or irradiated tumor cells did not induce T_{reg} cell conversion because they produced low levels of TGF-β. We also observed a reduced tumor burden in animals receiving 1D11. This reduction was associated with a decrease in T_{reg} cell conversion. In summary, we demonstrated that tumor cells can directly convert $CD4^+CD25^-$ T cells to T_{reg} cells through production of high levels of TGF-β, suggesting a possible mechanism through which tumors evade the immune system.

Blockage of TGF-β signaling in immune cells using a dominant negative TGF-β type II receptor (TβRIIDN) resulted in resistant to tumor challenge and the development of inflammatory diseases. To resolve this issue, we incorporated the HSV-tk/ganciclovir (GCV) suicide system in our original approach. In the present study, we developed the fusion gene TβRIIDN-tk and tested its efficacy. In the presence of GCV, NIH3T3 cells and moue bone marrow cells transduced with TβRIIDN-tk showed no proliferation and were not viable, while vigorous proliferation was observed in cells infected with the control vector. In addition, NIH3T3 cells infected with TβRIIDN-tk were insensitive to TGF-β while the control vector infected cells were responsive. Taken together, the results of our studies demonstrated the potential of TβRIIDN-tk/GCV suicide system as a powerful tool in eradicating tumors as well as preventing the development of autoimmunity. The ultimate goal of this study is to use this approach in human clinical trial in the treatment of cancer.

For my mother, 童先玉, who gave me the gift of life and opened my eyes to all the beauties in the world; who had set me free and become the best part of myself; who loved me endlessly and accompanied me for 21 years of my life; who will forever be missed and loved, and whose beautiful soul will always be with me and my children.

For my little cousin, 李辉, who generously shared all 12 years of his life with me and showed me the wonderful magic of his world; whom I never had a chance to say "good-bye", and whose sweet little smile will always be in my heart.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

FUNDAMENTAL TGF-β BIOLOGY

TGF-β and latency

Transforming growth factor–beta (TGF-β) was first discovered in 1981. It was found to transform normal rat kidney and fibroblast cell lines to induce anchorage-independent growth, thus the name transforming growth factor (1, 2). It soon became apparent that TGF-β is a multifunctional growth factor with a variety of biological effects involving embryonic development, tumorigenesis, fibrosis, wound healing, hematopoiesis, and immunoregulation (3-5). The name "TGF-β" is a generic name for five structurally related growth factors known as TGF-β1-5. The common isoforms, TGF-β 1-3, were identified in mammalian cells, and TGF-β 4 and 5 were found in chicken and *Xenopus* cells, respectively (6-9). This family of proteins is highly conserved. They are 25kD dimers comprised of two disulfide-bonded mature polypeptide chains (10). TGF-β is secreted by many cell types with a broad spectrum of biological effects, but is primarily inhibitory in most cells (10, 11).

The production of TGF-β is highly regulated. When it is first secreted, TGF-β is usually in a "latent" form (11). Latency is one of the mechanisms to regulate the growth factor activity by maintaining it in an inactivated form to prevent the activation before it reaches target cells. Since latent TGF-β is unable to bind to its receptors, the activation of latent TGF-β represents a critical regulation mechanism of TGF-β activities (11, 12). TGF-β precursor, when synthesized,

is a dimeric protein that is later cleaved to produce a mature TGF-β during secretion. The mature TGF–β is non-covalently associated with latency-associated peptide (LAP) to confer latency of this cytokine (11-14). Another protein, latent TGF-β binding protein (LTBP), is also associated with the latent TGF-β complex (11, 12). The association of LTBP has been shown to promote the proper folding of the latent TGF-β molecule (15), and may prevent latent TGF-β from binding to cell surface (11).

Studies in epithelial and smooth muscle cells convincingly demonstrated that the activation of latent TGF-β is mediated by plasmin (13, 14, 16-18). Plasmin cleaves the latent TGF-β at the amino-terminal glycopeptide and this cleavage causes a disruption of tertiary structure and non-covalent bonds, therefore activating TGF-β (14). TGF-β can also be activated by thrombospondin-1 (TSP-1) through a protease-independent manner, evidently through the induction of a conformational change in LTBP which releases the active form of TGF-β (19, 20). Recent studies have shown that integrins play an important role in activation of latent TGF-β as well, apparently through the integrin-binding domain on the TGF-β (19, 20). Finally, it has been reported that treatments with physical and chemical agents, including heat and acid, can activate latent TGF-β as well (12).

TGF-β Signaling Transduction

The mechanism of TGF-β signaling to the nucleus is a fairly simple process. It is mediated through its membrane receptors (Type I and II) and the family of transcription factors

Smad (R-Smad, Co-Smad, and I-Smad) (3, 21, 22). TGF-β receptor I (TβRI) is kept inactive in the basal state by a wedge-shaped glycine- and serine-rich sequence, termed GS region, that blocks the kinase domain, dislocating its catalytic center (22). As illustrated in Figure 1, when TGF-β binds to TGF-β receptor II (TβRII), it induces the association of TβRI and TβRII. In this complex, TβRII phosphorylates the GS domain, of TβRI, thus activating this receptor (23). Activated TβRI then phosphorylates receptor Smads, R-Smads, such as Smad2 and 3. The phosphorylation increases R-Smads affinity for common (Co-) Smads like Smad4 to form a complex. The resulting Smad complex is then free to move to the nucleus to regulate gene expression through association with transcriptional co-activators or co-repressors (21, 22). Smad6 and 7 are inhibitory Smads (I-Smads) that inhibit TGF-β induced signaling transduction by competing binding of R-Smads to Co-Smads. Termination of TGF-β signaling involves Smad ubiquitination in the nucleus followed by proteasome-mediated degradation of Smad proteins (22). In addition to type I and type II receptors, TGF-β can also signal through type III receptor (TβRIII or betaglycan) and endoglin, which are believed to influence the availability of activated TGF-β to the type II receptor (24). TβRIII binds to activated TGF-β and therefore regulates the availability to TβRII, while endoglin has been shown to down-regulate TGF-β signaling on certain cell types (24).

It is important to notice that although TβRII lacks the intracellular cytoplasmic tail and therefore the kinase activity, it is still capable of binding to TGF-β; however, it is unable to form a complex with TβRI. Consequently, this truncated TβRII is unable to elicit downstream

signaling effects of TGF-β. The truncated TβRII blocks TGF-β signaling and is known as a dominant negative TGF-β type II receptor, TβRIIDN. TβRIIDN has been utilized in gene therapy experiments as demonstrated by earlier studies in our laboratory (25, 26). In these studies, mice receiving either bone marrow cells (BMCs) or $CD8^+$ T cells that had been rendered insensitive to TGF-β by TβRIIDN expression were able to reject tumor challenge, while control mice succumbed to tumors. Both studies demonstrated the potential of TβRIIDN as a powerful tool in the treatment of cancer.

ROLE OF TGF-β IN IMMUNE REGUALTION

TGF-β and Immune System

Members of TGF-β family have been extensively studied in the field of immunology due to their potent negative immunoregulatory effects. Although TGF–β can sometimes stimulate immune cells, as in the case of macrophage activation, the overall function of TGF-β in regulating the immune system is negative. In the case of T cell regulation, studies have demonstrated that TGF-β inhibits interleukin-2 (IL-2) production by T lymphocytes, thus inhibiting T cell proliferation (27). In addition, TGF-β has been shown to inhibit the ability of naive CD4 and CD8 T cells to develop into mature effector T cells, T_H1 or T_H2 (28), and cytotoxic lymphocyte (CTL) respectively (29). Furthermore, TGF-β can decrease the production of pore-forming protein (PFP) and Granzyme B, both critical for CTL functions (30, 31).

Overall, the results of these studies demonstrated a critical role of TGF-β in regulating T cell activation and functions.

Over the past two decades, the important function of TGF-β in regulating the immune system has been best demonstrated in the knockout animal studies. These studies showed that mice lacking TGF-β develop a rapid wasting syndrome and die by 3-4 weeks of age, despite the normal growth of first 2 weeks (32-34). The presence of elevated antibody levels in sera against dsDNA, ssDNA, and ribonucleoproteins indicated a systemic lupus erythematosus (SLE)-like lymphophroliferative syndrome (35). The massive inflammation was revealed by histopathological analysis in multiple organs of these mice including heart, stomach, liver, lung, pancreas, salivary glands, and striated muscles (32-34). The infiltrates were identified as lymphocytes and macrophages. The massive inflammatory syndromes were later characterized as autoimmunity (33). In a double knockout study, TGF-$\beta^{-/-}$ MHCII$^{-/-}$ mice had an increased survival rate and decreased tissue inflammation, indicating the role of CD4^{+} T cells in inflammation of TGF-β knockout mice (36, 37). Of note, knockout studies in mice lacking either TGF-β2 or TGF-β3 revealed a role of these cytokines more in the embryonic development (38-40). Furthermore, transgenic studies also demonstrated the crucial role of TGF-β in regulating autoreactive T cells. In these studies, a conditionally expressed dominant negative TGF-β type II receptor was used to block the TGF-β signaling specially in T cell compartment. The results from these studies showed spontaneous differentiation of most T cells in these transgenic mice to a CD44^{+} memory-like phenotype (41, 42). These mice eventually developed inflammation of lung and GI tract about 6 months of age, suggesting a role for TGF-β signaling

in suppressing autoimmunity, particularly in T_H cells. Taken together, these *in vivo* studies have demonstrated a critical role of TGF-β in regulating the immune system.

The role of TGF–β in regulating macrophage functions has been shown to be both stimulatory and inhibitory (5). Earlier studies have demonstrated that TGF–β is a potent chemoattractant for human blood monocytes and it can activate the phagocytic activity of macrophages (5, 43). In addition, TGF-β stimulates mononuclear cells to produce interleukin 1 (IL-1) which in turn attracts more mononuclear cells (5, 43). Furthermore, the phagocytic activity of macrophages is also activated by TGF–β (5). However, TGF–β can also deactivate macrophages by suppressing the production of nitric oxide (NO) and reactive oxygen intermediates (44, 45), and therefore decrease macrophage killing ability in parasitic infections (46). The deactivation of macrophages by TGF–β is significant in that macrophages have been shown to have anti-tumor activities both *in vitro* and *in vivo* (47-51). Taken in sum, these studies have established the critical role of TGF–β as a potent negative regulator of the immune system.

ROLE OF TGF-β IN CANCER

TGF-β and Cancer

The relationship between TGF-β signaling pathway and cancer cells is complex owing to the seemingly contradictory fact that TGF-β is suppressive at the early state of tumor development, yet it promotes tumor progression during the later malignant state (52, 53). Overall, functions of TGF-β are suppressive to cell growth. Therefore, the TGF-β signaling

pathway is expected to provide protection against malignant transformation. Indeed, animal studies have shown TGF-β as a tumor suppressor. Transgenic mice producing a constitutively active form of TGF-β1 were shown to be resistant to 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumor formation (54). Conversely, TGF-β1 null mouse embryos have been shown to be highly susceptible to malignant conversion by v-ras_{Ha} oncogene (55). Moreover, heterozygous (TGF-$\beta^{+/-}$) animals were more prone to develop malignant lung and mammary tumors or pituitary adenomas than the wild-type (TGF-$\beta^{+/+}$) animals (56, 57). These studies have established the role of TGF-β as a tumor suppressor gene during the early state of tumor development.

During the late malignant state, TGF-β promotes the tumor progression. This is because many types of tumors have mutations in TβRI and TβRII genes, as seen in prostatic carcinoma cell line, pancreatic and biliary carcinomas, and breast carcinomas (52). Therefore the tumors lose the sensitivity to TGF-β-induced cell growth inhibition (52). Furthermore, it has been shown in ample examples that most tumor cells produce high levels of TGF-β, including brain tumors (58), head and neck tumors (59), lung cancer (60), breast cancer (56), digestive tract organ malignancies (61), and prostate cancer (62). The high levels of tumor-derived TGF-β can impair immune responses against tumor cells, therefore conferring a growth advantage to tumors.

TGF-β and Tumor Evasion of the Immune System

Tumor cells evade the immune system through various mechanisms, which can be generally summarized in three categories: down regulation of tumor associated antigens (TAAs), tumor-derived soluble factors to modulate the immune response, and functional impairment of immune cells in the tumor microenvironment (63, 64). Major histocompatibility complex (MHC) molecules are often down regulated in many types of tumors because they are crucial in antigen presentation and T cell priming and activation (65-68). Besides MHC molecules, transport associated peptide (TAP), which is a critical component of antigen presentation in the class I pathway, is also down regulated in some types of tumors (69-72). Among TAAs, decreased expression of gp100 and MART-1 were shown to be associated with tumor progression (73, 74). The down regulation of these molecules on the tumor cell surface could render immune cells ineffective against tumors and this is a concern in immunotherapy that targeted against single TAA.

Secreting soluble factors that negatively regulate the immune system such as TGF-β and IL-10 is another mechanism through which tumor cells evade the immune system. These soluble factors serve as broad spectrum immunosuppressants that modulate the anti-tumor immunity (63, 75). Many types of tumors have been shown to produce high levels of TGF-β (58-62). As it has been well established, TGF-β is a potent immunosuppressant (5). Consequently, high concentrations of TGF-β could create a favorable microenvironment for tumor growth by suppressing immune responses. For instance, high concentrations of tumor-produced TGF-β could make a “firewall” surrounding the tumor. As a result, immune responses against tumor

cells, such as those of T lymphocytes and macrophages, would be significantly down-regulated by high levels of TGF-β in the tumor microenvironment, thus conferring a growth advantage to tumor cells (Fig. 2).

Lastly, tumor cells can impair T cell function by various mechanisms. Lack of B7 co-stimulatory molecules on tumor cell surface renders T cells anergic (76, 77), while expression of Fas ligand (FasL) on tumor cells triggers apoptosis in T cells (78, 79). Recently, the presence of $CD4^+CD25^+$ T regulatory cells has been reported in the tumor microenvironment and the presence of such T cells is correlated with poor prognosis in patients (80). The regulation of T regulatory cells in the tumor microenvironment is further discussed in the following session. In summary, ample evidence has suggested that the immune system is capable of eradicating tumors given favorable conditions to break the tolerance that is induced by tumor cells. The challenge, however, is how to fine-tune the immune system to generate effective responses against tumors as well as prevent the development of autoimmunity after the eradication of tumors.

$CD4^+CD25^+$ T REGULATORY CELL BIOLOGY

$CD4^+CD25^+$ T regulatory cells

$CD4^+CD25^+$ T regulatory (T_{reg}) cells are a unique lineage of T cell populations that exert their ability to regulate immune responses by suppressing $CD4^+CD25^-$ T cells proliferation. Their role in protecting animals against autoimmune diseases has been well established (81, 82).

Study of regulatory T cells started in the early 1970s. It was first proposed by Gershon that T lymphocytes could function as helper cells as well as regulatory cells to suppress immune responses (83-86). It was a prevailing idea from the 1970s to the 1980s that T_{reg} cells were a specialized lineage of T cells that exerted their suppressive ability on other T cell proliferation by secreting antigen-specific factors (84-86). However, the failure to identify such factors led to the demise of the entire field. Not until the mid-1990s did this field find its way back to become one of the hottest areas of study (84-86). The comeback of this field is attributed to studies conducted by Sakaguchi *et al*, which showed that fewer than 10% of $CD4^+$ T cells co-expressing interleukin-2 receptor (IL-2-R) α-chain (CD25) were crucial for control of autoimmunity *in vivo* (87, 88). Following these studies, several other groups have also shown the importance of $CD4^+CD25^+$ T_{reg} cells in autoimmunity development *in vivo* as well as their characteristics being both hyporesponsive and suppressive (84-86). Today, it has been well established that $CD4^+CD25^+$ T_{reg} cells are a major player in regulation of the immune system to prevent autoimmunity by suppressing harmful autoreactive immune cells.

Recently, emerging interest has focused on the association between T_{reg} cells and cancer development. T_{reg} cells have been shown to suppress immune responses against tumors and depletion of these T cells *in vivo* enhances anti-tumor immunity and facilitates tumor rejection (89-92). The relationship between $CD4^+CD25^+$ T_{reg} cells and cancer development will be discussed more in detail in the following session.

ROLE OF $CD4^+CD25^+$ T REGULATORY CELLS IN CANCER DEVELOPMENT

$CD4^+CD25^+$ T regulatory cells and cancer

A key aspect of tumor immunology is immune surveillance in the tumor-bearing host. It has been almost half a century since the concept of "immune surveillance" was first proposed by Burnet (93). This hypothesis has been debated and tested over the years. Despite the ability to generate immune reactivity against tumor antigens, the immune surveillance program can be overpowered by tumors with eventual tumor progression (94-97). Tumor-infiltrating lymphocytes (TILs) were identified in both human and murine tumors and were shown to recognize and reject tumor cells in immunotherapy (96, 98). Despite their ability to infiltrate into tumor sites, the presence of TILs is not always beneficial. Although a better prognosis in patients with elevated TILs has been observed in several studies, the mere presence of TILs is insufficient to reject tumors (99-101). Rather, the type of infiltrating cells is the essential factor in tumor rejection (80, 102-105). Numerous studies have shown that $CD4^+$ helper T cells and $CD8^+$ cytotoxic T lymphocytes (CTL) are beneficial in anti-tumor immunity whereas the presence of $CD4^+CD25^+$ T_{reg} cells is detrimental as the presence of T_{reg} cells is associated with a reduced survival rate in patients (80, 102-105).

The majority of earlier studies focused on the ability of $CD4^+CD25^+$ T_{reg} cells to suppress autoimmune diseases in animal models (84-86). Recently, however, studies have been concentrated on the association between $CD4^+CD25^+$ T_{reg} cells and cancer development. T_{reg} cells have been shown to suppress immune responses against tumors. Depletion of $CD4^+CD25^+$

T_{reg} cells *in vivo* enhances anti-tumor immunity and facilitates tumor rejection (90, 92, 106, 107). Moreover, T_{reg} cells can suppress activation and proliferation of $CD4^+$ T cells and NK cells as well as $CD8^+$ T cells (108).

$CD4^+CD25^+$ T_{reg} cells inhibit target cells through a wide range of cytotoxic cytokines. In addition to expressing TGF-β, these cells produce interleukin-10 (IL-10), T lymphocyte-associated antigen-4 (CTLA-4), glucocorticoid induced TNF receptor (GITR) and Foxp3 transcription factor (109-111). Interestingly, TGF–β plays a critical role in mediating the function of these $CD4^+CD25^+$ T_{reg} cells (112). When activated by the T-cell receptor (TCR) and stimulated by TGF-β, $CD4^+CD25^-$ T cells transform into $CD4^+CD25^+$ T suppressor cells (111, 113). Since tumor cells are a rich source of TGF-β, it is reasonable to speculate that $CD4^+CD25^+$ T_{reg} cells are present in the tumor site as shown in many studies, thus implementing tumor-mediated immune suppression (80, 104).

There are at least two subsets of T_{reg} cells. One subset, known as naturally occurring T_{reg} cells, develops during the normal process of T cell maturation in the thymus. The other subset develops as a consequence of activation of mature T cells under particular conditions in the periphery (92, 114). The negative regulatory role of T_{reg} cells has been amply demonstrated, suggesting that the presence of T_{reg} cells in the tumor microenvironment could indeed promote, instead of suppressing, tumor progression by inhibiting anti-tumor immunity (104, 105, 107, 115). However, the origin of these T_{reg} cells in the tumor sites remains elusive. It is unknown whether they are naturally occurring T_{reg} cells that are recruited to the tumor sites or whether

they arrive at tumor sites originally as $CD4^+$ T helper cells that are later converted to T_{reg} cells in the tumor microenvironment. Since the presence of T_{reg} cells in the tumor sites is associated with poor prognosis in patients, identification of the origin of these cells will provide valuable information in treatment of cancer. The present study explored the possible origin of such $CD4^+CD25^+$ T_{reg} cells present in tumor masses and provided evidence of the importance of tumor-derived TGF-β and its role in T_{reg} cell conversion in the tumor microenvironment

TGF-β AND CANCER GENE THERAPY

Several current approaches to cancer gene therapy focus on identification of tumor-specific antigens and the cytolytic T cells specific for these antigens (116). When such antigens are defined, therapeutic approaches could use these antigens as a target (117-119). In cases where the antigen for a particular tumor has not been identified, tumor associated immune cells were manipulated for adoptive transfer with various degrees of success (120). Given the fact that tumor cells produce high levels of TGF-β to evade the immune surveillance, theoretically, if TGF-β is removed from the tumor cells, their growth should be inhibited by the host immune system. Alternatively, one could render the host immune cells insensitive to TGF-β allowing them to eradicate tumor cells. Both approaches have been confirmed by the earlier studies in our laboratory (26, 121, 122).

One such study conducted by Shah *et al* has demonstrated that TGF-β insensitive immune cells can boost host immune function and eradicate tumor cells (26). BMCs from donor

mice were rendered insensitive to TGF-β via retroviral expression of a dominant negative TGF–β receptor II (TβRIIDN) construct and were transplanted in irradiated recipient mice prior to tumor challenge. Following intravenous administration of 5 x 10^5 B16-F10 murine melanoma cells into TβRIIDN-BMC transplanted recipients, survival at 45 days was 70% (7/10) vs. 0% (0/10) for vector-control treated mice. Surviving TβRIIDN-BMC mice showed a virtual absence of metastatic lesions in the lung. The study also investigated the utility of the TGF-β insensitive bone marrow cells in a mouse metastatic model of prostate cancer, TRAMP-C2. Treatment of male mice with TβRIIDN-BMC resulted in survival of 80% (4/5) of recipients vs. 0% (0/5) in GFP-BMC recipients or wild-type controls. These results suggest that a gene therapy approach inducing TGF-β insensitivity in immune cells may be a viable anti-cancer strategy. However, recipient animals treated by this approach eventually developed widespread multi-organ inflammatory conditions, suggesting autoimmune disease. This finding confirmed the hypothesis that, if the host immune cells are rendered insensitive to TGF-β, these immune cells would be able to eliminate tumor cells. These results are highly encouraging, as there is a possibility that this approach will be able to achieve a cure for cancer.

The TβRIIDN-transduced BMCs led to the myeloid expansion, primarily monocytes/macrophages, and inflammatory diseases associated with cachexia and mortality (122). The TβRIIDN mice appeared to be normal for about 1-2 months after bone marrow transplant, but began to exhibit a progressive cachexic phenotype at time points between 2-3 months, including ruffled fur, hunched posture, and dramatic weight loss of nearly 50%

compared to the littermate GFP control. The mortality of TβRIIDN mice was significantly increased compared to that of the control mice. H&E staining of lung sections revealed perivascular mononuclear and polymorphonuclear inflammatory infiltrate as well as disruption of normal alveolar architecture.

Surprisingly, T cell expansion was not observed in these recipient mice in contrast to the expansion observed in myeloid cells; however, most of the T cells were differentiated to memory phenotype. These results indicated that TGF-β acts as a negative regulator of the immune system, and the lack of TGF-β signaling leads to proliferation in myeloid cells and inflammatory diseases.

The above results are very encouraging in the field of cancer gene therapy and they might provide a possible cure for cancer. However, as it has been pointed out in other studies that blocked TGF-β signaling, the development of the autoimmunity presents a huge drawback of this approach (26, 33, 122). To resolve this issue, a regulated gene expression system could be incorporated into this approach, such as the Tet-on/off system and HSV-tk/GCV suicide system. The ultimate goal in the present study is to develop a TGF-β-based gene therapy employing the HSV-tk/GCV suicide system. In this system, TβRIIDN gene is fused to the HSV-tk gene that could eliminate the cells expressing TβRIIDN-tk by GCV treatment. The incorporation of the HSV-tk/GCV suicide system into the original approach should provide a possible way to eradicate tumor cells and eliminate the inflammation/autoimmunity developed in the TβRIIDN mice observed in the original studies.

HSV-TK/GCV SUICIDE SYSTEM

The potential of Herpes Simplex Virus thymidine Kinase (HSV-tk) in gene therapy to treat cancer was first described by Federic Moolten in 1998 (123). Since then this suicide system has been widely used in the gene therapy field, especially in the treatment of cancer (124-127). Ganciclovir (GCV) is an acyclic analog of the natural nucleoside 2'-dexoyguanosine and is an anti-viral agent used to treat human viral infections, such as cytomegalovirus, herpes simplex viruses, and Epstein–barr virus (128). In the HSV-tk/GCV suicide system, HSV-tk phosphorylates GCV to produce GCV triphosphate, which is a potent toxic metabolite for cells (Fig. 33, 129). Neither GCV nor HSV-tk alone is harmful to cells. Hence, this conditional effect is desirable in gene therapy in treating cancer due to the specificity. In fact, the most commonly used clinical models for gene therapy involve transduction of the HSV-tk gene to tumor cells, followed by GCV treatment (130, 131).

The mechanisms of HSV-tk/GCV-mediated cell killing are thought to be through both apoptotic and non-apoptotic pathways. In the apoptotic pathway, BCL-2 is involved in the cell killing (133). BCL-2 is an anti-apoptotic molecule, and the expression of this molecule inhibited the bystander cell death (133). In the non-apoptotic pathway, cell killing is associated with induction of heat-shock protein 70 (hsp70) (132). A powerful potential in the treatment of cancer using the HSV-tk/GCV suicide system is the bystander effect (BE). It was observed cells expressing the HSV-tk gene and treated with GCV could induce the sensitivity to neighboring non-transduced cells (127). In the *in vitro* studies, it has been shown that 10% of cells

expressing HSV-tk could lead to 100% of cell death. Moreover, this bystander effect was validated in the *in vivo* studies. Treatment of GCV to 10-50% of cells expressing HSV-tk resulted in 100% of cell death and complete tumor regression (134-136). The mechanisms that mediate bystander effect killing of the neighboring cells are thought to be via gap junctions (131, 137).

In addition to the application in cancer treatment, the HSV-tk/GCV suicide system has been used to deplete specific cell populations by various investigators (138, 139). In our laboratory, we proposed to use this system to conditionally deplete TGF-β insensitive BMCs in recipient mice when it is necessary to eliminate the transferred TGF-β insensitive BMCs in order to prevent the development of autoimmunity in these mice.

CHAPTER II

MATERIALS AND METHODS

Mice and cell lines

Male C57BL/6 and Balb/c mice (6-8 weeks of age, Harlan Indianapolis, IN) were maintained in pathogen-free facilities at the Center for Comparative Medicine at Northwestern University Feinberg School of Medicine in accordance with established guidelines of the Animal Care and Use Committee of Northwestern University. The mouse prostate tumor TRAMP-C2 cells were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA). The mouse renal cell carcinoma RENCA cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/Streptomycin. The NRP-152 cells are non-tumorigenic rat prostate epithelium cells and were cultured in DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin, 5 μg/ml insulin, and 0.1 μM dexamethasone. All cells were kept at 37°C/5% CO_2.

Isolation of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells

For studies with conditioned medium (CM), $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from adult mouse spleens were isolated using a Mouse $CD4^+CD25^+$ T Regulatory Cell Isolation Kit according to manufacturer's instructions (Miltenyi Biotech, Auburn, CA). For co-culturing studies, T cells were stained with rat anti-mouse CD4 and CD25 monoclonal Abs (BD PharMingen, San Jose, CA) after co-culturing with TRAMP-C2 or NRP-152 cells for 7 day and sorted on a FACS sorter into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations. All purified T cells had a purity of ≥ 90% as determined by FACS.

Co-culture of TRAMP-C2 or NRP-152 cells with $CD4^+CD25^-$ T cells

$CD4^+CD25^-$ T cells (4 $x10^6$) were isolated from the spleen of adult C57BL/6 mice (Miltenyi Biotech) and cultured with TRAMP-C2 or NRP-152 cells ($2x10^6$) in the presence of 1 μg/ml rat anti-mouse anti-CD3 mAb (BD PharMingen) and 3,000 rad-irradiated, T cell-depleted splenocytes ($1x10^7$) as antigen-presenting cells (APCs) for 7 days with replacing of TRAMP-C2 or NRP-152 cells every 2 days. T cells cultured in T cell medium with an anti-CD3 Ab and APCs were used as a control. After the end of the 7-day culture, cells were sorted on a FACS sorter according to the cell surface CD25 expression into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations. To determine the rate of conversion, isolated $CD4^+CD25^-$ T cells were co-cultured with TRAMP-C2 as described above for 5 days. T cells were collected each day and stained for CD4 and CD25 expression levels with Abs against CD4 and CD25 (BD PharMingen) and analyzed by FACS. The effect of number of TRAMP-C2 cells on conversion was determined by co-culturing TRAMP-C2 cells and isolated $CD4^+CD25^-$ T cells at different ratios (0:1, 0.25:1, 0.5:1, 0.75:1, and 1:1) for 5 days. At the end point, T cells were collected and stained for cell surface expression levels of CD4 and CD25 (BD PharMingen) and analyzed by FACS.

Culture of $CD4^+CD25^-$ T cells with CM

Isolated $CD4^+CD25^-$ T cells (1 x 10^6/well in a 24-well plate, Miltenyi Biotech) were cultured either in complete T cell medium (RPMI supplemented with 10% FBS, 1% Penicillin/Streptomycin, 10 mM HEPES buffer, 10 mM Non-essential Amino Acids, 1 mM sodium pyruvate, 4 mM L-glutamine, and 0.05 mM β-mercaptoethanol) or in CM collected from a 3-day culture of RENCA, TRAMP-C2, NRP-152, or 20,000 rad-irradiated TRAMP-C2 cells in

the presence of 1 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 Abs (BD PharMingen) for 5 days. T cells cultured in T cell medium were used as a control. In some cases, a neutralizing Ab against TGF-β, 1D11, (100 μg/ml, Genzyme) was added to CM to block TGF-β signaling. To determine the concentration of CM on conversion of $CD4^+CD25^-$ to $CD4^+CD25^+$ T cells, 0.01, 0.1, 0.2, 0.5, 0.9 ml of TRAMP-C2 CM was added to T cell medium in a final volume of 1 ml and isolated $CD4^+CD25^-$ T cells were cultured in diluted CM for 5 days as described above. Five days later, T cells were collected and stained for cell surface CD4 and CD25 expression levels (BD PharMingen) and analyzed by FACS.

Foxp3 RT-PCR

To detect Foxp3 expression, total RNA was extracted from T cells cultured under various conditions using a QiaEasy RNA Isolation kit (Qiagen, Valencia, CA). Two-step RT-PCR was performed by first reverse transcribing RNA to cDNA using a First Strand DNA Synthesis Kit (Invitrogen), and then PCR was carried out using Foxp3-specific primers as described previously (111). For the methylation studies, isolated $CD4^+CD25^-$ T cells were cultured at 1 x 10^6/well in a 24-well plate with 1 μg/ml plate-bound anti CD3 and 2 μg/ml soluble CD28 Abs (BD PharMingen) for 4 days with following treatments: 10 ng/ml TGF-β, 50 μM methylation inhibitor 5-Aza-2'-deoxycytidine (5-Aza, Sigma), or 50 μM ERK inhibitor U0126 (Sigma). For 2 day treatment, T cells were cultured with anti-CD3 and CD28 Abs for 2 days as above and 5-Aza and U0126 were added for an additional 48 hours. Two-step RT-PCR was performed as above to examine Foxp3 expression levels.

Intracellular Detection of Foxp3

To detect Foxp3 protein levels, T cells were stained using a Foxp3-specific mAb according to the manufacturer's instructions (eBioscience, San Diego, CA). Briefly, cells were fixed and permeablized in Fix/Perm Buffer and incubated overnight in the dark at 4°C. The next day an Fc Block (BD PharMingen) was used to block the non-specific binding before adding Fopx3 Ab, and cells were incubated in the dark at 4°C for 30 minutes. A rat IgG2a-FITC Ab was used as an isotype control. Cells were then washed and analyzed by FACS.

ELISA

Isolated $CD4^{+}CD25^{-}$ T cells were cultured with or without TRAMP-C2 CM. Five days later they were collected, washed extensively, plated at a density of 1 x 10^{5}/well in a U-bottom 96-well plate, and re-stimulated with 1 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 Abs (BD PharMingen) for 48 hours (for IL-2 and IL-10 assays) or 72 hours (TGF-β assay). ELISA assays were performed to measure the cytokine levels in the cell-free supernatants according to manufacturers' instructions (IL-2, IL-10 ELISA, Biosource, Camarillo, CA; TGF-β ELISA, R&D System, Minneapolis, MN). For TGF-β ELISA, serum-free medium was used. To measure TGF-β production in CM of various cell lines, all cells were plated at a density of 1 x 10^{6}/T-25 flask and cultured in serum free medium for 48 hours. Cell-free supernatant was collected and TGF-β production was measured using a TGF-β ELISA kit according to the manufacturer's instructions (R&D Systems). To detect TGF-β production in CM from irradiated TRAMP-C2 cells, TRAMP-C2 cells were irradiated (20,000 rads) and cultured in serum free medium (lacking 10% FBS) for 48 hours and CM was collected for TGF-β ELISA.

T cell suppression assay

T cells (5 x 10^4) from various culture conditions were cultured with freshly-isolated $CD4^+CD25^-$ T cells (5 x 10^4 or 1.5 x 10^4) from spleens of wild-type mice in the presence of 0.5 μg/ml an anti-CD3 Ab (BD PharMingen) and 3,000 rad-irradiated, T cell-depleted splenocytes (5 x 10^4) for 72 hours in a 96-well U-bottom plate. During the last 6 -18 hours, 1 μCi/well [^{3}H]thymidine (Amersham Bioscience, Piscataway, NJ) was added to the culture. Cells were harvested and counted in a scintillation counter. For the titration assay, $CD4^+CD25^-$ T cells were cultured with TRAMP-C2 CM for 5 days. Five days later T cells were collected and cultured at various concentrations with $CD4^+CD25^-$ T cells (5 x 10^4) for suppression assays. For the *in vivo* study, 1.5 x 10^4/well $CD4^+CD25^+$ T cells isolated from lung tissues were used.

Immunofluorescence Staining of Mouse Lung Tissues

C57BL/6 mice were injected intravenously via tail vein with 1 x10^6 TRAMP-C2 cells. Thirty days later animals were sacrificed and lung tissues were fixed in formalin and cut into 4 μm sections for immunofluorescence staining. CD4-FITC, CD25-PE (1:100 dilution, BD PharMingen), and Foxp3 (1:100 dilution, eBioscience) were used for the histological staining. For TGF-β staining, a 1D11 Ab was used as the primary Ab (1:100 dilution, Genzyme) and a goat-anti-mouse Texas Red (TR) Ab was used as the secondary Ab (1:400 dilution, Santa Cruz). Nuclei were counterstained with Hoechst 33342 dye (Sigma, St. Louis, MO). Sections were viewed under a confocal microscope.

Tumor induction and treatment with 1D11

Balb/c mice were injected with 1 x 10^6 RENCA cells intravenously via the tail vein. Five mice received a neutralizing Ab against TGF-β, 1D11, and 5 mice received control IgG Ab 13C4 (Genzyme) at a loading dose of 100 mg/kg intraperitoneally at the day of the injection and 5 mice received no treatment. The experimental and control groups received 1D11 and 13C4, respectively, every 3 days at a dose of 50 mg/kg over a course of 30 day experiment. Mice were sacrificed at day 30 and lung tissues were fixed with formalin and cut into 4 μm sections for Hemotoxin and Eosin (H&E) and immunofluorescence staining. Tumor burden was evaluated by counting numbers of tumor metastases of H&E stained lung tissue sections of each animal.

$CD4^+CD25^+$ T cell isolation from lung tissues

Balb/c mice were challenged with RENCA cells and treated with or without 1D11 or 13C4 as described above. At the end of the experiment, lung tissues from each animal were digested with 150 U/ml Collagenase (Sigma) for 1 hr at 37^oC. After digestion, lung tissues were cut into small pieces and single cell suspensions were obtained and filtered through 70 μm and 40 μm cell strainers (VWR, West Chester, PA). Lymphocytes were separated using Percoll (Amersham Bioscience). Briefly, cells were pooled from animals in the same group and resuspended in 8 ml 40% Percoll and loaded onto 3 ml of 70% Percoll in a 15 ml centrifuge tube. Tubes were spun at 2,500 rpm for 20 min at room temperature and the interface (lymphocytes) was removed using a 3 ml syringe with a 20 gauge needle. Cells were washed 3 times with PBS to remove the Percoll. $CD4^+CD25^+$ T cells were isolated from enriched lung lymphocytes using MACS

(Miltenyi Biotech) according to the manufacturer's instructions. The purity of isolated cell populations was ≥ 90% as determined by FACS.

Construct of TβRIIDN-tk and Trans-tk

TβRIIDN-tk construct was kindly provided by Dr. Isaac Kim. This construct is a fusion gene made of TβRIIDN and HSV-tk. The control construct of this vector is Trans-tk and it was designed by eliminating the extracellular domain of TβRIIDN-tk so that only transmembrane domain and HSV-tk remain. To construct this control vector, specific primers flanking transmembrane domain and HSV-tk were designed to generate XhoI and EcoRI restriction enzyme sites and PCR was performed to amplify the fragment. The resulting fragment is called Trans-tk and was inserted into the MSCV-GFP vector at XhoI/EcoRI sites to generate Trans-tk construct. The primers used to amply the Trans-tk fragment are: 5'-CCT GAC CTC GAG ATG TTG TTG CTA GTC ATA TTT-3' (forward) and 5'-ATT AAC GAA TTC TCA GTT AGC CTC CCC CAT-3' (reverse).

RT-PCR for HSV-tk Gene Expression

NIH3T3 cells and BMCs from C57Bl/6 mice were infected with TβRIIDN-tk and Trans-tk as described previously (26). After infection total RNA was isolated using QiaEasy RNA Isolation kit (Qiagen, Valencia, CA). Two-step RT-PCR was performed by firstly reverse transcribing RNA to cDNA using a First Strand DNA Synthesis Kit (Invitrogen, Carlsbad, CA), and then PCR was carried out using HSV-tk-specific primers as described (140).

Trypan Blue Cell Viability Assay

Wild-type NIH3T3 and NIH3T3 infected with TβRIIDN-tk and Trans-tk were treated with 10 μg/ml ganciclovir (GCV) for 3 days. Three days later cells were stained with Trypan blue and counted under a hemacytometer. Trypan blue stains the dead or dying cells whereas live cells repel the dye and are not stained. Both live and dead cells were counted and percent of viable cells were calculated using the following formula: % of viable cells = (No. of live cells)/(No. of live cells + No. of dead cells)*100%.

Thymidine Incorporation Assay

Wild-type NIH3T3 or BMCs and NIH3T3 or BMC infected with TβRIIDN-tk and Trans-tk (5 x 10^4/well) were treated with or without 10 μg/ml GCV for 72 days in a 96-well plate. One μCi/well [^{3}H]thymidine (Amersham Bioscience) was added to the culture at the last 6-18 hours. Cells were harvested and counted in a scintillation counter.

PAI-1/Luciferase Assay

TGF–β increases Plasminogen Activator Inhibitor-1 (PAI-1) expression. Therefore, when PAI-1 promoter is fused to the Luciferase gene, luciferase activity indicates the extent of TGF–β signaling (54, 55). Wild-type NIH3T3 and NIH3T3 infected with TβRIIDN-tk and Trans-tk were transfected with PAI-1/Luciferase and Renilla/Luciferase for 12 hours using Lipofectamine (Invitrogen, Carslbad, CA). After transfection, cells were treated with or without10 ng/ml TGF–β for 20 hours, and luciferase activity was measured using Dual-Luciferase Report Assay

according to the manufacturer's instructions with an illuminometer (Protégé, Madison, WI). PAI-1 promoter/luciferase activity was calculated by normalizing to Renilla luciferase activity.

Real-time RT-PCR for DNMT1, DNMT3a, and DNMT3b

Isolated $CD4^+CD25^-$ T cells were cultured at 1 x 10^6/well in a 24-well plate with 1 μg/ml plate-bound anti CD3 and 2 μg/ml soluble CD28 Abs (BD PharMingen) for 4 days with following treatments: 10 ng/ml TGF-β, 50 μM 5-Aza (Sigma), and 50 μM U0126 (Sigma). At the end of the experiment, total RNA was extracted and an equal amount (1 μg) of RNA was used to generate cDNA using a First Strand DNA Synthesis Kit (Invitrogen). Real-time PCR was performed using primers specific for mouse DNMT1, DNMT3a, and DNMT3b as described (141).

Statistical Analysis

Mean ± SD are given. For comparison of groups the one-way ANOVA with post test comparison was performed and a $p < 0.05$ was considered significant.

CHAPTER III

RESULTS

PROJECT I CD4$^+$CD25$^+$ T REGULATORY CELLS AND CANCER DEVELOPMENT

TUMOR CELL INDUCTION OF CD4$^+$CD25$^+$ T REGULATORY CELLS FROM CD4$^+$CD25$^-$ T CELLS

Presence of CD4$^+$CD25$^+$Foxp3$^+$T cells in tumor masses

Since Woo and colleagues (142) observed an increased number of T_{reg} cells in peripheral blood in patients with non-small cell lung tumor than in the normal donors in 2001, there have been numerous studies that reported a high frequency of T_{reg} cells in peripheral blood in various human cancers (104, 107, 143, 144). A recent study by Curiel *et al* reported the presence of CD4$^+$CD25$^+$, Foxp3$^+$CD3$^+$ T cells in human ovarian carcinoma (80). These interesting observations lead to many questions: Why do CD4$^+$CD25$^+$ T_{reg} cells preferentially accumulate in the tumor mass? Do they home to tumor sites or are they generated in the tumor microenvironment? To understand more about this phenomenon and to explore a potential connection between tumor cells and CD4$^+$CD25$^+$ T_{reg} cells, we used a mouse prostate cancer cell line, TRAMP-C2, in the present study. In this model, C57BL/6 mice were injected with 1 x 10^6 TRAMP-C2 cells intravenously (i.v.) through tail vein and 30 days later the mice were sacrificed. To examine whether CD4$^+$CD25$^+$ T cells are also present in TRAMP-C2 tumors, lung tissues from mice receiving TRAMP-C2 were used for immunofluorescence studies. Since naturally occurring CD4$^+$CD25$^+$ T_{reg} cells constitutively express CD25 on their cell surface, the presence of CD4$^+$CD25$^+$ T cells was examined in the lung tissues. Not surprisingly, CD4$^+$CD25$^+$

T cells were detected in the metastatic tumor sites in lung tissues from these mice, in agreement with the above-mentioned observations by other investigators (Fig. 3). While such cells were observed at the tumor parenchyma, they were non-detectable in normal lung tissues (data not shown).

The positive staining of CD4 and CD25 in the lung tissue sections suggested the possible presence of T_{reg} cells. However, CD25 is a not a unique marker for T_{reg} cells because activated $CD4^+$ T cells also express CD25 on their surface. Since Foxp3 is a crucial transcription regulator of T_{reg} cells, the expression of Foxp3 was examined in the same lung tissues to ensure that the $CD4^+CD25^+$ T cells observed were indeed T_{reg} cells (111). The staining of lung sections revealed that $Foxp3^+CD25^+$ T cells were also present in the tumor sites (Fig. 4). Since Foxp3 is exclusively expressed by T_{reg} cells (111), the positive staining of Foxp3 in the tumor sites suggested that these cells were T_{reg} cells. Furthermore, the presence of these T cells in tumor sites indicated a possible connection between tumor and T_{reg} cells.

It has been shown in other studies that the presence of such $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the tumor microenvironment is associated with suppression of anti-tumor immunity. However, the origin of these cells remains elusive. In the following studies, we explored the connections between tumor cells and T_{reg} cells and the possible origin of such T_{reg} cells in the tumor mass.

T cells co-cultured with TRAMP-C2 express Foxp3

T_{reg} cells were first thought to develop in the thymus only. However, studies demonstrated that specific microenvironment, such as a tumor microenvironment, can induce non-T_{reg} cells to differentiate into T_{reg} cells (106, 145-147). It has been shown that tumor-derived cytokines, such as IL-10, TGF-β, and vascular endothelial growth factor (VEGF) suppress dendritic cell (DC) maturation and function. As a result these dysfunctional DCs induce differentiation of T_{reg} cells in the tumor site (148, 149). However, it is unknown whether tumor cells can directly induce T_{reg} cell differentiation. To investigate the direct relationship between tumors and T_{reg} cells, TRAMP-C2 cells were used in a co-culture system with $CD4^+CD25^-$ T cells isolated from C57BL/6 mice (Fig. 5). In this study, isolated $CD4^+CD25^-$ T cells from C57BL/6 mouse spleen were used in a co-culture with TRAMP-C2 cells for 7 days in the presence of an anti-CD3 Ab and 3,000 rad-irradiated, T cell-depleted splenocytes as antigen presenting cells (APCs). TRAMP-C2 cells are fast growing tumor cells. In our co-culture system, to prevent tumor cells from outgrowing the T cell population, TRAMP-C2 cells grown in monolayer were passaged every 2 days to maintain a constant tumor and T cell ratio. After a 7-day co-culture, T cells were sorted on a FACS sorter according to the cells surface CD25 expression into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations. In order to determine whether these T cells were T_{reg} cells, Foxp3 expression was examined (111). Total RNA was extracted from individual T cell populations and two-step RT-PCR was performed using Foxp3-specific primers. Positive control $CD4^+CD25^+$ T_{reg} cells (Ctrl 25^+) as well as TRAMP-C2 co-cultured $CD25^+$ T cells expressed Foxp3 (Fig. 6). In contrast, neither negative control $CD4^+CD25^-$ T cells (Ctrl 25^-) nor TRAMP-C2 co-cultured $CD25^-$ T cells expressed Foxp3 (Fig. 6). In addition, T

cells cultured alone did not express Foxp3 (data not shown). Since Foxp3 is exclusively expressed in $CD4^+CD25^+$ T_{reg} cells, the findings that TRAMP-C2 co-cultured $CD4^+CD25^+$ T cells express Foxp3 suggested that these cells are T_{reg} cells. These encouraging observations prompted us to further investigate the potential connections between tumor cells and T_{reg} cells.

Kinetics of tumor conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T cells

To understand the kinetics of $CD4^+CD25^+$ T cell conversion when co-cultured with TRAMP-C2 cells, a series of experiments was conducted. First, the rate of conversion was examined. TRAMP-C2 cells were co-cultured with purified $CD4^+CD25^-$ T cells as described above for 0-5 days. T cells were collected each day and analyzed by FACS for CD25 expression levels. The results are shown in Fig. 7A. The percentage of $CD4^+CD25^+$ T cells increased in a time-dependent manner. This result suggested that the longer $CD4^+CD25^-$ T cells were co-cultured with TRAMP-C2 cells, the more these cells were converted to $CD4^+CD25^+$ T cells. In addition, the optimal ratio of cell types for promoting conversion was studied by co-culturing TRAMP-C2 and $CD4^+CD25^-$ T cells at 0.1:1, 0.25:1, 0.5:1, 0.75:1, and 1:1 for 5 days. At the end of the 5 day culture, T cells were collected and cell surface CD25 expression was analyzed by FACS. Again, the percentage of $CD4^+CD25^+$ T cells increased in a ratio-dependent manner (Fig. 7B). These results suggest that both the duration of co-culture and the ratio of tumor cells to $CD4^+CD25^-$ T cells affect the conversion of $CD25^-$ to $CD25^+$ T cells by tumor cells.

$CD4^+CD25^-$ T cells cultured with TRAMP-C2 conditioned medium express Foxp3

The co-culture system allows tumor cells to have direct contact with T cells. To investigate whether cell-cell contact between tumor and T cells is required for the induction of Foxp3-expressing T cells, instead of co-culturing cells together, TRAMP-C2 cell conditioned medium (CM) was used to culture these T cells. Figure 8 delineates the experimental design. CM was collected after a 3-day culture of TRAMP-C2 cells. On the same day purified $CD4^+CD25^-$ T cells were cultured in CM in the presence of anti-CD3 and CD28 Abs. Five days later, Foxp3 expression was examined in these T cells by RT-PCR. Of note, the T cells cultured with CM were not sorted because more than 85% of cultured $CD4^+$ T cells were CD25 positive (Fig. 10, d 0). As the presence of APCs is not essential in the generation of $CD4^+CD25^+$ T_{reg} cells, all the experiments with CM were carried out without APCs (150, 151). Freshly isolated $CD4^+CD25^+$ T_{reg} cells were used the positive control and $CD4^+CD25^-$ T cells were used as the negative control. Both the positive control (Ctrl 25^+) and T cells cultured with TRAMP-C2 CM expressed Foxp3 (Fig. 9A). However, Foxp3 expression was absent in the negative control (Ctrl 25^-) or $CD4^+CD25^-$ T cells cultured in T cell medium (Ctrl) (Fig. 9A). In addition, Foxp3 protein expression was evaluated by intracellular staining using an Ab against Foxp3 and analyzed by FACS (Fig. 9B). In agreement with RT-PCR results, FACS analysis demonstrated an increased protein expression level of Foxp3 in T cells cultured in TRAMP-C2 CM (CM). However, $CD4^+CD25^-$ T cells cultured with T cell medium (Ctrl) did not express Foxp3 (Fig. 9B). Taken together, the results from this proof-of-principle study suggest that tumor cells can directly convert $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ T cells, at least in our *in vitro* system; and this conversion is time and tumor:T cells ratio dependent. Furthermore, this

conversion does not require cell-cell contract as CM collected from TRAMP-C2 cells is sufficient for the conversion. Thus, all the following experiments were conducted with CM instead of the co-culture system. Finally, the expression of Foxp3 in tumor-converted $CD4^+CD25^+$ T cells indicates that the resulting T cells are potential T_{reg} cells.

Effect of CM dilution on $CD4^+CD25^+$ T cells conversion

The initial studies suggest that the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T cells by TRAMP-C2 is time and tumor:T cell ratio-dependent. In the current study, the effect of TRAMP-C2 CM concentration on the conversion of such cells was examined. TRAMP-C2 CM was collected after a 3-day culture and then mixed with T cell medium in a final volume of 1 ml in the following proportions: 0%, 1%, 10%, 33%, 50%, 90%, and 100%. Purified $CD4^+CD25^-$ T cells were cultured in the diluted TRAMP-C2 CM for 5 days and analyzed by FACS to examine CD25 expression. A representative FACS profile of the conversion is shown in Fig. 10. The starting purified $CD4^+CD25^-$ T cell population is designated as day 0. FACS analysis revealed that the conversion of $CD4^+CD25^+$ T cells was dependent on the concentration of TRAMP-C2 CM, as an increased concentration of CM resulted in an increased percentage of $CD4^+CD25^+$ T cells. The graph on the bottom of Fig. 10 represents the percentage of $CD4^+CD25^+$ T cells cultured in various diluted CM. The results suggest that the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T cells was CM concentration-dependent and that TRAMP-C2 CM alone was sufficient for this conversion, indicating that one or more soluble factors in the CM may play a critical role in the conversion.

Cytokine Production Profile of T cells cultured with TRAMP-C2 CM

$CD4^+CD25^+$ T_{reg} cells differ from other T cells in that they have been shown to predominantly secrete IL-10 or TGF-β, but not IL-2 (111, 152). To examine whether T cells cultured with TRAMP-C2 CM share the same characteristics as naturally occurring $CD4^+CD25^+$ T_{reg} cells, we first examined the cytokine production profile of these T cells. $CD4^+CD25^-$ T cells were cultured in CM for 5 days. At the end of the 5-day culture, T cells were collected and washed extensively then re-stimulated with anti-CD3 and CD28 Abs for 48 hours, at which time IL-2 and IL-10 production were assayed; or 72 hours, when TGF-β was measured. Of note, for TGF-β ELISA, T cells were cultured in serum free T cell medium as serum itself contains TGF-β. Supernatants were collected from various T cell cultures and the cytokine production profile of these cells was evaluated using ELISA. As a comparison, the cytokine production profile of naturally occurring $CD4^+CD25^+$ T_{reg} cells (Ctrl 25^+) and $CD4^+CD25^-$ T cells (Ctrl 25^-) was also measured and used as a positive and negative control, respectively. Comparable to the positive control, T cells cultured with TRAMP-C2 CM (CM) produced virtually non-detectable amounts of IL-2, while T cells cultured with T cell medium (Ctrl) produced high levels of IL-2 (Fig. 11). IL-10 is a cytokine that negatively regulates the immune system and is produced at high levels by $CD4^+CD25^+$ T_{reg} cells but at low levels by $CD4^+CD25^-$ T cells. When re-stimulated, T cells cultured with TRAMP-C2 CM produced levels of IL-10 as high as the positive control (Fig. 12). However, T cells cultured with T cell medium produced levels of IL10 comparable to the negative control (Fig. 12). Finally, T cells cultured with CM produced high levels of TGF-β as compared to T cells cultured with T cell medium (Fig. 13). These results reveal that T cells cultured with CM have a similar cytokine production profile to that of naturally occurring

$CD4^+CD25^+$ T_{reg} cells and that these similarities provide evidence to further support the notion that tumor cells can directly convert $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ T_{reg} cells (111, 152).

T cells cultured with tumor cell CM suppress $CD4^+CD25^-$ T cell proliferation

The results from earlier studies indicate that the T cells generated from culturing with TRAMP-C2 cell CM are T_{reg} cells because they possess characteristics similar to those of naturally occurring $CD4^+CD25^+$ T_{reg} cells, including expression of Foxp3, low expression of IL-2, and high expression of IL-10 and TGF-β. However, more direct evidence is needed to support this notion. Therefore, the suppressive ability of T cells cultured with CM was examined in an *in vitro* functional assay, as the most important characteristic of T_{reg} cells is their ability to suppress $CD4^+CD25^-$ T cell proliferation. In this study, CM was collected from two different types of tumor cells, RENCA or TRAMP-C2, and used to culture isolated $CD4^+CD25^-$ T cells for 5 days. A different tumor cell line was used here to evaluate whether the above observations were due to a cell line-specific effect. At the end of a 5 day culture, freshly isolated $CD4^+CD25^-$ T cells were stimulated with an anti-CD3 Ab and APCs and cultured with various T cell populations for 72 hours. Cell proliferation was measured by [^{3}H]thymidine incorporation. When T cells initially cultured with RENCA or TRAMP-C2 CM were subsequently cultured alone, they did not proliferate (CM, Fig.14A and 15A). This was perhaps due to the fact that T cells cultured with tumor cell CM do not produce high levels of IL-2, and therefore, lack the crucial cytokine for proliferation (Fig. 11). More importantly, the suppressive ability of T cells cultured with RENCA or TRAMP-C2 CM was evaluated. To examine their suppressive ability for $CD4^+CD25^-$ responder T cell proliferation/activation, suppression assays were performed using

different CM T cell:$CD4^+CD25^-$ T cell ratios. Freshly isolated $CD4^+CD25^-$ T cells (1.5 x 10^4) were cultured with individual T cells that had been previously cultured with RENCA or TRAMP-C2 CM for 5 days (5 x 10^4). When cultured together, T cells cultured with CM strongly suppressed $CD4^+CD25^-$ T cell proliferation ($CM/25^-$) whereas T cells cultured with T cell medium ($Ctrl/25^-$) did not (Fig. 14A and 15A). Furthermore, the suppressive ability of T cells cultured with RENCA or TRAMP-C2 CM was evaluated by co-culturing with freshly isolated $CD4^+CD25^-$ T cells at a different ratio (1:1, 5 x 10^4). Again, T cells cultured with CM demonstrated a potent suppressive ability at this ratio (Fig. 14B and Fig. 15B).

To study how T cells cultured with TRAMP-C2 CM compared to naturally occurring $CD4^+CD25^+$ T_{reg} cells in terms of suppression, a titration assay was performed to evaluate their suppressive ability. In this study, 5 x 10^4 freshly isolated $CD4^+CD25^-$ responder T cells were cultured with different numbers of T cells previously cultured with TRAMP-C2 CM (0, 0.25, 0.5. 1.25, 2.5, and 5 x 10^4) for 72 hours. At the end of the experiment, proliferation of $CD4^+CD25^-$ T cells was measured by [^{3}H]thymidine incorporation assay. As shown in Fig. 16, T cells cultured with TRAMP-C2 CM showed suppressive ability even at the low concentration of 0.25 x 10^4. As the concentration of T cell cultured with CM increased, the suppression of $CD4^+CD25^-$ T cell proliferation also increased, demonstrating a potent suppressive ability of T cells cultured with TRAMP-C2 CM. These results provide direct evidence that T cells cultured with tumor cell CM are indeed T_{reg} cells because they suppress $CD4^+CD25^-$ T cell proliferation *in vitro*, express Foxp3, produce low levels of IL-2, and produce high levels of IL-10 and TGF-β.

The role of tumor-derived TGF-β in converting $CD4^+CD25^-$ T cells to T_{reg} cells

The results from the above studies demonstrate that TRAMP-C2 cells can directly convert $CD4^+CD25^-$ T cells into T_{reg} cells. Furthermore, CM derived from either RENCA or TRAMP-C2 cells is sufficient for this conversion, indicating that cell-cell contact is not necessary. We reasoned that certain soluble factors produced by TRAMP-C2 and RENCA are responsible for this conversion. Many types of tumor cells produce high levels of TGF-β, and recent studies have shown that $CD4^+CD25^+$ T_{reg} cells can be generated *in vitro* by stimulating with exogenous TGF–β (145, 151, 153). Based on these observations, it is logical to hypothesize that TGF–β derived from tumor cells, RENCA or TRAMP-C2, plays an important role in converting $CD4^+CD25^-$ T cells into T_{reg} cells. To test this hypothesis, the level of TGF-β present in the CM from RENCA or TRAMP-C2 cells was examined using a TGF-β ELISA. The results indicated that tumor cells produced high levels of TGF-β (Fig. 17), suggesting TGF-β could be a crucial regulator in the conversion of T_{reg} cells when cultured with CM derived from RENCA or TRAMP-C2 cells.

Non-tumorigenic and irradiated TRAMP-C2 cells were unable to covert $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ T_{reg} cells

To further test the role of tumor cell-derived TGF-β in conversion of $CD4^+CD25^-$ T cells into T_{reg} cells, CM derived from a non-tumorigenic rat prostate epithelium cell line, NRP-152, and 20,000 rad-irradiated TRAMP-C2 were used in this study. TGF-β ELISA revealed that the CM from these two types of cells contained little TGF-β as compared to CM derived from tumor cells RENCA or TRAMP-C2 (Fig. 17). To examine whether co-culture of NRP-152 with

$CD4^+CD25^-$ T cells could induce Foxp3 expression, isolated $CD4^+CD25^-$ T cells were co-cultured with NRP-152 for 7 days as described in Fig. 5. Seven days later, T cells were collected and sorted into individual populations according to the cell surface CD25 expression. Total RNA was extracted from all T cell populations and a two-step RT-PCR was performed to detect Foxp3 expression levels in these T cells. Interestingly, neither NRP-152 co-cultured and sorted $CD4^+CD25^+$ nor $CD4^+CD25^-$ T cells expressed Foxp3, as compared to TRAMP-C2 co-cultured $CD4^+CD25^+$ T cells (Fig. 18A). In addition, $CD4^+CD25^-$ T cells cultured with CM derived from NRP-152 cells also lacked Foxp3 expression (Fig. 18B).

To further evaluate the phenotype of T cells cultured with NRP-152 CM, an *in vitro* suppression assay was performed to examine their ability to suppress $CD4^+CD25^-$ T cells proliferation. In this study, T cells cultured with NRP-152 CM were either cultured alone or with freshly isolated $CD4^+CD25^-$ T cells as responder cells in the presence of an anti-CD3 Ab and APCs for 72 hours, and the proliferation of $CD4^+CD25^-$ T cells was measured by [^{3}H]thymidine incorporation assay. In contrast to T cells cultured with CM derived from tumor cells TRAMP-C2 or RENCA, T cells cultured with CM derived from NRP-152 cells proliferated as vigorously as the responder T cells. More importantly, when cultured with $CD4^+CD25^-$ responder T cells, T cells cultured with NRP-152 CM showed no sign of suppression, indicating they did not possess suppressive ability (Fig. 19).

CM derived from 20,000 rad-irradiated TRAMP-C2 cells also lacked TGF-β as mentioned above (Fig. 17), thus this CM was also used in comparison to CM derived from

TRAMP-C2 and RENCA cells. T cells were cultured with irradiated TRAMP-C2 CM and their suppressive ability was evaluated. Not surprisingly, T cells cultured with irradiated TRAMP-C2 CM did not possess suppressive ability as compared to T cells cultured with TRAMP-C2 CM (Fig. 20). Taken together, these results show that CM derived from tumor cells, but not non-tumorigenic cells, can convert $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T_{reg} cells. Furthermore, CM derived from irradiated TRAMP-C2 cells, which lacks TGF-β, is not sufficient to induce T_{reg} cell generation, suggesting a crucial role of tumor cell derived TGF-β in the process of such conversion. In summary, these results demonstrate that TGF-β derived from tumor cells in CM is responsible for the conversion as CM derived from neither non-tumorigenic cell CM nor irradiated TRAMP-C2 is sufficient to induce the conversion due to the lack of TGF-β in the CM.

Neutralization of TGF-β abrogates the conversion of $CD4^+CD25^+Foxp3^+$ T_{reg} cells

To further demonstrate the role of TGF-β in the conversion of $CD4^+CD25^+$ T_{reg} cells, a more direct approach was used in this study. A neutralizing Ab against TGF-β, 1D11, was used to deplete TGF-β present in TRAMP-C2 CM. This Ab recognizes TGF-β1, 2 and 3 in human and mouse and neutralizes biological activities of TGF-β (154). When 1D11 was added to TRAMP-C2 CM, T cells cultured with CM did not express Foxp3 (Fig. 18B). In addition, neutralization of TGF-β completely changed the profile of cytokine production of these T cells, with cells expressing high levels of IL-2 and low levels of IL-10 and TGF-β (Fig. 11-13). More importantly, in the suppression assay, addition of 1D11 abrogated the ability of T cells cultured with TRAMP-C2 CM to suppress $CD4^+CD25^-$ T cell proliferation (Fig. 15A). Taken together,

these results reveal a critical role of tumor-derived TGF-β in the conversion of $CD4^{+}CD25^{-}$ T cells into T_{reg} cells. Noticeably, non-tumorigenic NRP-152 cells produce low levels of TGF-β (Fig. 17 and 155) and T cells cultured with CM derived from these cells are unable to suppress T cell proliferation (Fig. 19). Collectively, these studies demonstrate that tumor cells can directly convert $CD4^{+}CD25^{-}$ T cells into T_{reg} cells through the production of high levels of TGF-β, whereas non-tumorigenic cells NRP-152 and irradiated TRAMP-C2 were unable to convert such cells due to the low levels of TGF-β production.

CHAPTER IV

IN VIVO FUNCTIONAL ANALYSIS OF TUMOR-INDUCED $CD4^+CD25^+$ T REGULATORY CELLS AND TUMOR PROGRESSION

Many types of tumor cells produce high levels of TGF-β and this has been amply demonstrated in recent studies in brain tumors (58), head and neck tumors (59), lung cancer (60), breast cancer (56), digestive tract organ malignancies (61), and prostate cancer (62). TGF-β is a potent immunosuppressant and TGF-β derived from tumor cells in turn suppresses anti-tumor immunity by either direct or indirect mechanisms. The above *in vitro* studies demonstrate that tumor cells can directly convert $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T_{reg} cells through production of TGF-β. An anti-TGF-β Ab completely abolished this conversion. The present study investigated the role of tumor-derived TGF-β in the tumor microenvironment in terms of suppression of anti-tumor immunity, specifically, through conversion of tumor-infiltrating $CD4^+$ T cells into T_{reg} cells. It has been shown that tumor cells produce high levels of TGF-β, and that TGF-β in turn can convert $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T_{reg} cells. Therefore, when tumor-infiltrating $CD4^+CD25^-$ T cells are exposed to a TGF-β rich environment, they are converted into $CD4^+CD25^+$ T_{reg} cells. These cells, as demonstrated in the above *in vitro* study, possess the ability to suppress $CD4^+$ T cells and potential immune responses to tumor cells. In theory, if tumor-produced TGF-β is blocked, tumor-infiltrating $CD4^+$ T cells will not be exposed to a TGF-β rich environment, and $CD4^+CD25^+$ T_{reg} differentiation will not occur. The present study was designed to test this hypothesis by using a neutralizing Ab against TGF-β, 1D11, to

systematically deplete TGF-β in a mouse model. Tumor burden and suppressive function of $CD4^+CD25^+$ T cells from lung metastases were evaluated.

Efficacy of a neutralizing Ab against TGF-β in mice

To support the *in vitro* observations, an *in vivo* study was carried out using mouse renal cell carcinoma RENCA cells as they produce high levels of TGF-β (Fig. 17). The experimental design is delineated in Fig. 21. Balb/c mice were used in this study as they are the syngeneic host for RENCA cells. At day 0, Balb/c mice were injected with 1×10^6 RENCA cells intravenously via the tail vein and randomized into three groups. On the day of tumor injection, 5 mice received 1D11 and 5 mice received control Ab13C4 at a loading dose of 100 mg/kg. These mice continued receiving 1D11 or 13C4 at a dose of 50 mg/kg every 3 days for a course of 30 days. At the end of experiment all animals were sacrificed and TGF-β levels were measured in sera and lung tissues, and tumor burden was evaluated in all animals.

To evaluate the efficacy of 1D11, TGF-β levels were examined in mouse sera and lung tissues. TGF-β ELISA revealed that serum levels of TGF-β were significantly reduced in 1D11-treated animal, whereas sera from untreated and 13C4-treated animals contained levels of TGF-β comparable to those of tumor-free animals (Fig. 22 and data not shown). In addition, lung sections from all animals were stained with an anti-TGF-β Ab and evaluated for TGF-β expression levels. A representative staining pattern of TGF-β in lung tissue of mice receiving tumor challenge is shown in Figure 23. In tumor-bearing mice receiving 13C4 or no treatment, TGF-β was ubiquitously expressed in tumor and lung tissues whereas no TGF-β expression was

detected in lung tissues of mice receiving 1D11. The reduced levels of TGF-β in both serum and lung tissue from 1D11-treated mice demonstrated the efficacy of 1D11 in systematically neutralizing TGF-β in mice treated with this Ab.

Neutralization of TGF-β reduces tumor burden in lung tissue

After establishing the efficacy of 1D11, tumor burden in these mice was evaluated. Lung tissues from all mice were collected at the end point of the experiment, cut into 4 μm sections, and used for H&E staining. Tumor nodules were counted on each H&E stained sections of lung tissues and the number of tumor metastases in mice receiving different treatments was calculated. Analysis of tumor burden revealed that animals receiving 1D11 had significantly fewer tumor metastases in the lung tissues as compared to those receiving 13C4 or no treatment (Fig. 24). In addition, lung metastases in lung tissue revealed the difference in tumor nuclei organization. Tumor nuclei appeared to be organized and intact in lung metastases from mice receiving no treatment. The number of tumor metastases was greatly reduced in mice receiving 1D11 treatment. The few metastases in these mice showed pathological signs of condensed nuclei compared to those from mice receiving no treatment (Fig. 25). The addition of neutralizing Ab 1D11 was sufficient to deplete TGF-β systemically in mice and such treatment led to a reduction of tumor burden in these mice, suggesting a critical role of TGF-β in tumor progression. The following experiments were designed to elucidate a possible mechanism by which tumor cells evade the immune system through the production of high levels of TGF-β.

Neutralization of TGF-β abrogates the conversion of T_{reg} cells in the tumor microenvironment

Many types of tumors produce high levels of TGF-β (58-60). TGF-β is a potent immunosuppressant, and high levels of tumor-derived TGF-β can impair immune responses against tumor cells, conferring a growth advantage to the tumor (58-60). Therefore, removal of tumor-derived TGF-β could facilitate tumor rejection by the host immune system. Indeed, as shown in Fig. 24 and 25, animals receiving 1D11 had a reduced tumor burden as compared to animals receiving control Ab 13C4 and no treatment. One mechanism by which tumor cells might evade the immune system is through the conversion of tumor-infiltrating $CD4^+$ T cells into T_{reg} cells. *In vitro* studies discussed in Chapter I have shown that $CD4^+CD25^-$ T cells exposed to CM derived from tumor cells, which contains high levels of TGF-β, were induced to differentiate into $CD4^+CD25^+$ T_{reg} cells that possess the same suppressive capability as naturally occurring $CD4^+CD25^+$ T_{reg} cells. By the same token, if tumor-infiltrating $CD4^+$ T cells are exposed to high levels of tumor-derived TGF-β in the tumor microenvironment, the conversion to T_{reg} cells should take place. Consequently the resulting T_{reg} cells should be able to suppress the anti-tumor immunity and confer a growth advantage to the tumor.

To test this hypothesis, the phenotype of T cells isolated from lung tissues from different treatment groups was examined. Lymphocytes were enriched as described in the Materials and Methods and the percentage of $CD4^+CD25^+$ T cells was analyzed by FACS. As shown in Fig. 26 (top panel), all animals had a similar percentage of $CD4^+CD25^+$ T cells in their lung tissues regardless of treatment. As mentioned above, CD25 is not an exclusive marker for T_{reg} cells as

activated $CD4^+$ T cells also express CD25. Thus, Foxp3 expression levels were examined in the lung sections from mice receiving different treatments. Immunofluorescence staining revealed that Foxp3 expression was absent in animals receiving 1D11 but present in animals receiving no treatment or control Ab 13C4 (Fig. 26, bottom panel), suggesting that $CD4^+CD25^+$ T cells present in the lung tissue from 1D11 treated mice are not T_{reg} cells despite cell surface expression of CD25.

Finally, the suppressive ability of $CD4^+CD25^+$ T cells from tumor sites in the lung tissues of animals was evaluated. $CD4^+CD25^-$ responder T cells were stimulated with an anti-CD3 Ab and APCs and cultured with $CD4^+CD25^+$ T cells isolated from the lung tissues of animals receiving different treatments. $CD4^+CD25^+$ T cells from animals either receiving no treatment or 13C4 demonstrated suppressive ability when co-cultured with $CD4^+CD25^-$ T cells (Fig. 27). However, the $CD4^+CD25^+$ T cells isolated from animals receiving 1D11 were unable to suppress the $CD4^+CD25^-$ T cell proliferation (Fig. 27). As predicated from the absence of $Foxp3^+$ T cells in lung tissue from mice treated with 1D11, $CD4^+CD25^+$ T cells were indeed not T_{reg} cells because they lacked the ability to suppress $CD4^+CD25^-$ responder T cell proliferation. It is of importance to note that depletion of TGF-β also depleted $CD4^+CD25^+Foxp3^+$ T cells in the lungs of 1D11 treated mice. Such depletion was associated with a reduction in tumor burden in these mice. These results suggest that tumor-derived TGF-β in the tumor microenvironment can directly convert tumor infiltrating $CD4^+$ T cells into $CD4^+CD25^+Foxp3^+$ T_{reg} cells. Since such conversion does not occur in the absence of TGF-β, tumor cells progress. Taken together, these

results provide direct evidence that tumor cells can directly convert $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ T cells by producing high levels of TGF-β to evade the immune system.

A proposed model for tumor evasion of the immune system by converting $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T_{reg} cells through production of TGF-β.

The presence of T_{reg} cells in the tumor microenvironment is associated with a poor prognosis in patients, but the source of these cells still remains elusive. Recently, studies have revealed various sources for T_{reg} cells in the tumor microenvironment. It appears that there are at least two types of T_{reg} cells in the tumor microenvironment. One type is naturally occurring T_{reg} cells that are recruited to the tumor sites, and the other type is tumor-converted T_{reg} cells. The present study provides evidence that tumor cells can directly convert $CD4^+$ T cells into T_{reg} cells through production of high TGF-β.

Many types of tumor cells produce high levels of TGF-β, and consequently, tumor-derived TGF-β can aid the tumor in evasion of the immune system. We propose a model that summarizes the studies discussed in Chapter I and II (Fig. 28). In this model, when tumor-infiltrating $CD4^+$ T cells are exposed to TGF-β rich environment in tumor sites, $CD4^+CD25^+Foxp3^+$ T_{reg} cells conversion occurs. As a result, the converted T_{reg} cells can suppress anti-tumor immunity and confer an advantage to tumor cells through evasion of the immune system. Indeed, a significant reduction in lung metastases was observed in tumor-bearing animals treated with a neutralizing Ab against TGF-β, 1D11 (Fig. 24 and 25). This reduction in tumor burden, at least in part, was due to the absence of $CD4^+CD25^+Foxp3^+$ T_{reg}

cells in the tumor microenvironment of these animals (Fig. 26 and 27). Furthermore, the absence of Foxp3^{+} T_{reg} cells is likely due to the lack of conversion of normal CD4^{+} T cells to T_{reg} cells as these animals lack TGF-β.

CHAPTER V

ELUCIDATION OF MECHANISMS OF TGF-BETA INDUCED FOXP3 EXPRESSION IN $CD4^+CD25^-$ T CELLS

It has been well established that TGF-β induces $CD4^+CD25^+$ T_{reg} cell generation from $CD4^+CD25^-$ T cells (145, 150, 156, 157). However, the mechanism of this induction remains elusive. Among mechanisms that regulate gene expression, promoter methylation is a common process in gene silencing (158). Methylation patterns vary in animals ranging from *C. elegans* whose genome lacks detectable possible methylation sites and does not encode a conventional DNA methyltransferase (159), to vertebrates such as humans whose genome encodes 70% methylated CpG residues (160, 161). CpG islands are unmethylated CG-rich regions that possess high relative densities of CpG and are positioned at the 5' ends of many human genes (162, 163). It is well documented that DNA methylation is essential in embryonic development (164, 165), imprinting (166), X-chromosome inactivation (167), and suppression of parasitic DNA sequences (168). As essential as methylation is to mammalian cells, its presence also represents a burden to the genome as many of the tumor suppressor genes are hypermethylated through their promoter CpG islands, such as BRCA1 (breast cancer), APC (colorectal carcinoma), VHL (renal carcinoma) (161). In the present preliminary studies, we focused on the identification of potential CpG islands on the mouse Foxp3 gene promoter that could be methylated, thereby allowing for possible gene silencing in $CD4^+CD25^-$ T cells.

To explore possible mechanisms by which TGF-β induces Foxp3 gene expression in $CD4^+CD25^-$ T cells, the mouse Foxp3 gene promoter (Genbank ID AF277994) was analyzed for potential

CpG islands for methylation. Results from MethPrimer (169) analysis revealed five potential CpG islands on the Foxp3 promoter (Fig. 29). We hypothesize that Foxp3 gene promoter is methylated in $CD4^+CD25^-$ T cells and that treatment with TGF-β reverses the predicted methylation process to induce gene expression. To test this hypothesis, the methylation inhibitor 5-Aza-2′-deoxycytidine (5-Aza) was used. In this study, isolated $CD4^+CD25^-$ T cells were cultured with TGF-β and 5-Aza in the presence of anti-CD3 and CD28 Abs for either 2 or 4 days. At the end of the experiment, total RNA was extracted from individual T cell cultures and a two-step RT-PCR was performed to examine the gene expression levels of Foxp3. Freshly isolated $CD4^+CD25^+$ T_{reg} cells were used as a positive control and $CD4^+CD25^-$ T cells were used as a negative control. As expected, similar to the positive control, $CD4^+CD25^-$ T cells treated with TGF-β expressed Foxp3, whereas Foxp3 expression was absent in the negative control (Fig. 30). Interestingly, when cultured with the methylation inhibitor 5-Aza, $CD4^+CD25^-$ T cells expressed Foxp3 in both 2 and 4 day cultures (Fig. 30). These results demonstrate that inhibition of methylation is sufficient to induce Foxp3 expression in $CD4^+CD25^-$ T cells, indicating that methylation is involved in Foxp3 induction. In addition, TGF-β treatment also induces Foxp3 expression. Taken together, these results suggest that TGF-β-induced Foxp3 gene expression in $CD4^+CD25^-$ T cells involves a possible promoter demethylation process.

TGF-β has been linked to the ERK signaling pathway during T cell activation. When T cells are stimulated, three MAPK pathways, ERK, JNK, and p38 are activated (170, 171). TGF-β has been shown to either activate or inhibit ERK phosphorylation in a context-dependent manner. Studies conducted by Schiott *et al* demonstrated a decrease in ERK2 phosphorylation in

T cells when treated with TGF-β or an ERK2 inhibitor, thereby inhibiting T cell activation (172). In the context of $CD4^+CD25^-$ T cells, TGF-β also plays an inhibitory role. A decrease in ERK phosphorylation is also linked to decreased DNA Methyltransferase (DNMT) activity in T cells of Lupus patients (173-175). To explore the possible connection between TGF-β and Foxp3 gene expression, an ERK inhibitor, U0126, was used. Isolated $CD4^+CD25^-$ T cells were cultured with U0126 for either 2 or 4 days, and at the end of the experiment, a two step RT-PCR was performed to examine the expression levels of Foxp3. Interestingly, U0126 treated $CD4^+CD25^-$ T cells also expressed Foxp3 in both 2 and 4 day cultures (Fig. 30). The results from the initial studies suggest a possible demethylation process of Foxp3 promoter that involves interaction between TGF-β and ERK signaling pathways.

To further investigate this possibility, DNMT expression profiles were examined in individual T cell cultures using real-time RT-PCR. Five related DNMTs have been identified in the DNMT family (DNMT1, 2, 3a, 3b, and 3l), and they maintain genome-wide DNA methylation patterns (176, 177). The most abundant methyltransferase is DNMT1 with a primary role of DNA methylation maintenance (178, 179). It is unclear whether DNMT2 plays a role in methylation since it has a very low enzymatic activity *in vitro,* and knockout of this gene in mice produced no discernible phenotype (180-182). DNMT3a and DNMT3b are highly expressed during embryonic development and are regarded as *de novo* methyltransferases (183). Unlike DNMT1, DNMT3a and DNMT3b methylate hemimethylated and unmethylated DNA equally well (183, 184). To study mRNA expression levels of DNMT1, DNMT3a, and DNMT3b in $CD4^+CD25^-$ T cells when cultured with different conditions, real-time RT-PCR was

performed as described (141). Isolated $CD4^+CD25^-$ T cells were cultured as above and total RNA was extracted and equal amount of RNA was used for real-time RT-PCR to examine the expression profile of DNMTs in cultured T cell populations. Results revealed an overall decrease in expression level of all three DNMTs in TGF-β, 5-Aza, and U0126 treated $CD4^+CD25^-$ T cells as well as $CD4^+CD25^+$ T_{reg} cells in comparison with $CD4^+CD25^-$ T cells cultured with anti-CD3 and CD28 Abs only (Fig. 31). In summary, the above results demonstrate that inhibition of methylation allows for induction of Foxp3 gene expression in $CD4^+CD25^-$ T cells and that this process involves the TGF-β and ERK signaling pathways. In addition, TGF-β, 5-Aza, or U0126 treatment decreases DNMTs expression levels in $CD4^+CD25^-$ T cells, suggesting DNMTs are important in maintaining Foxp3 gene expression.

CHAPTER VI

PROJECT II A REGULATED SUICIDE SYSTEM IN CANCER GENE THERAPY

DESIGN AND EFFICACY TEST OF TβRIIDN-TK/GCV SUICIDE SYSTEM

The role of TGF–β in regulating autoimmunity has been best demonstrated by animal knockout studies. These studies have showed that TGF-β has a profound anti-proliferative effect on the immune system (185). TGF-β knockout mice grow normally for the first 2 weeks before they develop a rapid wasting syndrome and eventually die by 3-4 weeks of age (34). Pathological examination show an excessive inflammatory response with massive infiltration of lymphocytes and macrophages as well as immunoglobulin (Ig) deposits (33, 34). These syndromes were later characterized as autoimmunity (33). Transgenic studies using a dominant negative TGF-β receptor II to abrogate TGF-β signaling specifically in T cells (CD4-TβRIIDN) showed a development of autoimmunity in the mice (42). Therefore, blockage of TGF-β signaling in hematopoeitic stem cells, which develop into all immune cells, is expected to lead to the development of autoimmunity in the TβRIIDN mice (26, 122).

Results from the previous studies in our laboratory are very encouraging in the field of cancer gene therapy and might provide a possible therapy for cancer (26, 122). However, the development of autoimmunity resulting from blockage of TGF-β signaling presents a huge drawback to this approach (33, 122). To resolve this issue, a regulated gene expression system can be incorporated into this approach, such as the Tet-on/off system or HSV-tk/GCV suicide system. In an earlier attempt, a Tet-on/off system was used to make a fusion gene TβRIIDN-

Tet. However, due to the nature of this system, the regulation was rather leaky. This unsuccessful attempt turned our attention to the HSV-tk/GCV suicide system. The ultimate goal of this study is to develop a HSV-tk/GCV suicide system in which the TβRIIDN gene is fused to the HSV-tk gene, so that cells expressing this fusion gene can be eliminated by GCV treatment. The incorporation of the HSV-tk/GCV suicide system into the original approach should provide a possible way to eradicate tumor cells and eliminate the inflammation/autoimmunity developed in the TβRIIDN mice observed in the original studies.

Design MSCV-based retroviral vectors harboring TβRIIDN-tk gene

TGF-β signals through its type I and type II receptors on the cell surface. Upon binding to its Type II receptor, the intracellular portion of the receptor recruits and activates Type I receptor to form a receptor complex (Fig. 1). Taking advantage of this property, a dominant negative Type II receptor was designed to block TGF-β signaling by truncating the intracellular domain of Type II receptor (Fig. 32). The truncated form of this receptor is termed TβRIIDN. This truncated receptor still functions as a receptor in terms of binding of TGF-β. However, due to the lack of its intracellular domain, it is unable to form a receptor complex with Type I receptor. Consequently, cells expressing TβRIIDN are devoid of all the downstream effects that are normally elicited by binding of TGF-β. Therefore, blockage of TGF-β signaling is achieved (Fig. 32).

In collaboration with Dr. Isaac Kim, currently at the Cancer Institute of New Jersey, we have modified the original retroviral vector containing TβRIIDN by connecting the HSV-tk to

the TβRIIDN in lieu of the TβRII intracellular domain (26, 122). The incorporation of HSV-tk/GCV suicide system provides a potential treatment for cancer while preventing the development of autoimmunity elicited by blocking TGF-β signaling (Fig. 33). The original TβRIIDN and control vector GFP used in studies by Shah *et al*. are shown in Fig 34 A. This retroviral vector is a MSCV (mouse stem cell virus) based bicistronic vector that harbors the TβRIIDN gene with an IRES site enabling the expression of GFP. The control vector GFP is the same vector that lacks TβRIIDN gene (Fig 34A). The modified vector, termed TβRIIDN-tk, uses the same viral backbone with HSV-tk cloned to TβRIIDN as a fusion gene. This vector possesses two properties: it still functions as the dominant negative type II TGF-β receptor, which blocks TGF-β signaling, and it is also susceptible to GCV treatment (Fig. 34B). The control vector is termed GFP-tk in which TβRIIDN-tk gene is replaced with HSV-tk gene and is regulated by GCV but does not block TGF-β signaling (Fig. 34B). The development of the fusion gene TβRIIDN-tk provides a potential solution to prevent the autoimmunity development resulting from blockage of TGF-β signaling (122).

TβRIIDN-TK/GCV SUICIDE SYSTEM IN CANCER GENE THERAPY

Studies by Shah *et al*. have demonstrated TβRIIDN as a powerful tool in cancer treatment. Yet the issue of autoimmunity development remained unresolved. The ultimate goal of the present study is to utilize TβRIIDN-tk in cancer treatment. BMCs will be rendered insensitive to TGF-β so that they can eradicate tumors. And the presence of HSV-tk ensures elimination of the transduced BMCs and the immune cells derived from these BMCs by GCV

treatment before they cause autoimmunity in the host. The following studies were designed to test the efficacy of TβRIIDN-tk in both control NIH3T3 cells and BMCs.

Expression of HSK-tk gene in transduced NIH3T3 and BMCs

To assess HSV-tk gene expression after transduction of TβRIIDN-tk and GFP-tk constructs, HSV-tk mRNA expression in the infected cells were examined using RT-PCR. Control NIH3T3 cells and BMCs were infected as described in the Materials and Methods, total RNA was isolated, and RT-PCR was performed using HSV-tk-specific primers. As expected, transduced NIH3T3 cells (Fig. 35A) and BMCs (Fig. 35B) expressed HSV-tk. This control experiment established the efficient TβRIIDN-tk and GFP-tk gene transfer and expression in both control NIH3T3 cells and BMCs.

Efficacy of TβRIIDN-tk/GCV suicide system in cell killing

We then tested the efficacy of TβRIIDN-tk/GCV suicide system in NIH3T3 cells. Cells were infected with TβRIIDN-tk or the control vector GFP-tk. After infection, cells were cultured in the presence or absence of GCV (10 μg/ml) for 72 hours. After treatment with GCV, a proliferation assay was performed. Wild-type NIH3T3 cells and NIH3T3 cells infected with TβRIIDN or GFP vectors were used as controls. NIH3T3 cells infected with TβRIIDN-tk did not proliferate in the presence of GCV but proliferated vigorously in the absence of GCV (Fig. 36). As expected, treatment of GCV had no effect on proliferation of wild-type NIH3T3 cells and NIH3T3 cells infected with TβRIIDN or GFP (Fig. 36). Interestingly, GCV treatment elicited only a minimal effect on proliferation of NIH3T3 cells infected with control vector GFP-

tk (Fig. 36). This was also observed in BMCs infected this vector (Fig. 37). However, BMCs infected with TβRIIDN-tk did not proliferate in the presence of GCV while proliferation was vigorous without the treatment (Fig. 37).

The minimal effect of GFP-tk on proliferation of cells infected with this vector in responding to GCV treatment was intriguing, as HSV-tk gene is obviously present in these cells (Fig. 35). To resolve this issue, a new control vector was designed and used in the following studies.

Redesign and development of a new control vector Trans-tk

For unknown reasons, cells infected with the control vector GFP-tk did not behave like those infected with TβRIIDN-tk when treated with GCV (Fig. 36 and 37). Since the difference between TβRIIDN-tk and GFP-tk is that HSV-tk is anchored to the TβRIIDN molecule and HSV-tk is free in the GFP-tk construct, we reason that if HSV-tk in GFP-tk construct is also anchored to the cell membrane, as it is in TβRIIDN-tk, cells infected with this new control vector should also respond to GCV treatment. The new vector was designed by excising the extracellular domain of the TβRIIDN but retaining the transmembrane domain so that the HSV-tk is anchored into the membrane. This new control vector is termed Trans-tk. This construct lacks the extracellular domain of TβRIIDN, so cells infected with this vector can not bind TGF-β. Since HSV-tk is present in the infected cells, these cells should be efficiently eliminated by GCV treatment (Fig. 38).

Efficacy of the new control vector Trans-tk in NIH3T3 cells

To examine the efficacy of the new control vector Trans-tk, RT-PCR was performed to evaluate the level of gene expression in the infected control cell line NIH3T3. NIH3T3 cells were infected as described in the Materials and Methods, and HSV-tk expression was examined in these cells (Fig. 39). High infection efficiency was achieved in NIH3T3 cells (> 97%) as determined by FACS (Fig. 39A). RT-PCR revealed that infected NIH3T3 cells expressed HSV-tk gene (Fig. 39B). To measure the proliferation of cells after infection in the presence or absence of GCV treatment, a [^{3}H]thymidine incorporation assay was performed. As expected, NIH3T3 cells infected with TβRIIDN-tk showed no proliferation when treated with GCV, while wild-type NIH3T3 cells proliferated vigorously both in the presence or absence of GCV (Fig. 40A). As predicted, cells infected with Trans-tk did not proliferate in the presence of GCV but they proliferated vigorously in the absence of GCV (Fig. 40 A). As seen in earlier studies, however, cells infected with GFP-tk vector again showed a minimal reduction in proliferation when treated with GCV (Fig. 40A). The results demonstrate the efficacy of the new control vector Trans-tk in decreasing proliferation of infected cells when treated with GCV. GCV-mediated effect on proliferation was many times more effective in cells infected with Trans-tk than cells infected with GFP-tk.

Next, cell viability was measured using a Trypan blue exclusion assay. Trypan blue enters the membrane of dead or dying cells and stains these cells, whereas live cells repel the dye because of the intact membrane. Infected NIH3T3 cells were treated with or without GCV for three days then stained with Trypan blue. The percentage of viable cells was calculated as

described in the Materials and Methods. Viability of wild-type NIH3T3 cells did not decrease when treated with GCV, while cells infected with either TβRIIDN-tk or Trans-tk had a dramatic decrease in cell viability in the presence of GCV (Fig. 40B). GCV treatment had a minimal affect on cell viability in cells infected with GFP-tk vector (Fig. 40B). Taken together, these experiments validate the efficacy of the HSV-tk/GCV suicide system in our MSCV retroviral model, with the redesigned control vector Trans-tk displaying comparable cell killing to the TβRIIDN-tk vector in NIH3T3 cells when treated with GCV.

Verification for TGF-β sensitivity

After establishing the efficacy in cell killing, we examined the sensitivity to TGF–β in NIH3T3 cells infected with TβRIIDN-tk or Trans-tk. A Plasminogen Activator Inhibitor-1 (PAI-1)/Luciferase assay was used to measure TGF–β signaling. TGF-β increases PAI-1 expression, therefore, when PAI-1 promoter is fused to the Luciferase gene, luciferase activity correlates to the extent of TGF–β signaling (54, 55). Wild-type NIH3T3 and NIH3T3 infected with Trans-tk or GFP-tk exhibited elevated Luciferase activity when treated with TGF-β, while TβRIIDN-tk-infected NIH3T3 cells lost their sensitivity to TGF-β, as indicated by the lack of an increase in Luciferase activity (Fig. 41). These experiments successfully demonstrated the efficacy of the TβRIIDN-tk/GCV suicide system in cell killing as well as blocking TGF-β signaling. The results provide strong support to our hypothesis that incorporation of a HSV-tk/GCV suicide system in TβRIIDN-mediated gene therapy could offer a potential solution in prevention of autoimmunity in the host with TβRIIDN-tk infected immune cells.

Efficiency of HSV-tk gene expression in BMCs

Finally, the efficiency of gene transfer was tested in BMCs. BMCs were infected as described in the Materials and Methods and examined for gene transfer efficiency. Greater than 75% of TβRIIDN-tk or Trans-tk gene transfer efficiency was achieved in BMCs (Fig. 42A). HSV-tk gene was efficiently expressed in infected BMCs as determined by RT-PCR (Fig. 42B). The efficiency of gene transfer allows us to further pursue this study in an *in vivo* mouse model. In the future studies as discussed in the following session, BMCs infected with TβRIIDN-tk will be transferred to lethally-irradiated recipient mice. This approach will then allow us to efficiently eradicate tumor cells as well as eliminate potential harmful BMCs before the onset of autoimmunity in the recipient mice. Detailed experimental designs are further discussed in the Discussion and Future Directions session.

CHAPTER VII

DISCUSSION AND FUTURE DIRECTIONS

$CD4^+CD25^+Foxp3^+$ T_{reg} cells are known for their ability to suppress proliferation of T cells and autoimmunity in animals (84-86). They are also linked to cancer development, as depletion of T_{reg} cells facilitates tumor rejection (89-92). T_{reg} cells preferentially accumulate in tumor draining lymph nodes and tumor masses, and the presence of such cells is correlated with a poor prognosis in patients (80, 104, 147). Depletion of T_{reg} cells is beneficial in tumor rejection in animal studies, identification of the origin of T_{reg} cells in the tumor microenvironment will provide valuable information to successfully eliminate T_{reg} cells and thus the suppression of anti-tumor immunity by these cells (91, 130, 186). Thus far, the origin of T_{reg} cells in the tumor microenvironment still remains elusive. However, recent evidence has suggested at least four possible sources of these T_{reg} cells (149). First is trafficking. Tumor cells produce chemokines, such as CCL22, that specifically recruit T_{reg} cells to tumor sites (80). Second is differentiation through contact with tumor-induced immature dendritic cells (DCs) (148). Third is the expansion of T_{reg} cells through DCs stimulation (187). Lastly, T_{reg} cells can also be converted from normal $CD4^+$ T cells in tumor-bearing animals (147). It appears that there are two distinct types of T_{reg} cells in the tumor microenvironment. Naturally occurring T_{reg} cells that are either recruited to the tumor sites or stimulated to expand represent one type, and converted T_{reg} cells in the tumor microenvironment represent the second type. Although the phenotype of these two types of T_{reg} cells is virtually indistinguishable (188), the origin of these T_{reg} cells differs as described above. TGF-β plays a critical role in extrathymic generation/conversion of T_{reg} cells either through direct or indirect mechanisms. The direct

conversion of T_{reg} cells by tumor-derived TGF-β is demonstrated in the present study, while the indirect mechanism involves induction of immature DCs by tumor-derived TGF-β which in turn increase T_{reg} cell conversion (148). Neither direct expansion of naturally occurring T_{reg} cells by DCs nor trafficking of such cells to the tumor microenvironment requires TGF-β (80, 187). And because a direct treatment with TGF-β induces T_{reg} cell conversion and neutralization of TGF-β reduced T_{reg} cells in the tumor microenvironment (Chapter I and II), it is logical to reason that $CD4^+CD25^+Foxp3^+$ T_{reg} cells observed in the tumor masses, at least some of them, are converted by tumor-produced TGF-β. These converted T cells possess the suppressive ability of immune cells, thus giving tumor a growth advantage by evading the immune system.

T cells obtained from the *in vitro* conversion studies are *bona fide* regulatory T cells in terms of Foxp3 expression, cytokine production (low IL-2, high IL-10 and TGF-β) and suppression of $CD4^+CD25^-$ T cells (Chapter I). Additional studies revealed a critical role of TGF-β in the conversion as tumor cells (TRAMP-C2 and RENCA) produce high levels of this cytokine, and neutralization of TGF-β completely abrogated the conversion of $CD4^+CD25^-$ T cells to T_{reg} cells (Chapter I). The importance of TGF-β in this conversion was further demonstrated by the studies of $CD4^+CD25^-$ T cells cultured in CM derived from a non-tumorigenic cell line NRP-152 or irradiated TRAMP-C2, as these cells lack all the characteristics of T_{reg} cells due to the absence of TGF-β in CM (Chapter I).

Tumor cells are a rich source of TGF-β in the tumor microenvironment, and tumor-derived TGF-β becomes a source that influences the conversion of normal $CD4^+$ T cells into T_{reg} cells. Indeed, studies by Kretschmer *et al* have demonstrated that addition of TGF-β enhances the conversion of naïve $CD4^+$ T cells into T_{reg} cells when stimulated by a minute antigen dose with suboptimal dendritic cell activation (189). The present study suggests that tumor-derived TGF-β can convert subset of normal $CD4^+$ T cells to T_{reg} cells in the tumor sites. We thus propose that tumor cells evade the immune system by conversion of T_{reg} cells through production of high levels of TGF-β. When tumor-infiltrating $CD4^+$ T cells are exposed to a TGF-β rich environment in tumor sites, $CD4^+CD25^+Foxp3^+$ T_{reg} cell conversion occurs. As a result, the converted T_{reg} cells suppress anti-tumor immunity, conferring a growth advantage to tumor cells. Indeed, a significant reduction in lung metastases was observed in tumor-bearing animals treated with a neutralizing Ab against TGF-β, 1D11 (Fig. 24 and25). This reduction in tumor burden, at least in part, was due to the absence of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the tumor microenvironment of these animals (Fig. 27). The absence of $Foxp3^+$ T_{reg} cells is likely due to the lack of conversion of normal $CD4^+$ T cells because of the absence of TGF-β in these animals. Moreover, it is of interest to point out that TGF-β produced by host tissues also could play a role in T_{reg} cell conversion. Studies by Thomas *et al* showed that blockage of host-produced TGF-β aids tumor rejection (190). In their studies, they engineered tumor cells to produce a soluble form of TGF-β type II receptor that systemically neutralizes TGF-β. When these engineered tumor cells were injected into the mice, animals were able to reject the tumors. Mice injected with tumor cells which produce minimal levels of TGF-β eventually succumbed to tumor

burden, suggesting host-produced TGF-β could also play an important role in tumor rejection as an alternative source of TGF-β in T_{reg} cell conversion.

The present and other recent studies have revealed various sources of T_{reg} cells found in the tumor microenvironment. Although the possibility still remains that some T_{reg} cells observed in the tumor mass in the present study, are recruited by tumor-derived CCL22, as demonstrated by Curiel *et al* (80), it is likely that the population of T_{reg} cells present in the tumor microenvironment consists of a heterogeneous mixture of naturally occurring T_{reg} cells and converted T_{reg} cells. Cytokines produced by tumor cells, such as CCL22, IL-10, and TGF-β, can work in concert to aid tumors in evading the immune system, either by recruitment of naturally occurring T_{reg} cells to tumor sites or through direct or indirect conversion of normal $CD4^+$ T cells to T_{reg} cells (80, 147-149). Furthermore, it has been shown that functional $CD4^+CD25^+$ T_{reg} cells can be expanded by DCs (187). Finally, T_{reg} cell conversion could also occur independently of TGF-β, as shown in the study by Kretschmer *et al* in which T cells with antigen (Ag) specificity can be efficiently induced to T_{reg} cells with minute amount of Ag stimulation along with suboptimal DC activation (189). However, addition of TGF-β further enhances such conversion. It appears that extrathymic generation/conversion of T_{reg} cells could be Ag-specific or non-specific. In either case, TGF-β plays an essential role in this conversion. It is therefore important to identify the source of T_{reg} cells in the tumor mass in order to provide a better understanding and more efficient measurement in the treatment of existing tumors, as depletion of $CD25^+$ T cells will not prevent the conversion of normal $CD4^+$ T cells into T_{reg} cells by tumor cells. Combination of $CD25^+$ T cell depletion and blockage of TGF-β should further improve

the outcome of treatment of existing tumors. However, because TGF-β is a very potent immunosuppressant, long term systematic blockage of TGF-β may result in autoimmunity in patients. Therefore, it is critical to find the delicate balance between tumor immunity and autoimmunity that is maintained by T_{reg} cells in the treatment of tumors. In summary, the present study provides evidence that tumor cells can directly convert normal $CD4^+$ T cells into $CD4^+CD25^+Foxp3^+$ T_{reg} cells through production of TGF-β, thus providing a possible mechanism whereby tumors evade the host immune surveillance program through generation of T_{reg} cells.

It is well documented that TGF-β induces Foxp3 expression in $CD4^+CD25^-$ T cells *in vitro* (145, 191). We have demonstrated in our laboratory that tumor cells can directly convert $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T_{reg} cells through action of tumor-derived TGF-β (Chapter I and II). The converted T_{reg} cells express the crucial transcription factor Foxp3. Although ample evidence in the literature supports the notion that TGF-β induces Foxp3 expression, the precise mechanism remains elusive. In our initial studies, we have made an attempt to address this issue by exploring possible de-methylation of the Foxp3 promoter in $CD4^+CD25^-$ T cells. First of all, using Methprimer (169) we have successful predicted 5 possible CpG islands on the mouse Foxp3 promoter (Fig. 29). This finding prompted us to further investigate the potential mechanisms of Foxp3 induction by TGF-β. Using a methylation inhibitor 5-Aza, we were able to show that global inhibition of methylation induced Foxp3 expression in $CD4^+CD25^-$ T cells (Fig. 30). In addition, an ERK inhibitor U0126 was able to induce Foxp3 expression these T cells (Fig. 30). Furthermore, real-time RT-PCR revealed an overall decrease in expression level

of DNMTs in $CD4^+CD25^-$ T cells treated with TGF-β, 5-Aza, or U0126 (Fig. 31). These results suggest a possible de-methylation process of the Foxp3 promoter in $CD4^+CD25^-$ T cells treated with TGF-β.

From the above observations, we have derived a hypothesis: TGF-β induces Foxp3 promoter demethylation in $CD4^+C25^-$ T cells through an ERK/DNMT-dependent pathway. According to this hypothesis, when $CD4^+CD25^-$ T cells are treated with TGF-β, ERK2 phosphorylation is down regulated. As a result, this down regulation of ERK2 activation leads to decreased activity of DNMTs and therefore decreased methylation of Foxp3 promoter, and consequent gene expression. Thus far, the initial experiments can only serve as indirect evidence to support this hypothesis. To provide direct evidence to support the hypothesis, a methylation specific PCR (MSP PCR) is needed to examine the methylation status of potential CpG islands on the Foxp3 promoter (Fig. 29). If the MSP PCR reveals methylation on the predicted CpG islands, it will provide direct support to the hypothesis that the mouse Foxp3 gene expression is regulated by promoter methylation. To further elucidate the mechanism, phospho-ERK2 levels need to be examined by western blot in $CD4^+CD25^-$ T cells when treated with TGF-β. Studies conducted by Deng *et al* showed that ERK2 phosphorylation is down regulated in T cells treated with TGF-β (173, 174), but this phenomenon is yet to be proven in our hands. Finally, the DNMT responsible for methylation of Foxp3 promoter can be determined by using siRNA to knock down specific DMNTs one at the time. Methylation status will be examined in the absence of one or more DNMNTs. The above critical experiments will provide evidence to

support the hypothesis that TGF-β influences Foxp3 promoter methylation through down regulation of ERK phosphorylation.

Many tumors produce high levels of TGF−β. Since TGF−β is a potent immunosuppressant, tumor-produced TGF−β can suppress the immune responses against tumor cells and therefore facilitate tumor evasion of the immune surveillance (34, 58-62). In theory, if TGF−β production is inhibited in tumor cells, the immune system can eliminate tumors in the absence of the suppression by TGF-β. Alternatively, if the immune system could be rendered insensitive to TGF−β, it would then be possible for the immune system to eradicate tumors. Indeed, in the previous studies carried out in our laboratory, we tested both hypotheses and found that both approaches were successful in eliminating tumor growth in animal models (26, 121) However, such approaches, although successful, also revealed their limitations. It is not realistic to eliminate TGF-β production in already-established tumors. Therefore it is not clinically feasible. In the case of rendering the immune system insensitive to TGF-β, the biggest limitation is the development of autoimmunity in animals receiving the TβRIIDN BMCs after tumor eradication (122).

Fortunately, this limitation could possibly be resolved through a conditional gene expression, such as the TβRIIDN-tk/GCV suicide system. In this approach, TβRIIDN is fused to HSV-tk and it is under regulation of HSV-tk. When treated with GCV, cells expressing HSV-tk will undergo apoptosis. The ultimate goal of this study is to efficiently eradicate tumors while preventing the development of autoimmunity. We have successfully demonstrated the efficacy

of TβRIIDN-tk/GCV in killing NIH3T3 cells. We have also shown that cells infected with TβRIIDN-tk are insensitive to TGF-β signaling while the control vector Trans-tk infected cells remain responsive to TGF-β (Fig. 40-41).

As gene transfer efficiency has been established in the BMC, BMCs infected with TβRIIDN-tk or Trans-tk will be transferred into lethally-irradiated recipient mice. The experimental design of future studies is outlined in Fig. 43. In this *in vivo* study, instead of transferring BMCs before tumor challenge as described in an earlier study in our laboratory conducted by Shah *et al*. (26), tumor cells will be injected I.V. via tail vein to mice prior to BMCs transplant to mimic a more realistic clinical setting where tumor cells are already established. As outlined in Fig. 43, 5 days after Fluorouracil (5-FU) injection of the donor mice to enrich the stem cells, BMCs will be isolated as described previously (26). After collecting viral particles harboring TβRIIDN-tk or Trans-tk from GP293 cells, these viral particles will be used to infect 2 and 3 days post isolation primary bone marrow cells via spin infection. BMCs infected with the TβRIIDN-tk or Trans-tk will be transplanted into the lethally irradiated recipient mice that were challenged with tumor cells prior to BMCs transplant. The resulting chimeric mice will be subjected to the administration of GCV i.p. at 150 mg/kg twice a day for 6 days and the development of autoimmunity and tumor metastases will be monitored in these animals.

In conclusion, we expect the combination of TβRIIDN and HSV-tk would provide a powerful tool in cancer therapy where tumor cells are efficiently eradicated. The potential

harmful TβRIIDN-expressing cells could be eliminated using the HSV-tk/GCV suicide system before they cause any damage to the host. In addition, in this study, we will test the efficacy of TβRIIDN-tk BMC to eradicate already-established tumors by challenging the mice with tumor cells before BMC transplant, which mimics the realistic clinical setting. The results from our initial *in vitro* experiments are very encouraging, and we are hopeful to develop this technique and eventually apply it as a potential human gene therapy in cancer treatment.

FIGURES

Figure 1. TGF-β signaling transduction pathway. TGF-β signals through its Type I (TβRI) and type II (TβRII) receptors. Binding of TGF-β to TβRII induces the association and activation of TβRI. The activated TβRI then phosphorylates and activates receptor-associated Smads, R-Smads (Smad2 and 3). Phosphorylated R-Smads have increased affinity for common Smads, Co-Smad (Smad 4) to form a complex. The resulting complex then translocates into nucleus to associate with transcriptional co-activators or co-repressors to regulate gene expression in a context-specific manner.

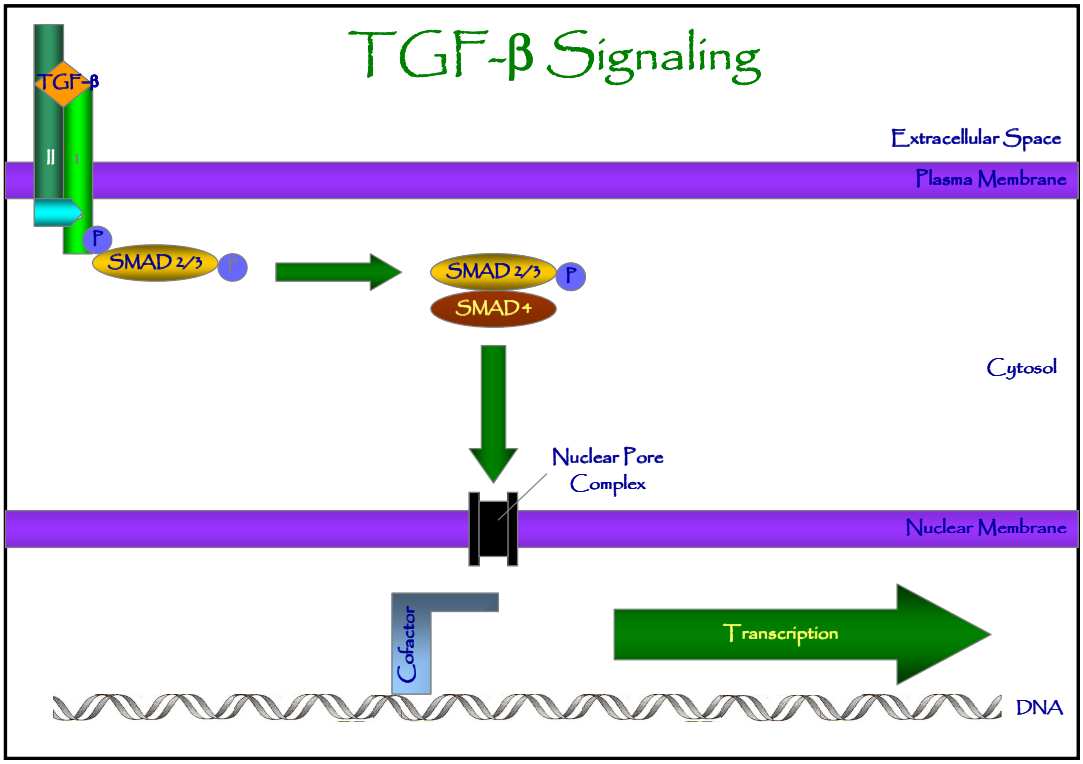

TGF-β Signaling
TGF-β
II
I
P
SMAD 2/3
P
SMAD 2/3
P
SMAD4
Extracellular Space
Plasma Membrane
Cytosol
Nuclear Pore
Complex
Nuclear Membrane
Cofactor
Transcription
DNA

Figure 2. Tumor evasion of the immune system by producing high levels of TGF-β. Many types of tumor cells produce high levels of TGF-β. Consequently, tumor-derived TGF-β forms a "firewall" around the tumor cells in the tumor microenvironment. Since TGF-β is a potent immunosuppressant, tumor-produced TGF-β can suppress the anti-tumor immunity, therefore conferring a growth advantage to tumor cells.

Tumor microenvironment

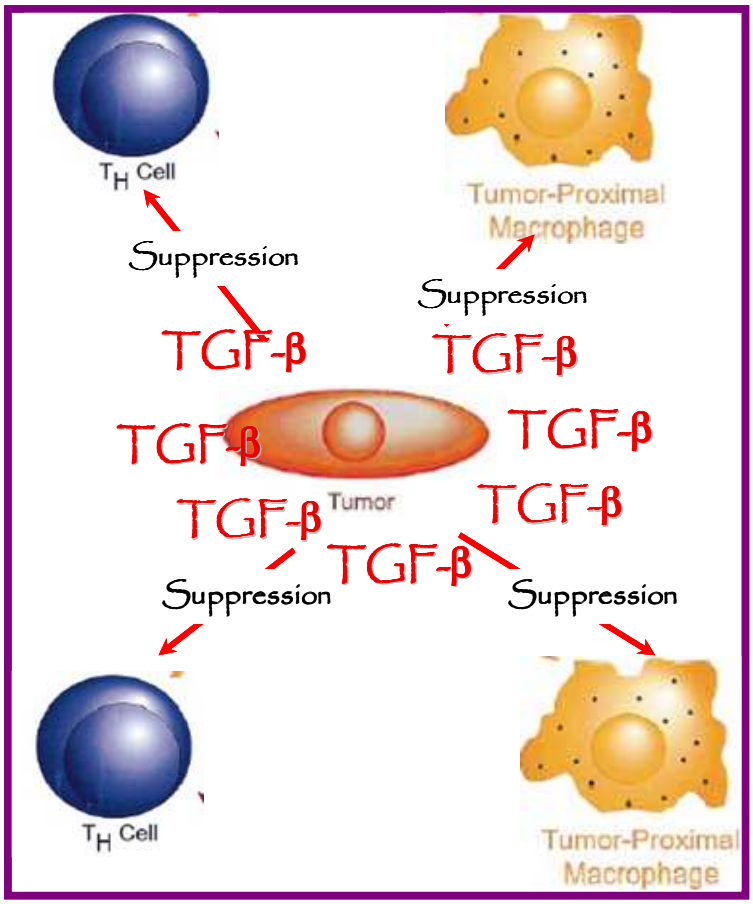


Figure 3. $CD4^+CD25^+$ T cells present in the tumor mass. TRAMP-C2 cells (1×10^6) were injected into C57BL/6 mice intravenously via tail vein. Mice were sacrificed at day 30 and lung tissues were fixed in formalin, cut into 4 μm sections, and used to examine CD4 and CD25 expression. A representative section is shown here. Red color indicates CD25 positive T cells, green, CD4 positive T cells, and double positive T cells are shown as yellow. Tumor nuclei were counterstained with Hoechst 33342 dye and are shown as blue. Scale bars, 20 μm.

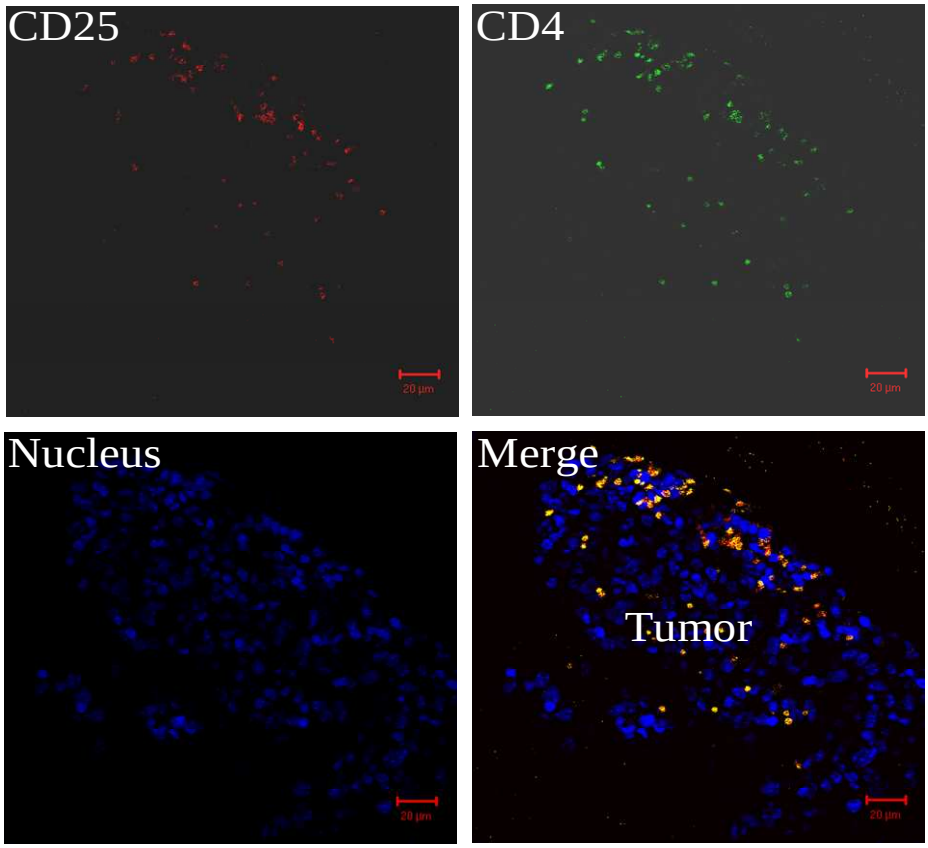

CD25
CD4
Nucleus
Merge
Tumor

Figure 4. $CD25^+Foxp3^+$ T cells present in the tumor mass. TRAMP-C2 cells (1×10^6) were injected into C57BL/6 mice intravenously via tail vein. Mice were sacrificed at day 30 and lung tissues were fixed in formalin, cut into 4 μm sections, and used to examine CD25 and Foxp3 expression. A representative section is shown here. Red color indicates CD25 positive T cells, green, Foxp3 positive T cells, and double positive T cells are shown as yellow. Tumor nuclei were counterstained with Hoechst 33342 dye and are shown as blue. Scale bars, 20 μm.

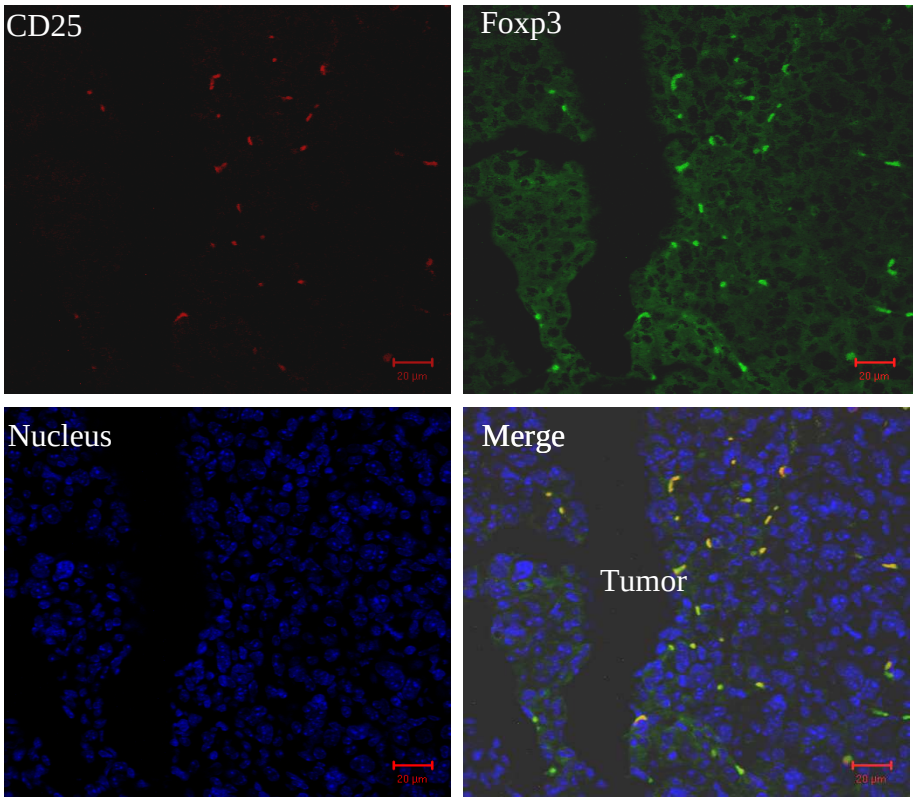

CD25
Foxp3
Nucleus
Merge
Tumor

Figure 5. Experimental design of TRAMP-C2 and T cell co-culture system. TRAMP-C2 cells were co-cultured with isolated $CD4^{+}CD25^{-}$ T cells in the presence of 1 μg/ml anti-CD3 Ab and APCs for 7 days with replacement of TRAMP-C2 cells every two days. Seven days later T cells were sorted on a FACS sorter according to cell surface CD25 expression into $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{-}$ T cell populations. The sorted T cell populations were used for Foxp3 RT-PCR.

Experimental Design

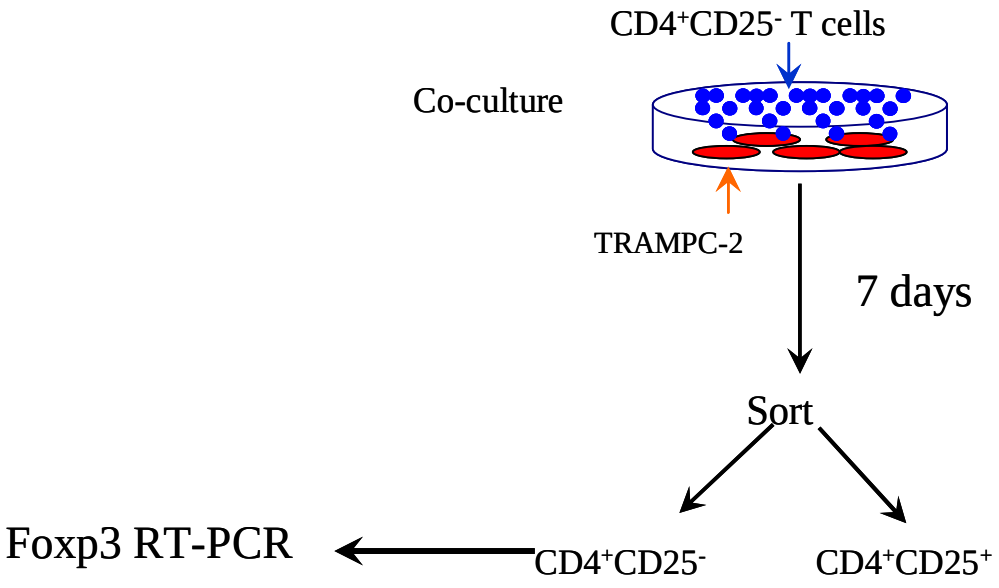


Figure 6. Expression of Foxp3 in $CD4^+CD25^-$ T cells co-cultured with TRAMP-C2 cells. Isolated $CD4^+CD25^-$ T cells were cultured and sorted as described in Fig. 5. Two-step RT-PCR was performed to evaluate Foxp3 mRNA expression levels in sorted T cell populations. Freshly isolated $CD4^+CD25^+$ T_{reg} cells were used as the positive control and freshly-isolated $CD4^+CD25^-$ T cells were used the negative control.

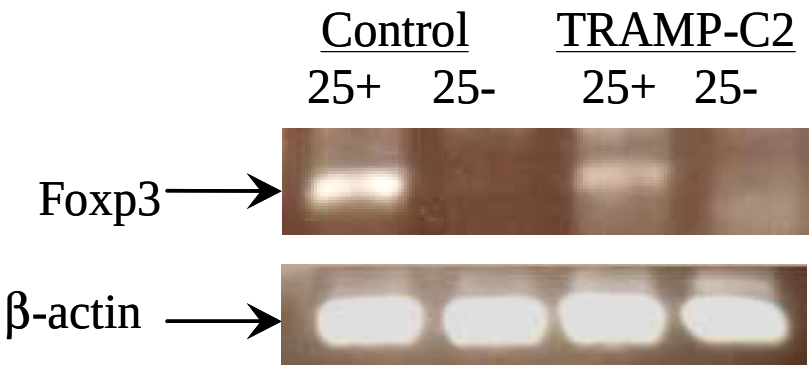
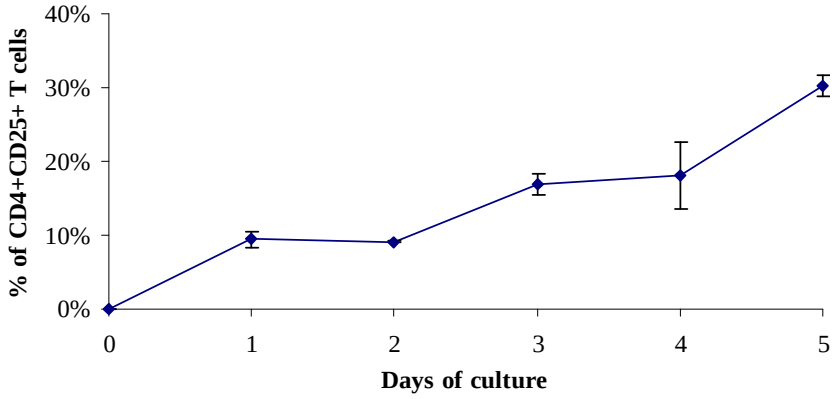

Control
TRAMP-C2
25+
25-
25+
25-
Foxp3
β-actin

Figure 7. Conversion kinetics of $CD4^+CD25^-$ T cells co-cultured with TRAMP-C2 cells. A. Rate of conversion. Isolated $CD4^+CD25^-$ T cells were co-cultured with TRAMP-C2 cells for 0-5 days. T cells were collected each day and stained for cell surface expression of CD4 and CD25 and analyzed by FACS. B. Ratio of conversion. The effect of number of TRAMP-C2 cells on $CD4^+CD25^-$ T cell conversion was determined by co-culturing TRAMP-C2 cells and $CD4^+CD25^-$ T cells at different ratios (0.1:1, 0.25:1, 0.5:1, 0.75:1, and 1:1) for 5 days. At the end of the experiment, T cells were collected and stained for cell surface expression of CD4 and CD25 and analyzed by FACS. All conditions were performed in triplicate and reported as the mean ± S.D. Results shown are representative of at least three independent experiments.

A


40%
30%
20%
10%
0%
% of CD4+CD25+ T cells
0
1
2
3
4
5
Days of culture

B

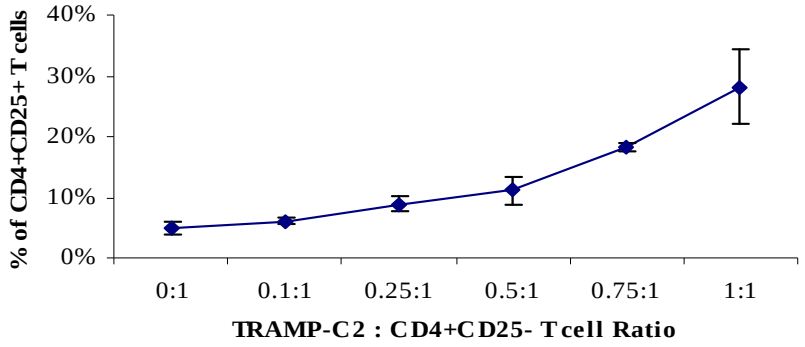

40%
30%
20%
10%
0%
% of CD4+CD25+ T cells
0:1
0.1:1
0.25:1
0.5:1
0.75:1
1:1
TRAMP-C2 : CD4+CD25- T cell Ratio

Figure 8. Experimental design of culturing $CD4^+CD25^-$ T cells with TRAMP-C2 CM. To determine whether cell-cell contact is required for TRAMP-C2 cells to induce Foxp3 expression in $CD4^+CD25^-$ T cells, another set of experiments were designed. Instead of co-culturing TRAMP-C2 cells with $CD4^+CD25^-$ T cells, CM was collected from a 3-day culture of TRAMP-C2 cells. CM was then used to culture isolated $CD4^+CD25^-$ T cells for 5 days in the presence of anti-CD3 and CD28 Abs. Five days later, the resulting T cells were used for phenotype analysis.

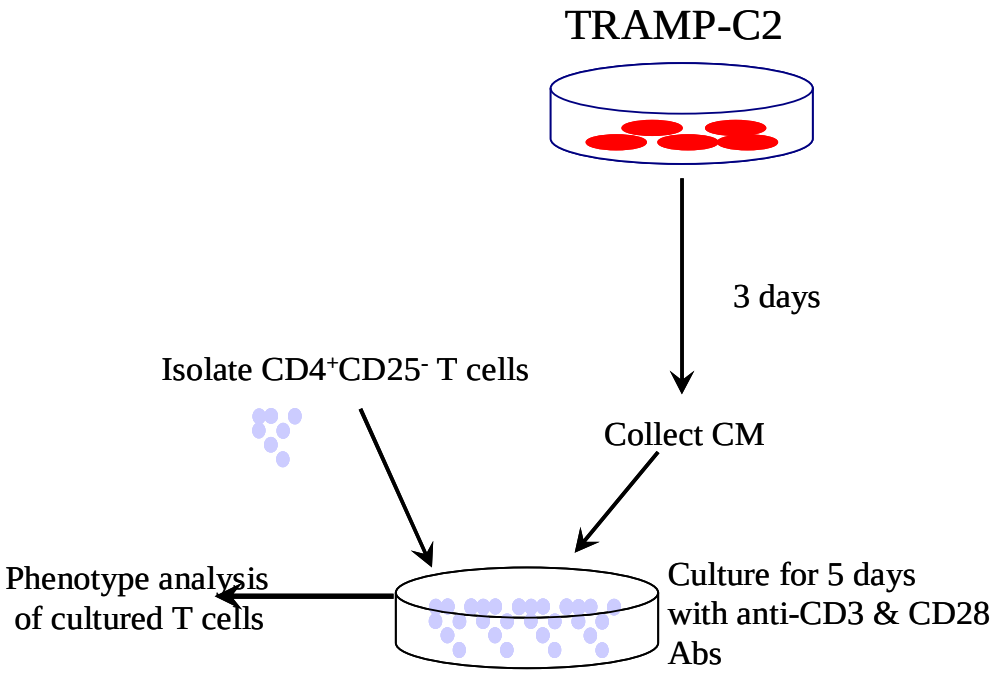
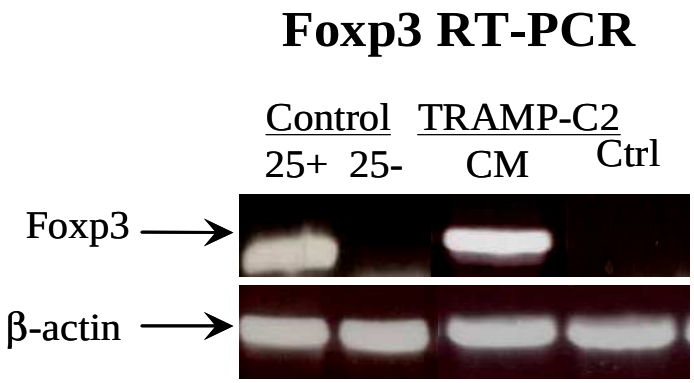

TRAMP-C2
3 days
Isolate CD4+CD25- T cells
Collect CM
Phenotype analysis
of cultured T cells
Culture for 5 days
with anti-CD3 & CD28
Abs

Figure 9. Expression of Foxp3 in $CD4^+CD25^-$ T cells cultured with TRAMP-C2 CM. Isolated $CD4^+CD25^-$ T cells were cultured as described in Fig. 7 for 5 days. The resulting T cells were used to evaluate mRNA (A) and protein (B) levels of Fox3 expression. A. Foxp3 RT-PCR. Two-step Foxp3 RT-PCR was performed to measure mRNA levels of Foxp3 expression in $CD4^+CD25^-$ T cells cultured with TRAMP-C2 CM. Freshly isolated $CD4^+CD25^+$ T_{reg} cells were use as the positive control and freshly isolated $CD4^+CD25^-$ T cells were used as the negative control. B. Intracellular staining of Foxp3. $CD4^+CD25^-$ T cells were cultured as above and protein levels of Foxp3 were measured by intracellular staining using an Ab specific to Foxp3 and analyzed by FACS. Left panel shows the intracellular Foxp3 expression of positive and negative controls. Bottom panel shows the intracellular Foxp3 expression of $CD4^+CD25^-$ T cells cultured with T cell medium or TRAMP-C2 CM. Isotype control rat IgG2a is shown as a gray area.

A


Foxp3 RT-PCR
Control
TRAMP-C2
25+
25-
CM
Ctrl
Foxp3
β-actin

B

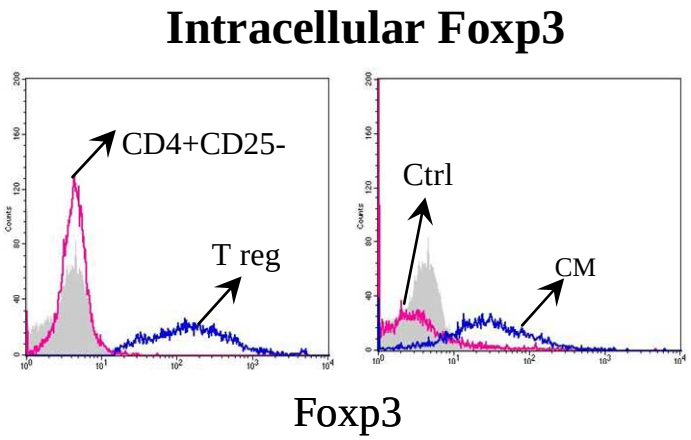

Intracellular Foxp3
CD4+CD25-
T reg
Ctrl
CM
Foxp3

Figure 10. The dilution effect of TRAMP-C2 CM on conversion of $CD4^+CD25^-$ T cells. Isolated $CD4^+CD25^-$ T cells were cultured with TRAMP-C2 CM diluted at various concentrations for 5 days. At the end of the experiment, T cells were stained for cell surface expression of CD4 and CD25 and analyzed by FACS. The starting isolated $CD4^+CD25^-$ T cell population is shown as Day 0 (d 0). A graph representation is shown at the bottom. Results shown are representative of at least three independent experiments.

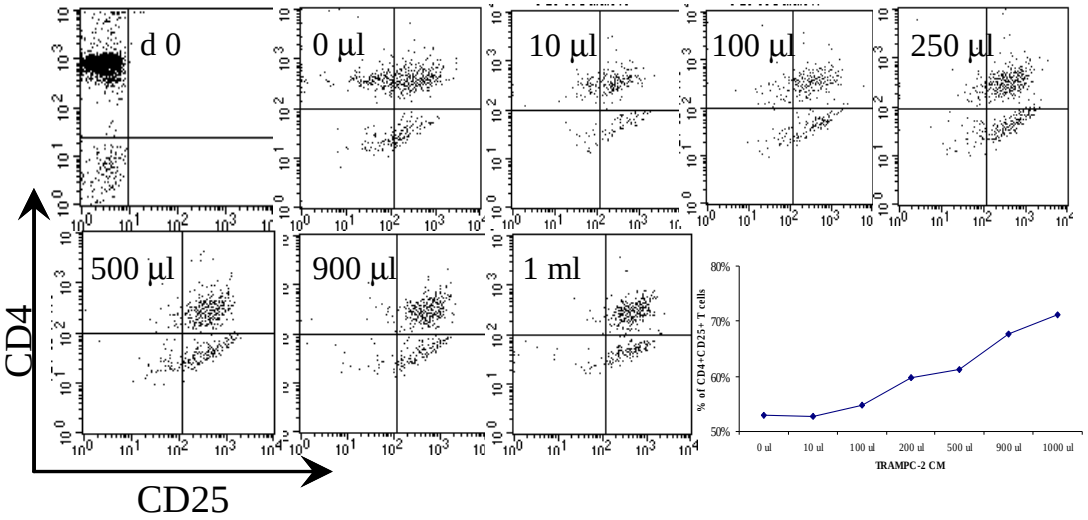
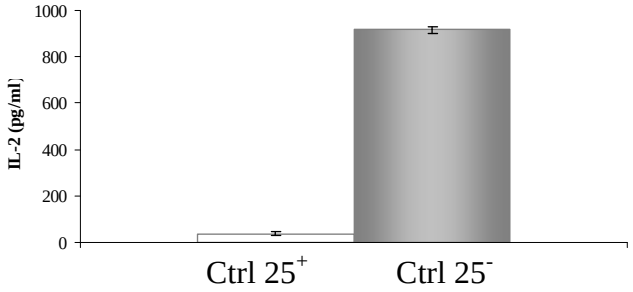

d 0
0 µl
10 µl
100 µl
250 µl
500 µl
900 µl
1 ml
CD4
CD25
% of CD4+CD25+ T cells
80%
70%
60%
50%
0 ul
10 ul
100 ul
200 ul
500 ul
900 ul
1000 ul
TRAMPC-2 CM

Figure 11. IL-2 production of $CD4^+CD25^-$ T cells cultured with TRAMP-C2 CM. Isolated $CD4^+CD25^-$ T cells were cultured with TRAMP-C2 CM for 5 days as described in Fig. 7. At the end of 5 days, T cells were washed extensively and re-stimulated with anti-CD3 and CD28 Abs for another 48 hours in T cell medium. IL-2 production by T cells was measured using IL-2 ELISA. A. IL-2 ELISA of $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ T cells. Freshly isolated $CD4^+CD25^+$ T_{reg} cells were used as the positive control and freshly isolated $CD4^+CD25^-$ T cells were used as the negative control. T cells were stimulated with anti-CD3 and CD28 Abs for 48 hours and IL-2 production was measured by ELISA. B. IL-2 ELISA of $CD4^+CD25^-$ T cells cultured with TRAMP-C2 CM. All conditions were performed in triplicate and reported as the mean ± S.D. Results shown are representative of at least three independent experiments.

A


1000
800
600
400
200
0
IL-2 (pg/ml)
Ctrl 25+
Ctrl 25-

B

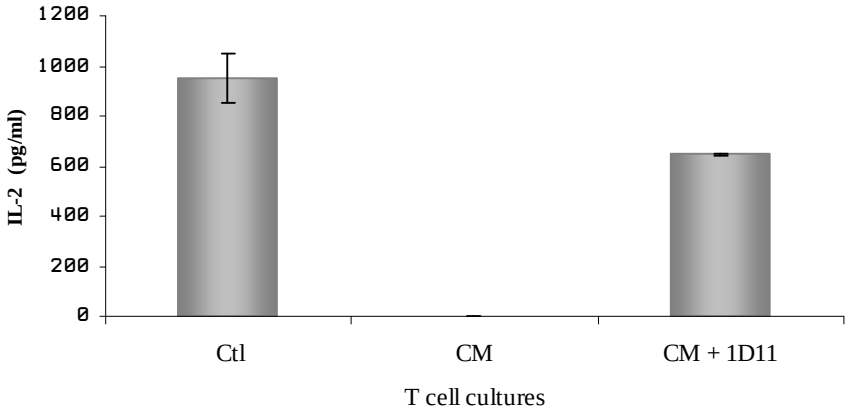
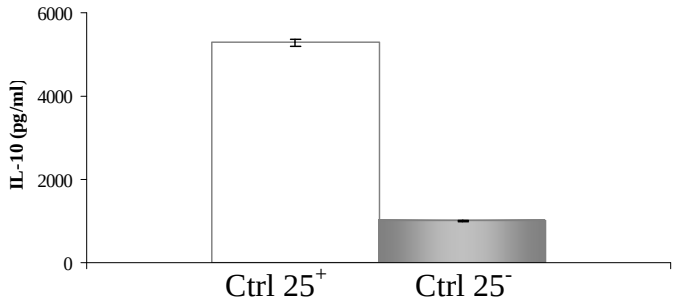

1200
1000
800
600
400
200
0
IL-2 (pg/ml)
Ctl
CM
CM + 1D11
T cell cultures

Figure 12. IL-10 production of $CD4^+CD25^-$ T cells cultured with TRAMP-C2 CM. Isolated $CD4^+CD25^-$ T cells were cultured with TRAMP-C2 CM for 5 days as described in Fig. 7. At the end of 5 days, T cells were washed extensively and re-stimulated with anti-CD3 and CD28 Abs for another 48 hours in T cell medium. IL-10 production by T cells was measured using a IL-10 ELISA. A. IL-10 ELISA of $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ T cells. Freshly isolated $CC4^+CD25^+$ T_{reg} cells were used as the positive control and freshly isolated $CD4^+CD25^-$ T cells were used as the negative control. T cells were stimulated with anti-CD3 and CD28 Abs for 48 hours and IL-10 production was measured by ELISA. B. IL-10 ELISA of $CD4^+CD25^-$ T cells cultured with TRAMP-C2 CM. All conditions were performed in triplicate and reported as the mean ± S.D. Results shown are representative of at least three independent experiments.

A


6000
4000
2000
0
IL-10 (pg/ml)
Ctrl 25+
Ctrl 25-

B

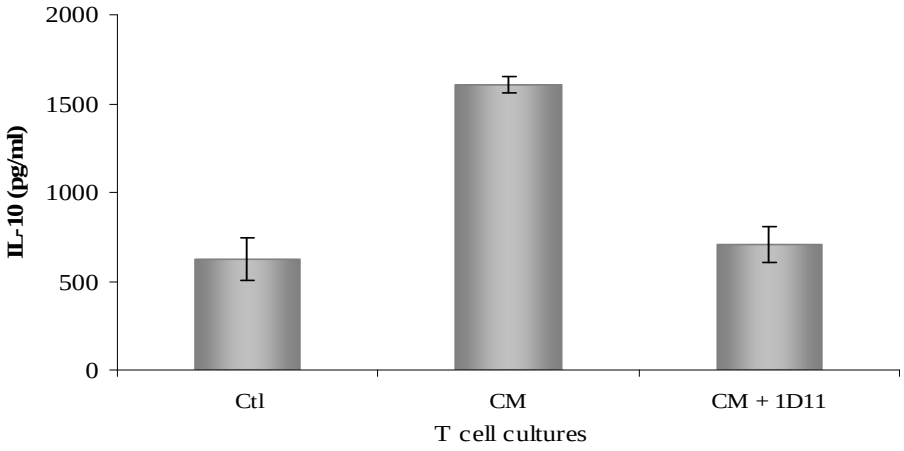
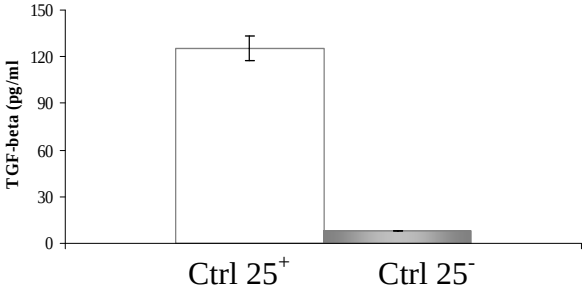

2000
1500
1000
500
0
IL-10 (pg/ml)
Ctl
CM
CM + 1D11
T cell cultures

Figure 13. TGF-β production of $CD4^+CD25^-$ T cells cultured with TRAMP-C2 CM. Isolated $CD4^+CD25^-$ T cells were cultured with TRAMP-C2 CM for 5 days as described in Fig. 7. At the end of 5 days, T cells were washed extensively and re-stimulated with anti-CD3 and CD28 Abs for another 72 hours in serum free T cell medium. TGF-β production by T cells was measured using a TGF-β ELISA. A. TGF-β ELISA of $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ T cells. Freshly isolated $CC4^+CD25^+$ T_{reg} cells were used as the positive control and freshly isolated $CD4^+CD25^-$ T cells were used as the negative control. T cells were stimulated with anti-CD3 and CD28 Abs for 72 hours in serum free medium and TGF-β production was measured by ELISA. B. TGF-β ELISA of $CD4^+CD25^-$ T cells cultured with TRAMP-C2 CM. All conditions were performed in triplicate and reported as the mean ± S.D. Results shown are representative of at least three independent experiments.

A


150
120
90
60
30
0
TGF-beta (pg/ml
Ctrl 25+
Ctrl 25-

B

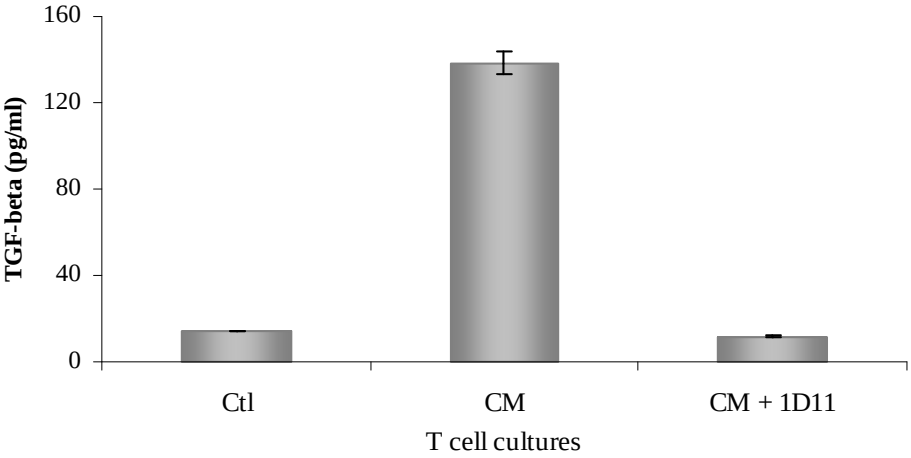
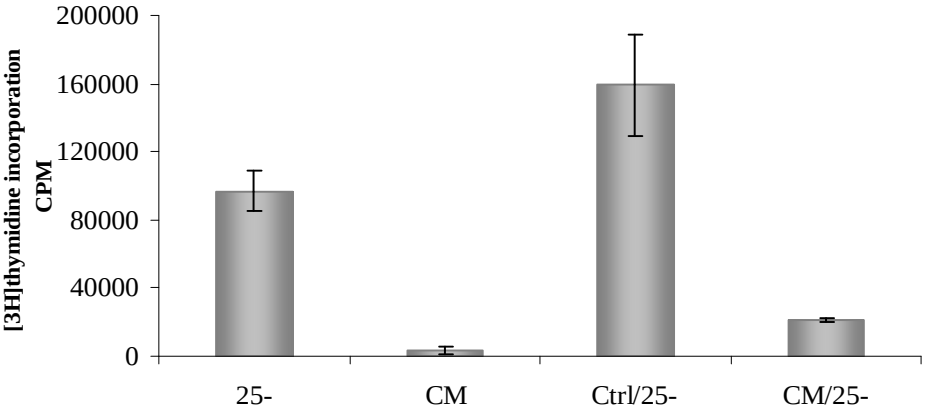

160
120
80
40
0
TGF-beta (pg/ml)
Ctl
CM
CM + 1D11
T cell cultures

Figure 14. Suppression assay of $CD4^+CD25^-$ T cells cultured with RENCA CM. A. Suppression of $CD4^+CD25^-$ T cell (1.5×10^4) proliferation. $CD4^+CD25^-$ T cells were purified and cultured in RENCA CM for 5 days. At the end of the 5-day culture, 1.5×10^4 freshly isolated $CD4^+CD25^-$ responder T cells were stimulated with an anti-CD3 Ab and APCs either alone or with 5×10^4 T cells previously cultured with RENCA CM for 72 hours. At the last 6-18 hours, 1 μCi/well of [3H]thymidine was added to each well and cells were harvested and counted in a scintillation counter. B. Suppression of $CD4^+CD25^-$ T cell (5×10^4) proliferation. 5×10^4 freshly isolated $CD4^+CD25^-$ T cells were stimulated with an anti-CD3 Ab and APCs and cultured with 5×10^4 T cells previously cultured with RENCA CM. All conditions were performed in triplicate and reported as the mean ± S.D. Results shown are representative of at least three independent experiments.

A


200000
160000
120000
80000
40000
0
[3H]thymidine incorporation
CPM
25-
CM
Ctrl/25-
CM/25-

B

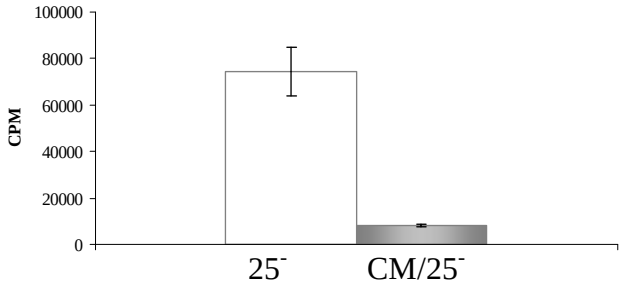
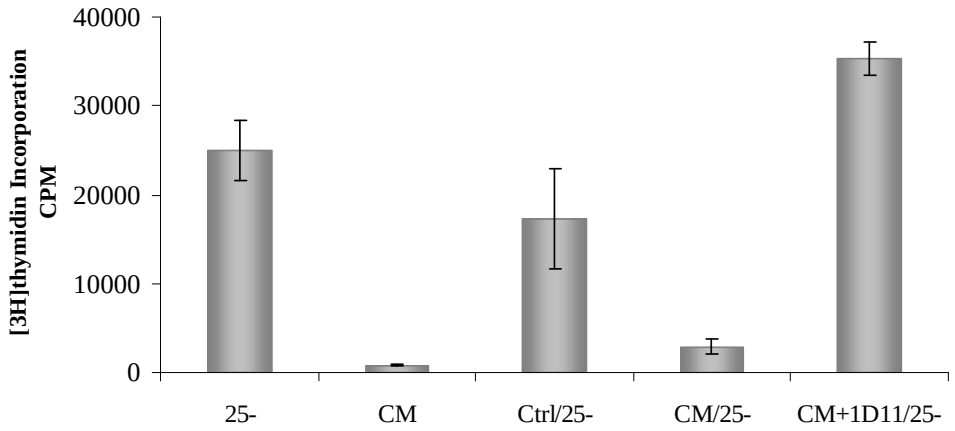

100000
80000
60000
40000
20000
0
CPM
25-
CM/25-

Figure 15. Effects of addition of 1D11 on suppressive ability of $CD4^+CD25^-$ T cells cultured with TRAMP-C2 CM. A. Suppression of $CD4^+CD25^-$ T cell proliferation in the presence or absence of 1D11. $CD4^+CD25^-$ T cells were purified and cultured in TRAMP-C2 CM for 5 days. At the end of the 5-day culture, 1.5 x 10^4 freshly isolated $CD4^+CD25^-$ responder T cells were stimulated with an anti-CD3 Ab and APCs either alone or with T cells previously cultured with TRAMP-C2 CM for 72 hours. At the last 6-18 hours, 1 μCi/well of [3H]thymidine was added to each well and cells were harvested and counted in a scintillation counter. In some cases, a neutralizing Ab against TGF-β, 1D11, was added to the TRAMP-C2 CM used to culture $CD4^+CD25^-$ T cells. B. Suppression of $CD4^+CD25^-$ T cell (5 x 10^4) proliferation. 5 x10^4 freshly isolated $CD4^+CD25^-$ T cells were stimulated with an anti-CD3 Ab and APCs and cultured with T cells previously cultured with TRAMP-C2 CM. All conditions were performed in triplicate and reported as the mean ± S.D. Results shown are representative of at least three independent experiments.

A


[3H]thymidin Incorporation
CPM
40000
30000
20000
10000
0
25-
CM
Ctrl/25-
CM/25-
CM+1D11/25-

B

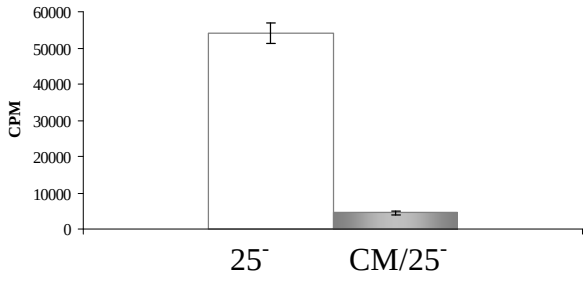

CPM
60000
50000
40000
30000
20000
10000
0
25-
CM/25-

Figure 16. **Titration assay of $CD4^+CD25^-$ T cells cultured with TRAMP-C2 CM**. $CD4^+CD25^-$ T cells were purified and cultured in TRAMP-C2 CM for 5 days. At the end of the 5-day culture, 5 x 10^4 $CD4^+CD25^-$ freshly isolated responder T cells were stimulated with an anti-CD3 Ab and APCs and cultured with various concentrations of T cells previously cultured with TRAMP-C2 CM (0 to 5 x 10^4) for 72 hours. $CD4^+CD25^-$ T cell proliferation was measured using [^{3}H]thymidine incorporation assay. All conditions were performed in triplicate and reported as the mean ± S.D. All results shown are representative of at least three independent experiments.

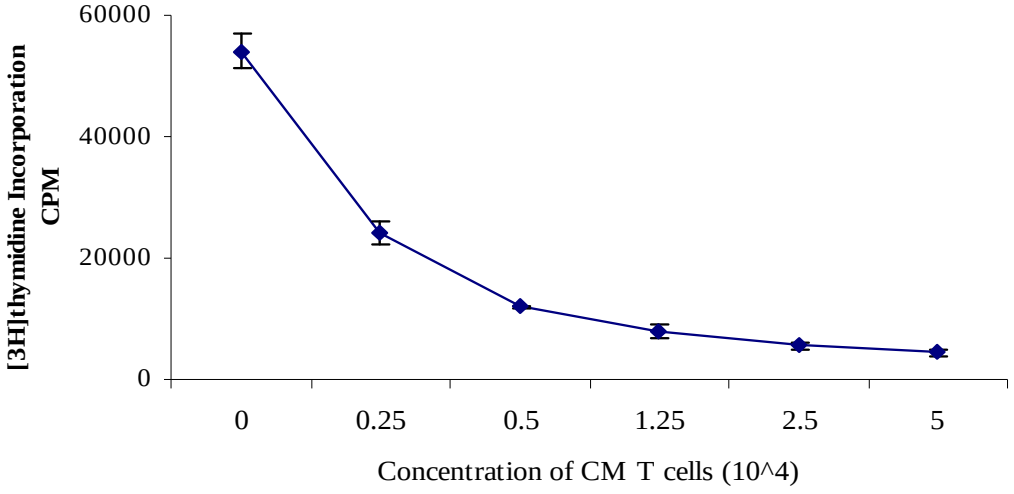

60000
40000
20000
0
[3H]thymidine Incorporation
CPM
0
0.25
0.5
1.25
2.5
5
Concentration of CM T cells (10^4)

Figure 17. TGF-β production of various cell lines. TRAMP-C2, RENCA, NRP-152, and irradiated TRAMP-C2 cells were cultured in serum free media for 3 days and CM was collected. TGF-β production in the CM was measured by ELISA. All conditions were performed in triplicate and reported as the mean ± S.D. All results shown are representative of at least three independent experiments.

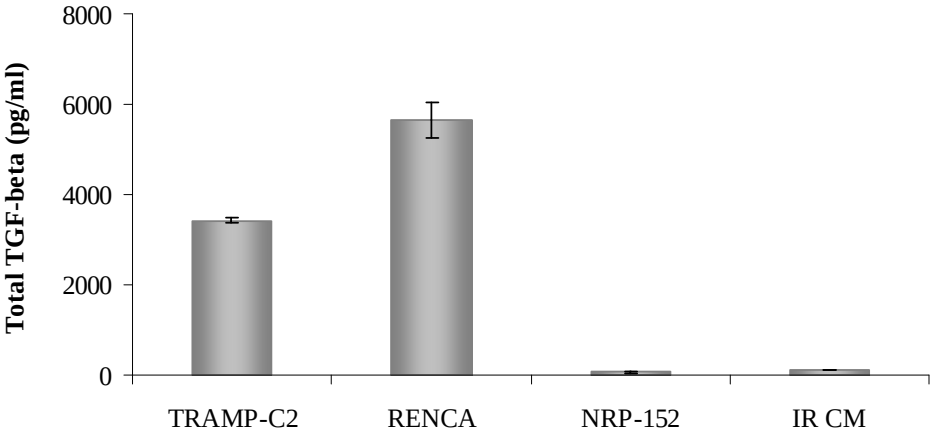
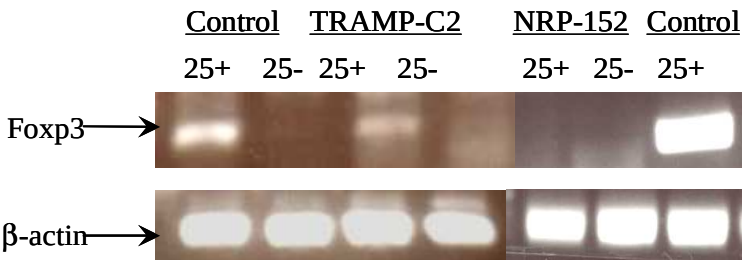

8000
6000
4000
2000
0
Total TGF-beta (pg/ml)
TRAMP-C2
RENCA
NRP-152
IR CM

Figure 18. The role of TGF-β in induction of Foxp3 expression in $CD4^+CD25^-$ T cells. A. Induction of Foxp3 expression by tumor, but not non-tumorigenic cells. $CD4^+CD25^-$ T cells were cultured with either TRAMP-C2 or NRP-152 cells for 7 days as described in Fig. 5. After sorting, total RNA was extracted from individual T cell populations and two step RT-PCR was performed to detect the Foxp3 expression. B. Abrogation of Foxp3 induction. $CD4^+CD25^-$ T cells were cultured with CM from TRAMP-C2 and NRP-152 for 5 days as described in Fig. 7. Two step RT-PCR was performed to measure the Foxp3 expression levels in the resulting CM cultured T cells. In some cases, 1D11 was added to the TRAMP-C2 CM used to cultured $CD4^+CD25^-$ T cells.

A


Control
TRAMP-C2
NRP-152
Control
25+
25-
25+
25-
25+
25-
25+
Foxp3
β-actin

B

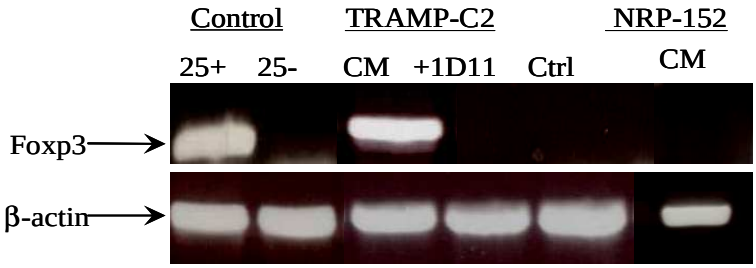

Control
TRAMP-C2
NRP-152
25+
25-
CM
+1D11
Ctrl
CM
Foxp3
β-actin

Figure 19. Suppression assay of $CD4^+CD25^-$ T cells cultured with NPR-152 CM. $CD4^+CD25^-$ T cells were purified and cultured in NPR-152 CM for 5 days. At the end of the 5-day culture, 1.5 x 10^4 freshly isolated $CD4^+CD25^-$ responder T cells were stimulated with an anti-CD3 Ab and APCs either alone or with T cells previously cultured with NRP-152 CM for 72 hours. At the last 6-18 hours, 1 μCi/well of [^{3}H]thymidine was added to each well and cells were harvested and counted in a scintillation counter. All conditions were performed in triplicate and reported as the mean ± S.D. Results shown are representative of at least three independent experiments.

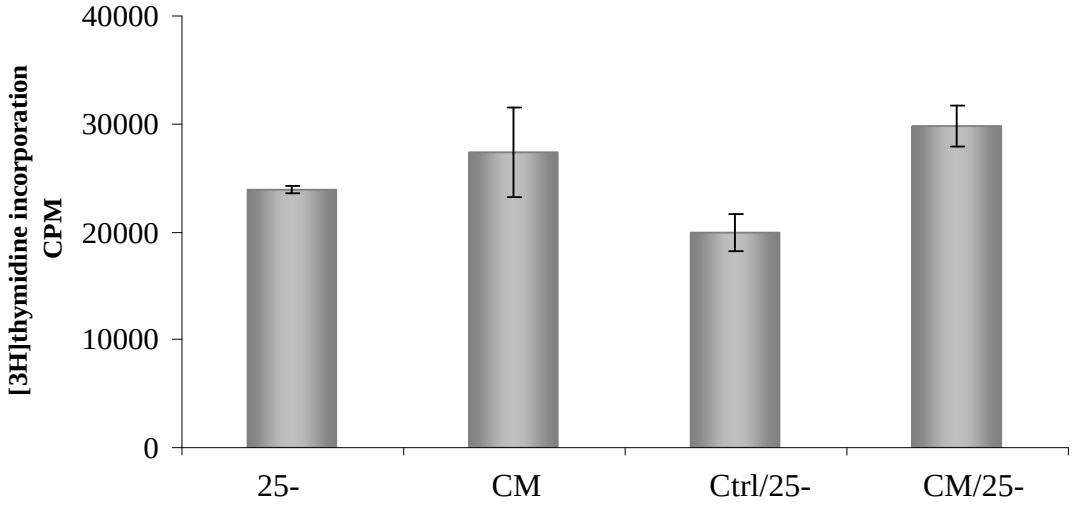

[3H]thymidine incorporation
CPM
40000
30000
20000
10000
0
25-
CM
Ctrl/25-
CM/25-

Figure 20. Suppression assay of $CD4^+CD25^-$ T cells cultured with irradiated TRAMP-C2 CM. TRAMP-C2 cells were irradiated with 20,000 rads and CM was collected from a 3-day cultured of irradiated cells. $CD4^+CD25^-$ T cells were purified and cultured with the CM for 5 days. At the end of the 5-day culture, 1.5 x 10^4 freshly isolated $CD4^+CD25^-$ responder T cells were stimulated with an anti-CD3 Ab and irradiated APCs either alone or with T cells previously cultured with irradiated TRAMP-C2 CM for 72 hours. At the last 6-18 hours, 1 μCi/well of [^{3}H]thymidine was added to each well and cells were harvested and counted in a scintillation counter. All conditions were performed in triplicate and reported as the mean ± S.D. Results shown are representative of at least three independent experiments.

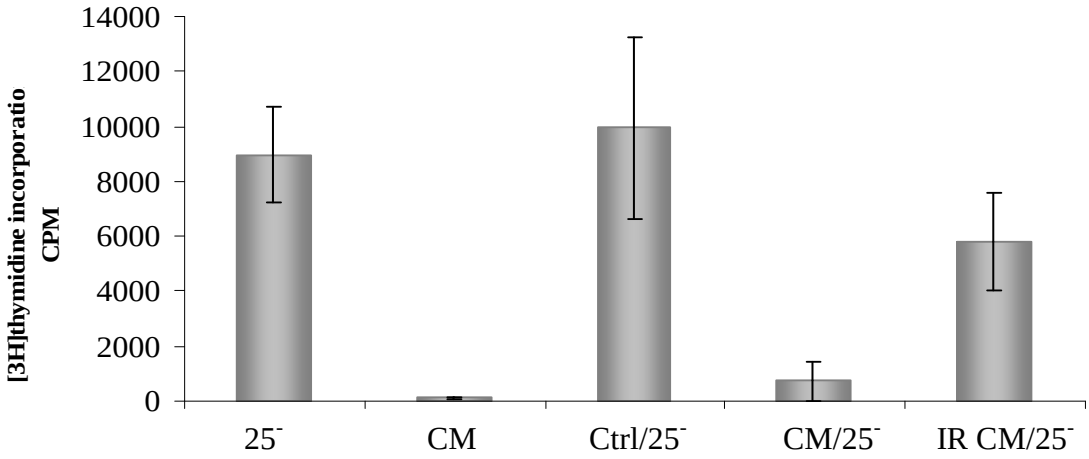

[3H]thymidine incorporatio
CPM
14000
12000
10000
8000
6000
4000
2000
0
25-
CM
Ctrl/25-
CM/25-
IR CM/25-

Figure 21. Experimental design of *in vivo* studies using a neutralizing Ab against TGF-β. Balb/c mice were injected with 1 x 10^6 RENCA cells intravenously via tail vein. Five mice received a neutralizing Ab against TGF-β, 1D11, and 5 mice received the control IgG Ab13C4 (Genzyme) at a loading dose of 100 mg/kg intraperitoneally at the day of the injection. Five mice received no treatment. Mice continuously receiving 1D11 or 13C4 treatment every 3 days at a dose of 50 mg/kg over a course of 30 day experiment. At day 30, mice were sacrificed, lung tissues were fixed with formalin, and cut into 4 μm sections for Hemotoxin & Eosin (H&E) and immunofluorescence staining. Tumor burden was evaluated by counting numbers of tumor metastases of H&E stained lung tissue sections of each animal.

Experimental Design

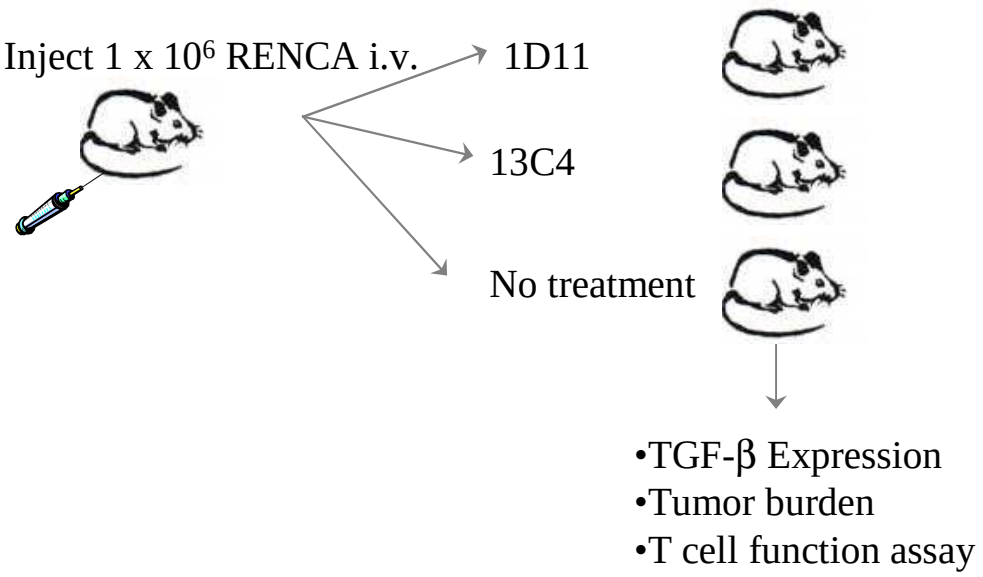


Figure 22. Serum TGF–β ELISA. Balb/c mice were treated as described in Fig. 21. At the end of the experiment, mouse sera were collected and serum TGF-β levels were measured using a TGF-β ELISA Kit. All conditions were performed in triplicate and reported as the mean ± S.D. One of two representative experiments is shown.

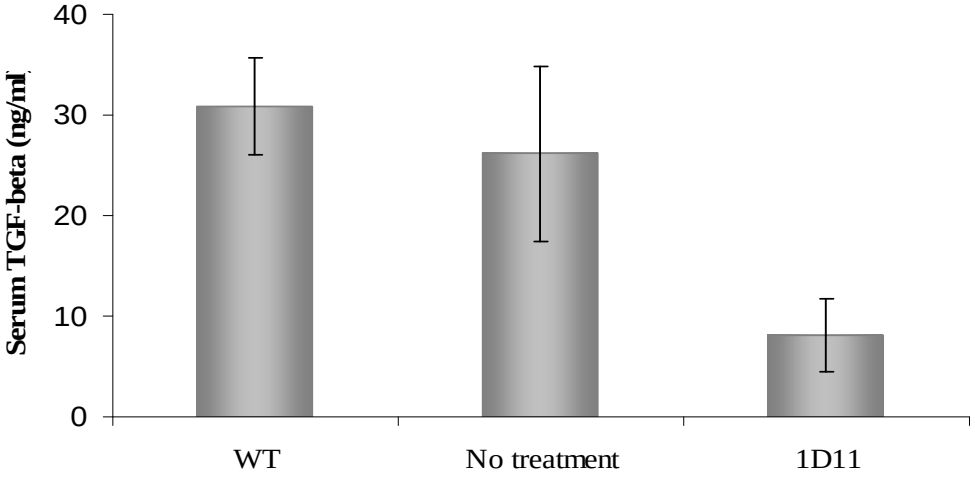
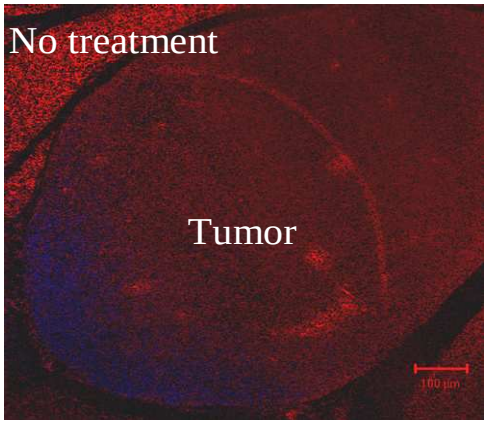
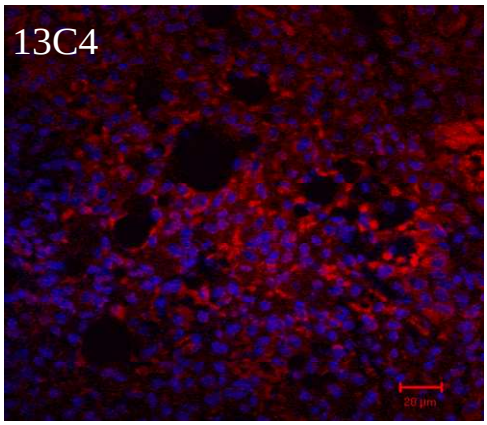

Serum TGF-beta (ng/ml)
40
30
20
10
0
WT
No treatment
1D11

Figure 23. Immunofluorescence staining of TGF-β in lung tissues. Balb/c mice were treated as described in Fig. 21. At the end of experiment, lung tissues were collected, cut into 4 μm sections, and used for immunofluorescence staining for TGF-β. A representative staining pattern is shown. Red color indicates the positive staining of TGF-β, blue, nuclei. Scale bars: Top and bottom panels, 100 μm and the middle panel, 20 μm.


No treatment
Tumor
100 µm


13C4
20 µm

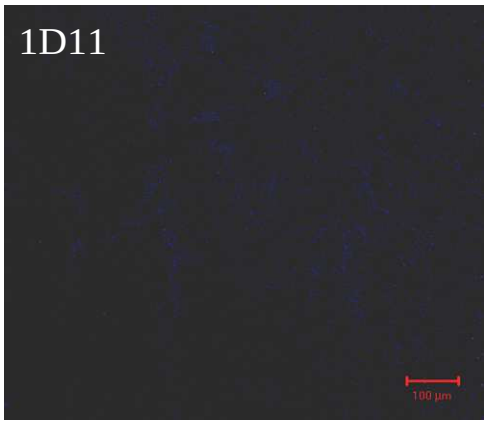

1D11
100 µm

Figure 24. Numbers of lung metastases. Balb/c mice were treated as described in Fig. 21. At the end of experiment, lung tissues were collected, cut into 4 μm sections, and used for H&E staining. Lung metastases were counted in H&E stained lung sections and the number of lung metastases of each mouse was plotted. The graph represents pooled data from 2 independent experiments. Each dot represents one animal and the lines represent the mean. *, $p < 0.05$; **, $p < 0.01$ using 1 way ANOVA with post test comparison.

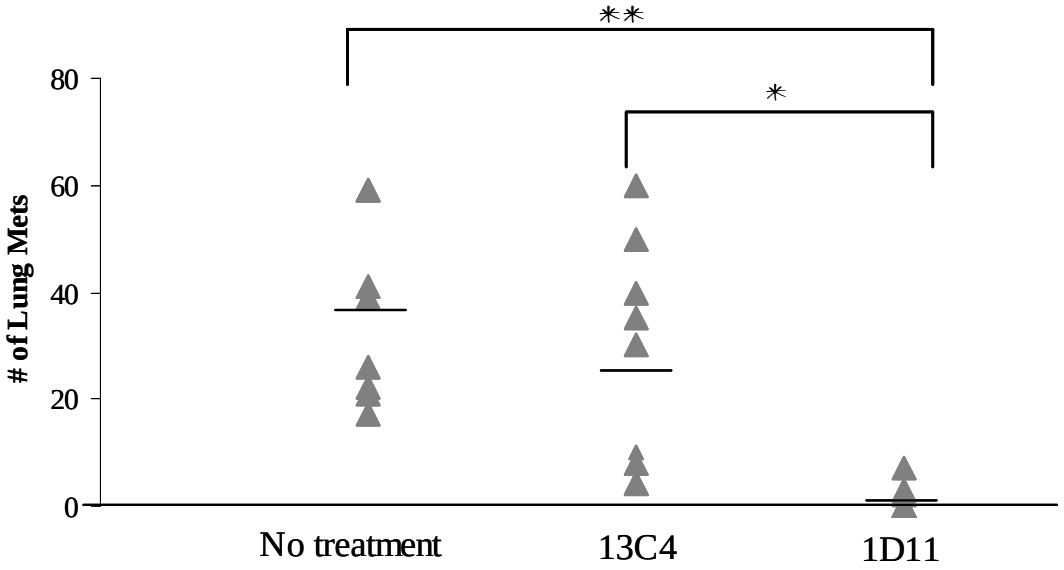

**
*
80
60
40
20
0
# of Lung Mets
No treatment
13C4
1D11

Figure 25. H&E staining of lung tissues from tumor-bearing animals. Balb/c mice were treated as described in Fig. 21. At the end of the experiment, lung tissues were collected, cut into 4 μm sections, and used for H&E staining. A representative staining section is shown in this figure. Arrows indicate lung metastases.

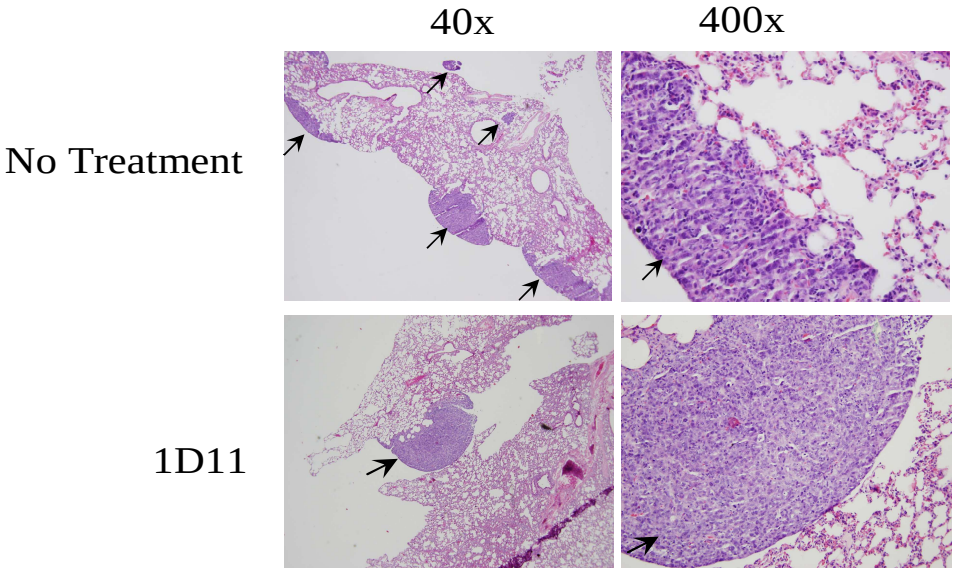

40x
400x
No Treatment
1D11

Figure 26. Absence of Foxp3^{+} T cells in the tumor mass of animals receiving 1D11. Balb/c mice were treated as describe in Fig. 21. At the end of the experiment, T lymphocytes were first enriched using Percoll from the lung tissues of animals receiving different treatments. CD4^{+}CD25^{+} T cells in lung tissues were purified from Percoll-enriched lymphocytes and analyzed by FACS (top panel). Lung tissues were collected, cut into 4 μm sections, and used for immunofluorescence staining for Foxp3. Bottom panel shows a representative staining pattern for Foxp3 in the tumor mass. Fopx3 positive cells are shown in green and nuclei are shown in blue. Scare bars, 20 μm.

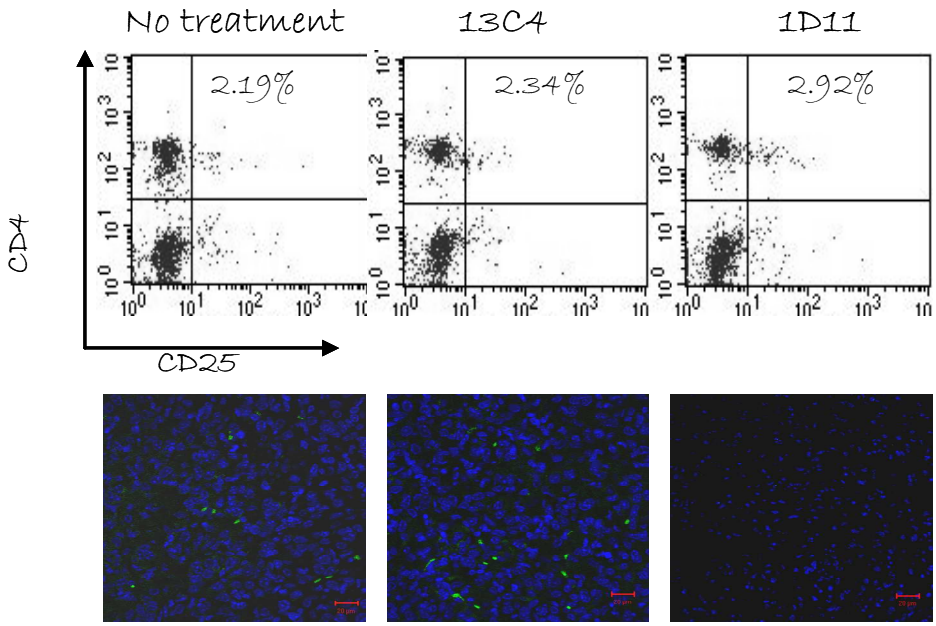

No treatment
13C4
1D11
2.19%
2.34%
2.92%
CD4
CD25

Figure 27. Loss of suppressive ability of $CD4^+CD25^+$ T cells from mice receiving 1D11. Balb/C mice were treated as described in Fig. 21. At the end of the experiment, $CD4^+CD25^+$ T cells were isolated from the lung tissues (see Materials and Methods) and used for an *in vitro* suppression assay to measure their suppressive ability. 5×10^4 freshly isolated responder $CD4^+CD25^-$ T cells were stimulated with an anti-CD3 Ab and APCs either alone or cultured with $CD4^+CD25^+$ T cells isolated from lung tissues from mice receiving different treatments. All conditions were performed in triplicate and reported as the mean ± S.D. One of two representative experiments is shown.

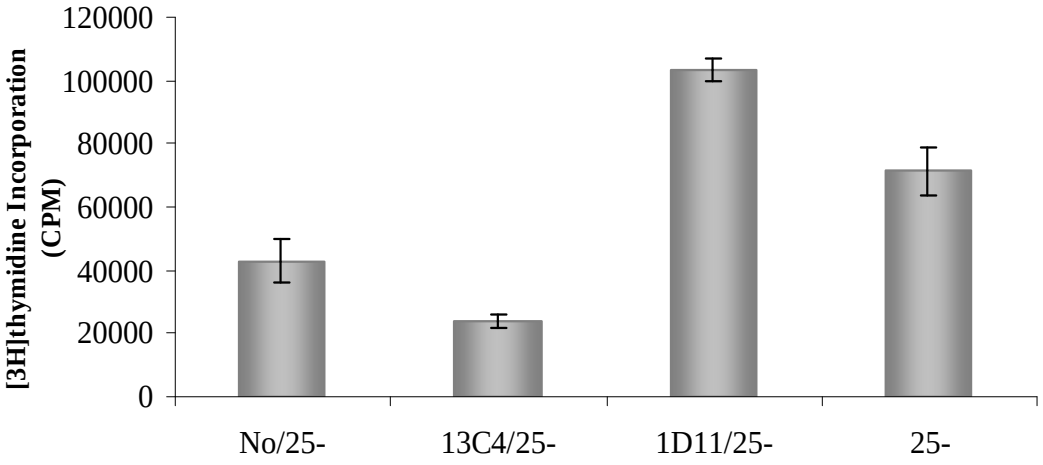

[3H]thymidine Incorporation (CPM)
120000
100000
80000
60000
40000
20000
0
No/25-
13C4/25-
1D11/25-
25-

Figure 28. A proposed model for tumor evasion of the immune system by converting $CD4^+CD25^-$ T cells into T_{reg} cells through production of TGF−β. The presence of T_{reg} cells in the tumor microenvironment is associated with a poor prognosis in patients. The source of these cells still remains elusive. However, the present and other studies have revealed various sources for T_{reg} cells in the tumor microenvironment. It appears that there are two types of T_{reg} cells. Naturally occurring T_{reg} cells that are recruited to the tumor sites represent one type, and converted T_{reg} cells in the tumor microenvironment represent the other type (See Discussion). The present study provides evidence that tumor cells can directly convert $CD4^+$ T cells into T_{reg} cells through production of high TGF-β. In this proposed model, normal $CD4^+$ T cells in the tumor microenvironment encounter the high levels of TGF-β produced by tumor cells. As a result, they are converted into $CD4^+CD25^+Foxp3^+$ T_{reg} cells. Consequently, these tumor converted T_{reg} cells suppress the anti-tumor immunity and therefore confer a growth advantage to tumor cells.

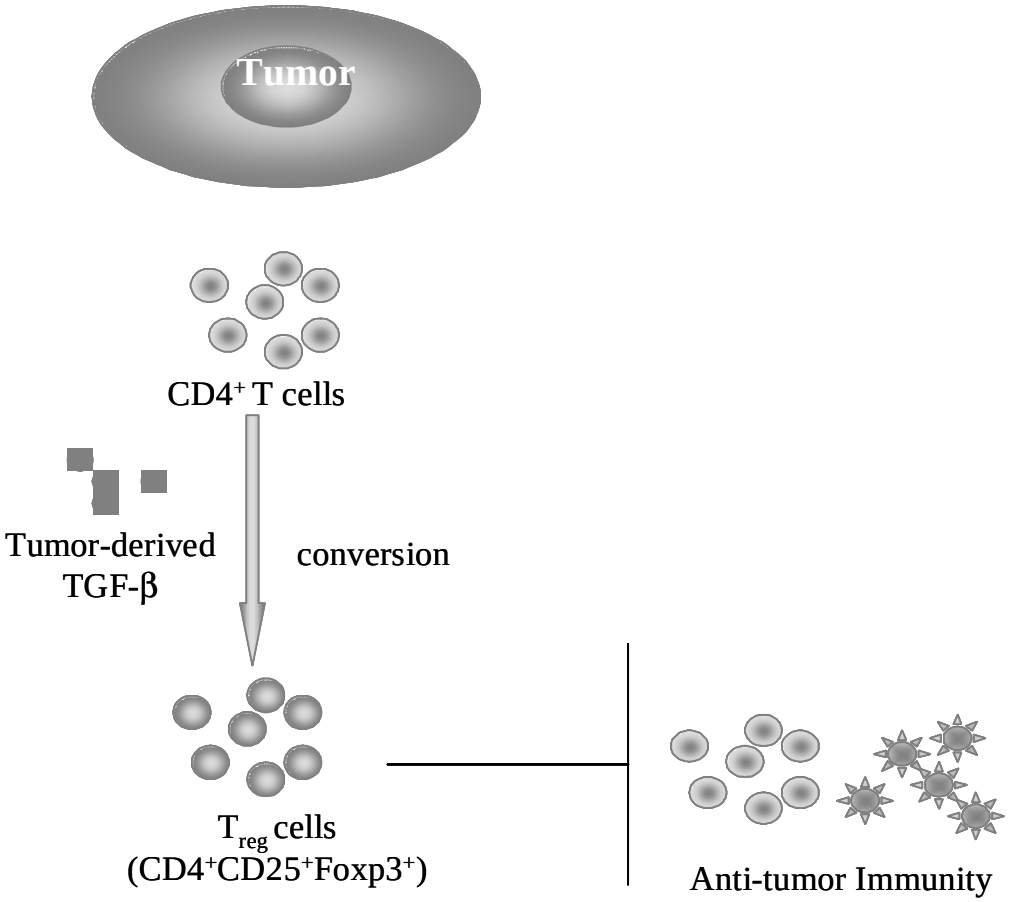

Tumor
CD4+ T cells
Tumor-derived
TGF-β
conversion
Treg cells
(CD4+CD25+Foxp3+)
Anti-tumor Immunity

Figure 29. Predicted potential CpG islands of the mouseFoxp3 promoter by MethPrimer. Mouse Foxp3 promoter sequence was obtained from NCBI nucleotide sequence database (AF277994) and was analyzed by MethPrimer (http://www.urogene.org/methprimer/index.html, 169). Five potential CpG islands are predicted by the software, shown as blue areas in the map.

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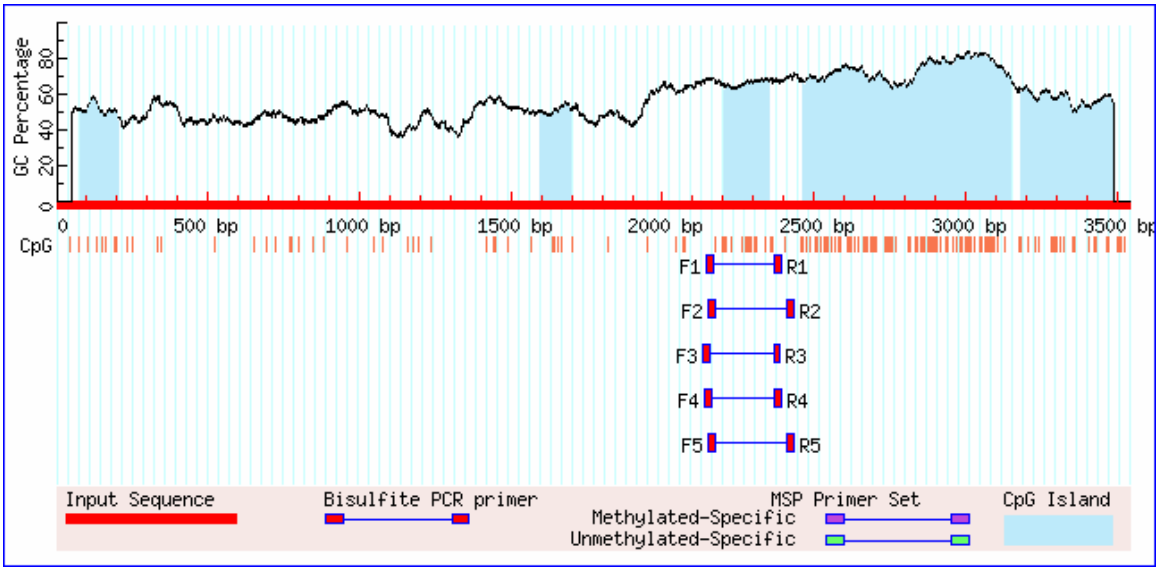


MethPrimer (http://www.urogene.org/methprimer/index.html (169)

Figure 30. Expression of Foxp3 in $CD4^+CD25^-$ T cells treated with 5-Aza or U0126. Isolated $CD4^+CD25^-$ T cells were cultured with TGF-β, 5-Aza, and U0126 for either 2 or 4 days in the presence of anti-CD3 and CD28 Abs. At the end of the experiment, total RNA was isolated and two-step RT-PCR was performed as described in the Materials and Methods. Freshly isolated $CD4^+CD25^+$ T_{reg} cells were used as the positive control and $CD4^+CD25^-$ T cells cultured with anti-CD3 and CD28 Abs only were used as the negative control.

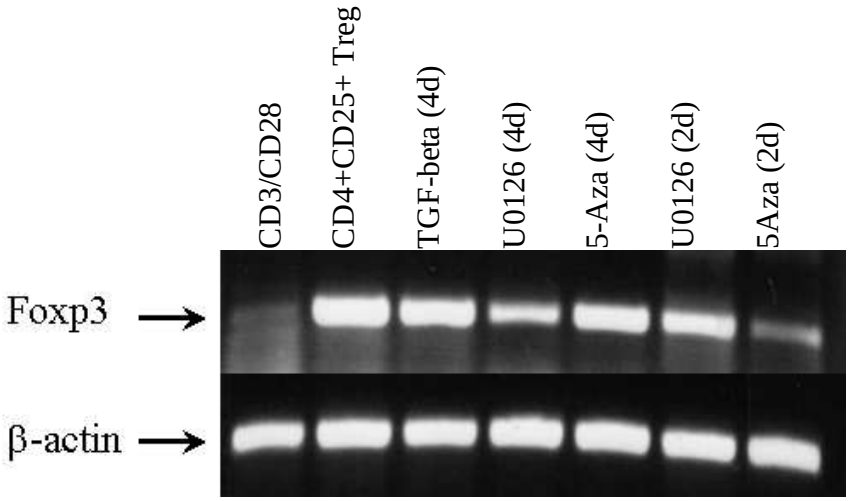
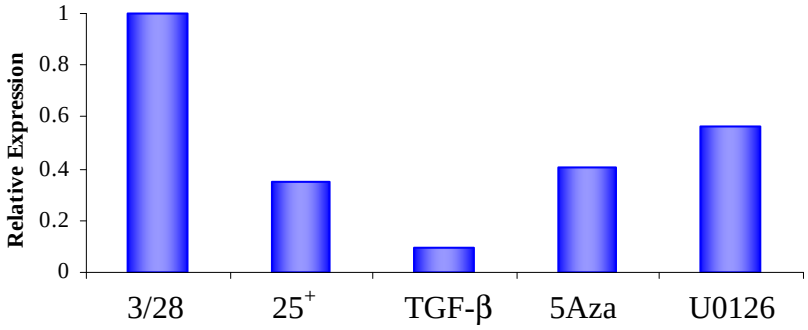
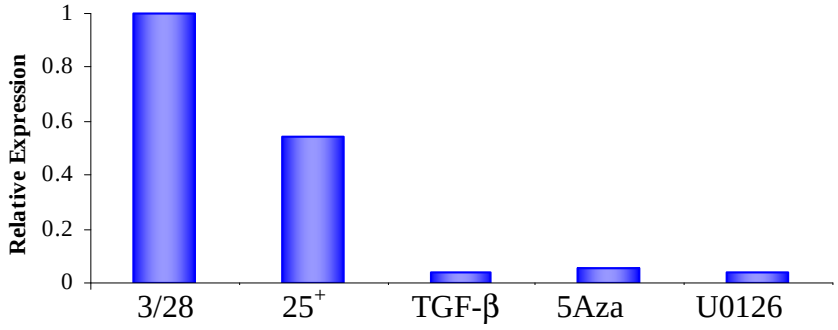

CD3/CD28
CD4+CD25+ Treg
TGF-beta (4d)
U0126 (4d)
5-Aza (4d)
U0126 (2d)
5Aza (2d)
Foxp3
β-actin

Figure 31. DNMT real-time RT-PCR. Isolated $CD4^{+}CD25^{-}$ T cells were cultured with TGF-β, 5-Aza, and U0126 for 4 days in the presence of anti-CD3 and CD28 Abs. At the end of the experiment, total RNA was isolated and equal amount of RNA was used to generate cDNA. Real-time PCR was performed to examine the expression levels of DNMNT1, DNMT3a, and DNMT3b (see the Materials and Methods).

DNMT-1 QRT-PCR



DNMT-3a QRT-PCR



DNMT-3b QRT-PCR

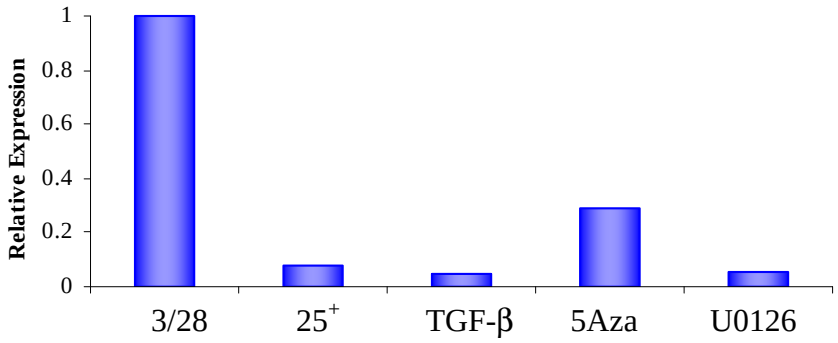


Figure 32. Blockage of TGF-β signaling using a dominant negative type II TGF-β receptor (TβRIIDN). TβRIIDN is a type II TGF-β receptor that lacks the intracellular portion. This truncated receptor can still bind to TGF-β but is unable to form a receptor complex with type I TGF-β receptor. Therefore it lacks the ability to elicit the downstream effects that are otherwise carried out in response to TGF-β signaling.

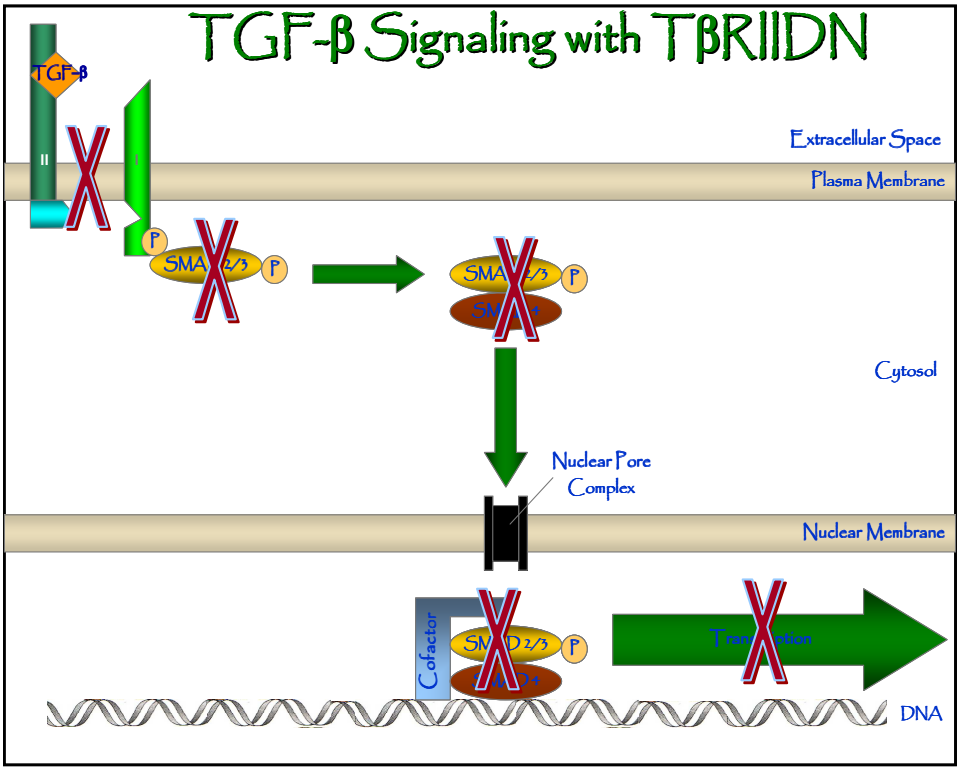
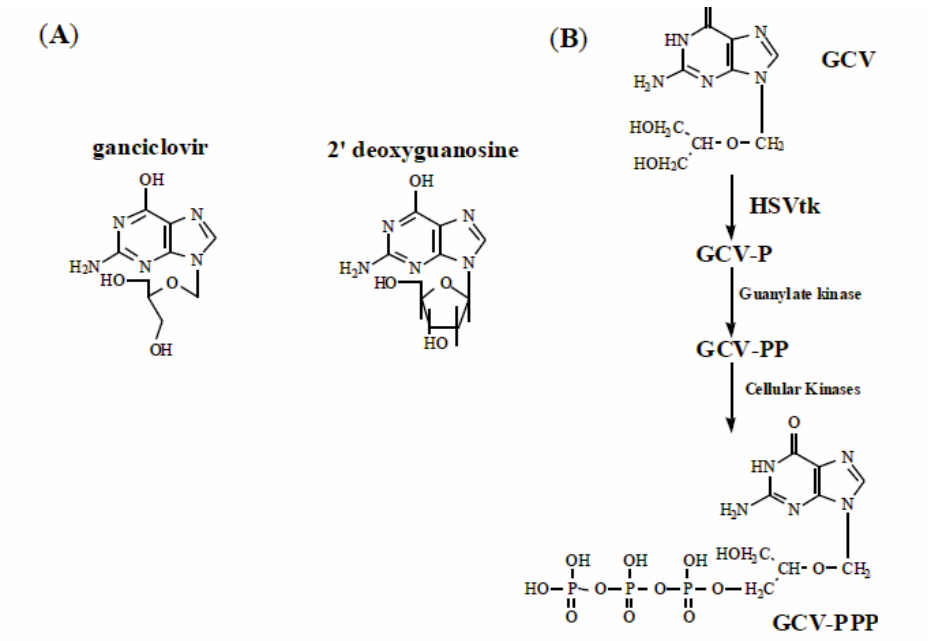

TGF-β Signaling with TβRIIDN
TGF-β
II
I
Extracellular Space
Plasma Membrane
P
SMAD 2/3
P
SMAD 2/3
P
SMAD 4
Cytosol
Nuclear Pore
Complex
Nuclear Membrane
Cofactor
SMAD 2/3
P
SMAD 4
Transcription
DNA

Figure 33. Mechanism of HSV-tk/GCV suicide system in cell killing. A. Structure of GCV. B. Enzymatic conversion of GCV-ppp by HSV-tk. HSV-tk catalyzes GCV to ganciclovir monophosphate. The subsequent formation of the diphosphate and triphosphate ganciclovir is catalyzed by cellular enzymes. GCV triphosphate is analogous to 2'-deoxyguanosine triphosphate. Although it has hydroxyl groups, it lacks a complete sugar ring that makes GCV a poor substrate for continuing chain elongation. Consequently, chain termination almost always occurs immediately after GCV incorporation into DNA and leads to cell death. C. Mechanism of cell killing. Cells are infected by retroviral vector harboring HSV-tk gene. When treated with GCV, HSV-tk catalyzes the GCV to a toxic form and consequently this conversion leads to cell death.


(A)
ganciclovir
2' deoxyguanosine
(B)
GCV
HSVtk
GCV-P
Guanylate kinase
GCV-PP
Cellular Kinases
GCV-PPP

C

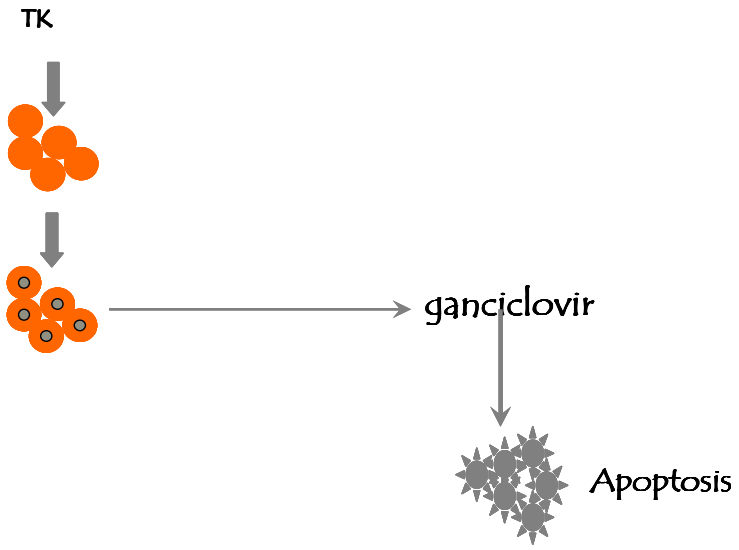
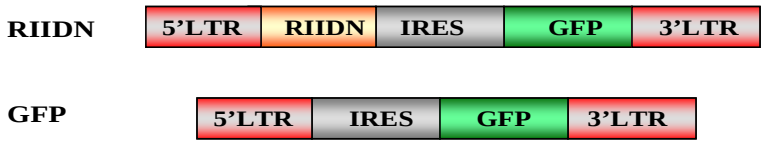

TK
ganciclovir
Apoptosis

Figure 34. TβRIIDN-tk and GFP-tk constructs. A. The original retroviral constructs used in studies conducted by Shah *et al* (26, 122). A bicstronic construct employing an IRES to ensure gene express of both TβRIIDN and GFP. GFP construct was used as the control. B. TβRIIDN-tk and GFP-tk constructs. In the present study, the HSV-tk gene is used to make the fusion gene with TβRIIDN to form TβRIIDN-tk construct that blocks the TGF-β signaling and is susceptible to GCV treatment. GFP-tk is used as the control vector.

A


RIIDN
5'LTR
RIIDN
IRES
GFP
3'LTR
GFP
5'LTR
IRES
GFP
3'LTR

B

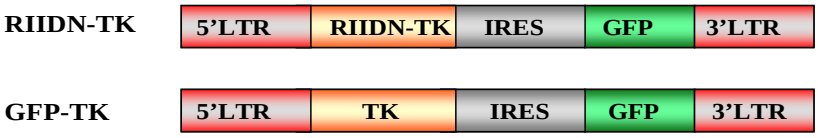
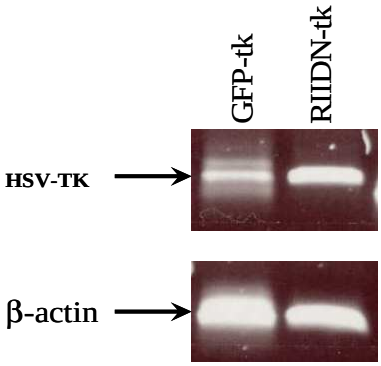

RIIDN-TK
5'LTR
RIIDN-TK
IRES
GFP
3'LTR
GFP-TK
5'LTR
TK
IRES
GFP
3'LTR

Figure 35. Expression of HSV-tk gene in cells transduced with TβRIIDN-tk and GFP-tk. NIH3T3 cells (A) and BMCs (B) were infected with TβRIIDN-tk or GFP-tk as described in the Materials and Methods. After infection, total RNA was isolated and two-step RT-PCR was performed to detect the HSV-tk expression using HSV-tk-specific primers. A. HSV-tk RT-PCR of infected NIH3T3 cells. B. HSV-tk RT-PCR of infected BMCs.

A


GFP-tk
RIIDN-tk
HSV-TK
β-actin

B

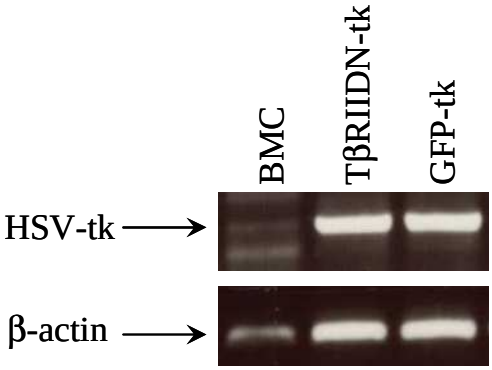

BMC
TβRIIDN-tk
GFP-tk
HSV-tk
β-actin

Figure 36. Effect of TβRIIDN-tk/GCV suicide system on NIH3T3 cell proliferation.

NIH3T3 cells were infected with TβRIIDN-tk or GFP-tk and cultured in the presence or absence of 10 μg/ml GCV for 72 hours. Cell proliferation was measured by [^{3}H]tymindine incorporation assay. All conditions were performed in triplicate and reported as the mean ± S.D. All results shown are representative of at least three independent experiments.

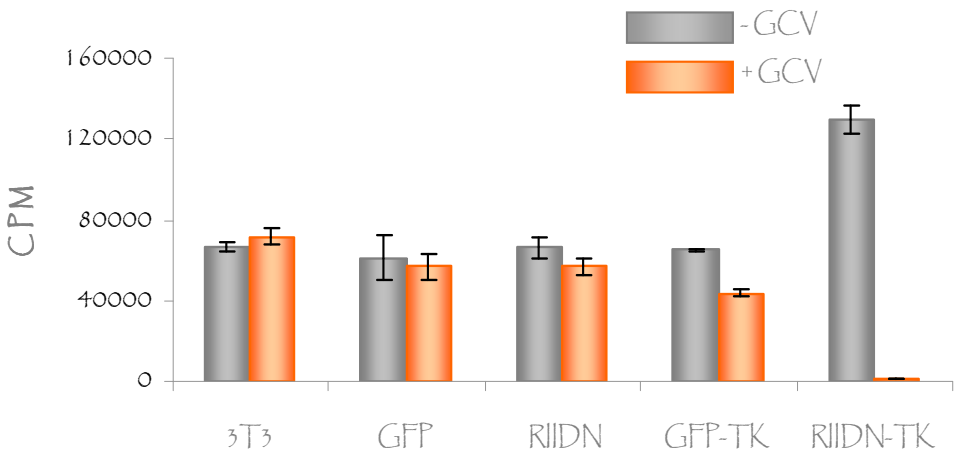

- GCV
+ GCV
160000
120000
80000
40000
0
CPM
3T3
GFP
RIIDN
GFP-TK
RIIDN-TK

Figure 37. Effect of TβRIIDN-tk/GCV suicide system on BMC proliferation. BMCs were infected with TβRIIDN-tk or GFP-tk and treated in the presence or absence of 10 μg/ml GCV for 72 hours. Cell proliferation was measured by [^{3}H]tymindine incorporation assay. Non-infected BMCs were used as a control as the treatment of GCV should have no effect on them. All conditions were performed in triplicate and reported as the mean ± S.D. All results shown are representative of at least three independent experiments.

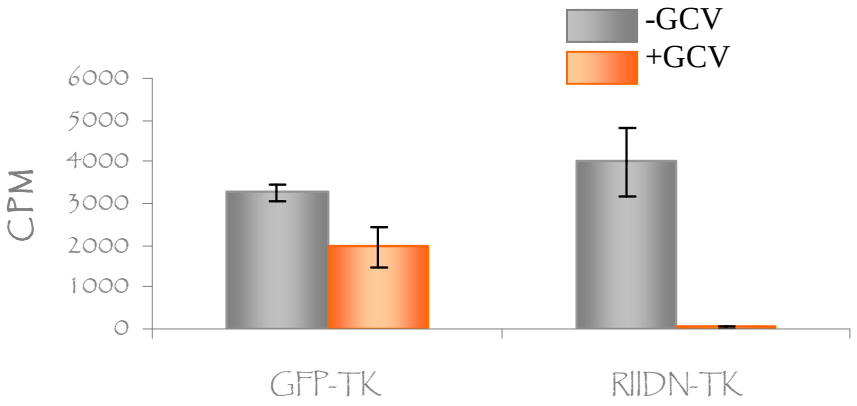

-GCV
+GCV
CPM
6000
5000
4000
3000
2000
1000
0
GFP-TK
RIIDN-TK

Figure 38. TβRIIDN-tk and Trans-tk constructs. A. TβRIIDN-tk construct. Truncated TGF−β type II receptor TβRIIDN is fused to the HSV-tk gene to generate TβRIIDN-tk. B. Trans-tk construct. Extracellular domain of TβRIIDN is deleted in this construct, only the transmembrane domain is intact and fused to the HSV-tk gene. The resulting construct, Trans-tk, lacks the the ability to bind to TGF−β and is susceptible to GCV treatment.

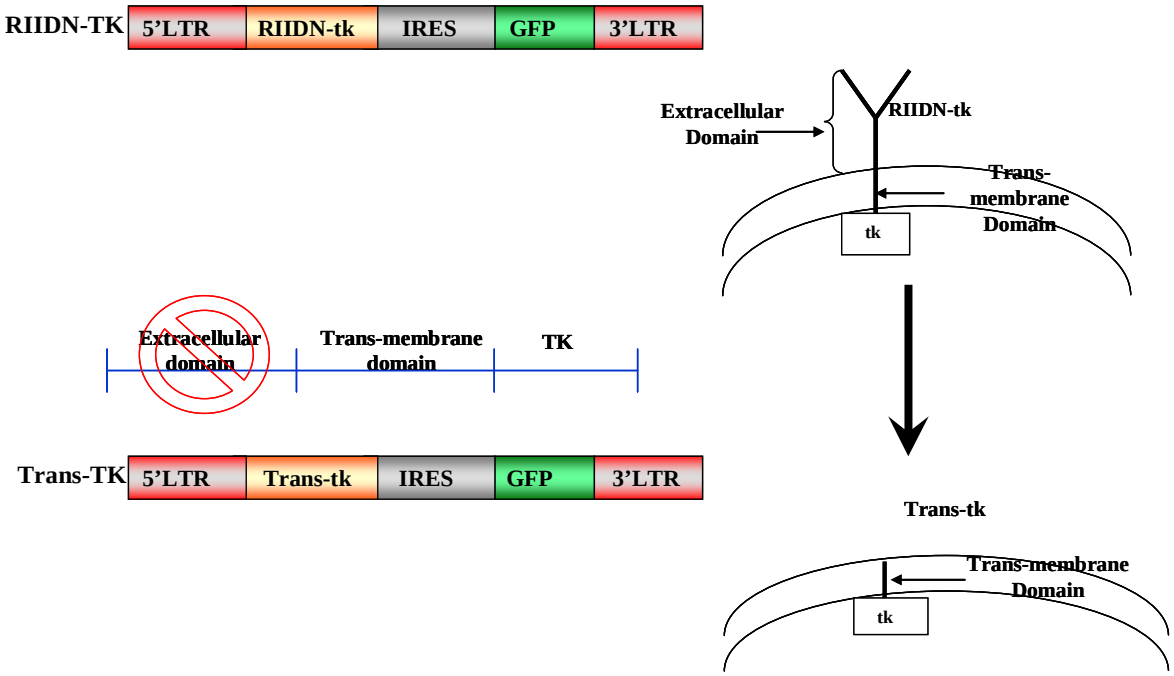
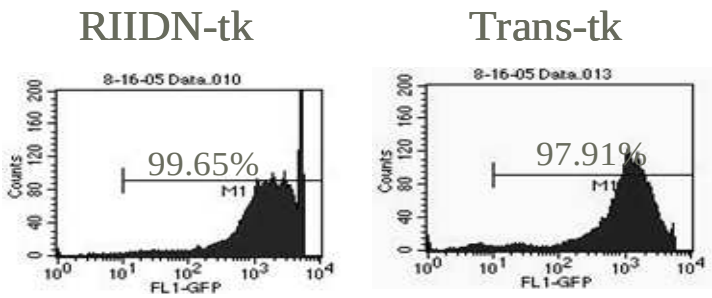

RIIDN-TK
5'LTR
RIIDN-tk
IRES
GFP
3'LTR
Extracellular Domain
RIIDN-tk
Trans-membrane Domain
tk
Extracellular domain
Trans-membrane domain
TK
Trans-TK
5'LTR
Trans-tk
IRES
GFP
3'LTR
Trans-tk
Trans-membrane Domain
tk

Figure 39. Efficiency of TβRIIDN-tk and Trans-tk gene transfer in NIH3T3 cells. NIH3T3 cells were infected with TβRIIDN-tk or Trans-tk as described in the Materials and Methods. After infection, cells were examined for gene transfer efficiency (A) and HSV-tk expression (B). A. Gene transfer efficiency. NIH3T3 cells were analyzed for GFP expression by FACS. The percentage of GFP positive cells represents the percentage of cells that are infected with the constructs. A representative graph is shown here. B. HSV-tk RT-PCR. Total RNA was extracted from infected NIH3T3 cells and two-step RT-PCR was performed to measure the HSV-tk expression.

A


RIIDN-tk
Trans-tk
8-16-05 Data.010
8-16-05 Data.013
99.65%
97.91%
M1
Counts
FL1-GFP

B

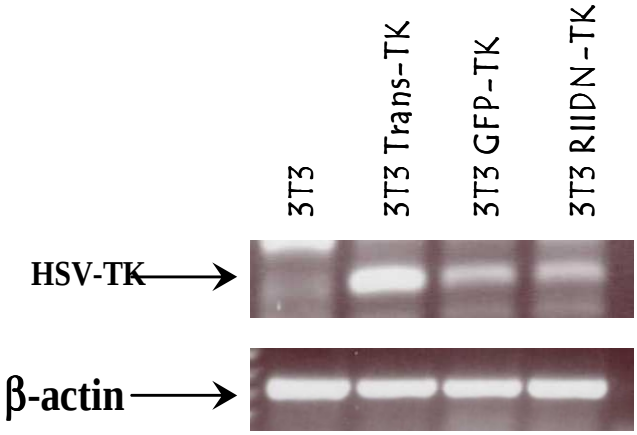
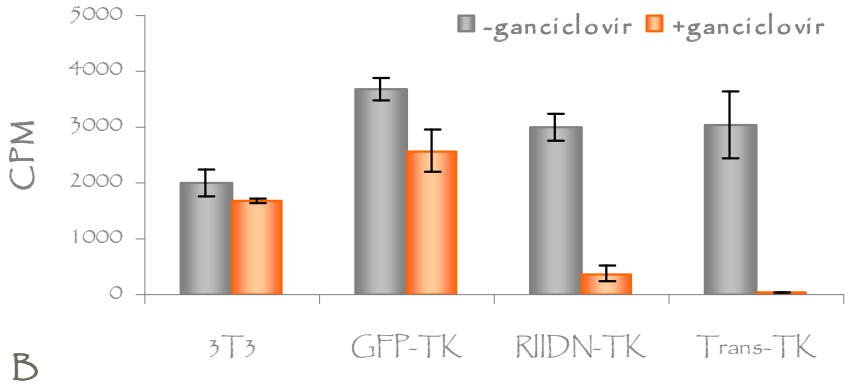

3T3
3T3 Trans-TK
3T3 GFP-TK
3T3 RIIDN-TK
HSV-TK
β-actin

Figure 40. Efficiency of TβRIIDN-tk/GCV suicide system in cell killing. NIH3T3 cells were infected with TβRIIDN-tk, GFP-tk, or Trans-tk and cultured in the presence or absence of 10 μg/ml GCV for 72 hours. A. Effect of TβRIIDN-tk and Trans-tk/GCV on NIH3T3 cell proliferation. Cells were plated in a 96 well plate and treated with or without 10 μg/ml GCV for 72 hours. Cell proliferation was measured using [^{3}H]thymidine incorporation assay. B. Effect of TβRIIDN-tk and Trans-tk/CGV on cell viability. Cells were infected and treated as above. At the end of the experiment, cells were stained with Trypan blue and both dead and live cells were counted with a hemacytometer. The percentage of viable cells was calculated as described in the Materials and Methods. All conditions were performed in triplicate and reported as the mean ± S.D. All results shown are representative of at least three independent experiments.

A


-ganciclovir
+ganciclovir
CPM
5000
4000
3000
2000
1000
0
3T3
GFP-TK
RIIDN-TK
Trans-TK

B

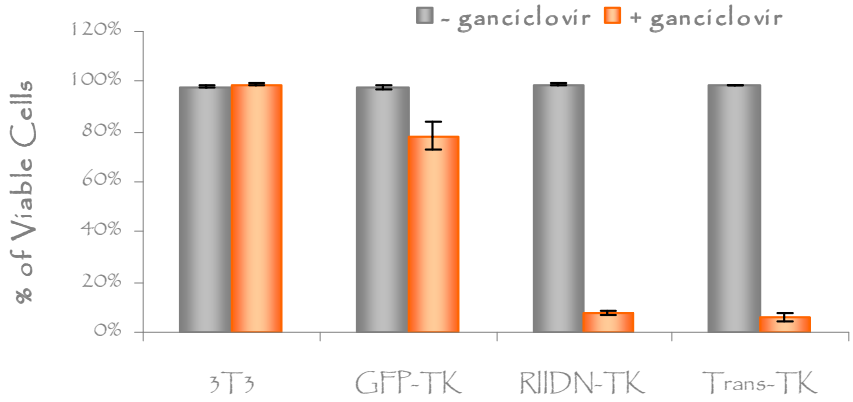

- ganciclovir
+ ganciclovir
% of Viable Cells
120%
100%
80%
60%
40%
20%
0%
3T3
GFP-TK
RIIDN-TK
Trans-TK

Figure 41. Efficiency of TβRIIDN-tk in blockage of TGF-β signaling. NIH3T3 cells were infected with TβRIIDN-tk, GFP-tk, or Trans-tk. After infection, cells were plated and transfected with PAI-1/Luciferase and Renilla/Luciferase for 12 hours. Cells were then treated with or without 10 ng/ml TGF−β for 20 hours and Luciferease activity was measured using an illuminometer. All conditions were performed in triplicate and reported as the mean ± S.D. All results shown are representative of at least three independent experiments.

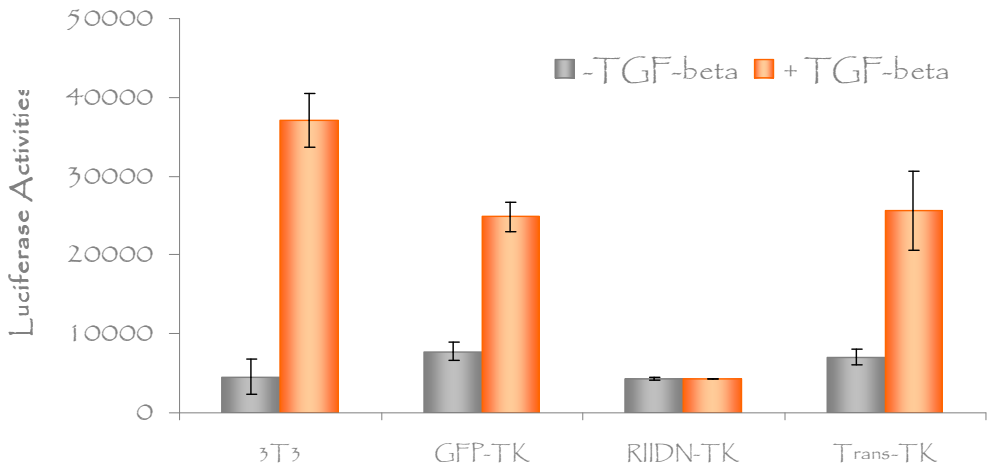
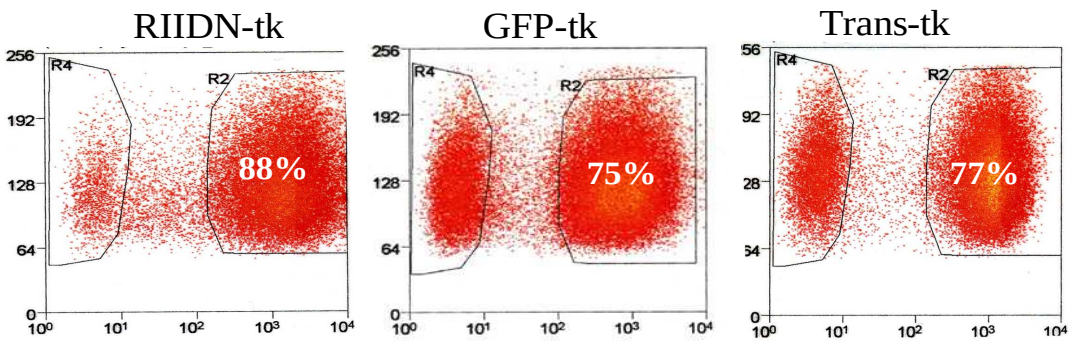

50000
40000
30000
20000
10000
0
Luciferase Activities
-TGF-beta
+TGF-beta
3T3
GFP-TK
RIIDN-TK
Trans-TK

Figure 42. Efficiency of TβRIIDN-tk and Trans-tk gene transfer in BMCs. BMCs were infected with TβRIIDN-tk and Trans-tk as described in the Materials and Methods. After infection, cells were examined for gene transfer efficiency (A) and HSV-tk expression (B). A. Gene transfer efficiency. BMCs were analyzed for GFP expression by FACS. The percentage of GFP positive cells represents the percentage of infected BMCs. A representative graph is shown here. B. HSV-tk RT-PCR. Total RNA was extracted from infected BMCs and two-step RT-PCR was performed to measure the HSV-tk expression.

A


RIIDN-tk
GFP-tk
Trans-tk
R4
R2
88%
75%
77%
256
192
128
64
0
92
28
10^0
10^1
10^2
10^3
10^4

B

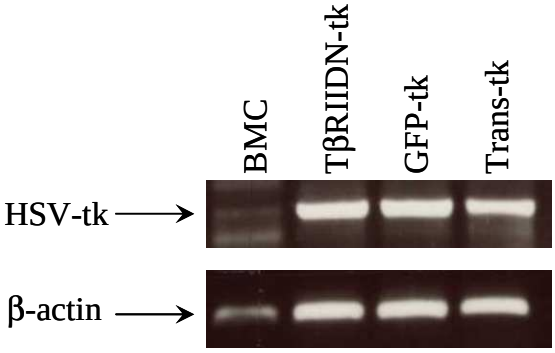
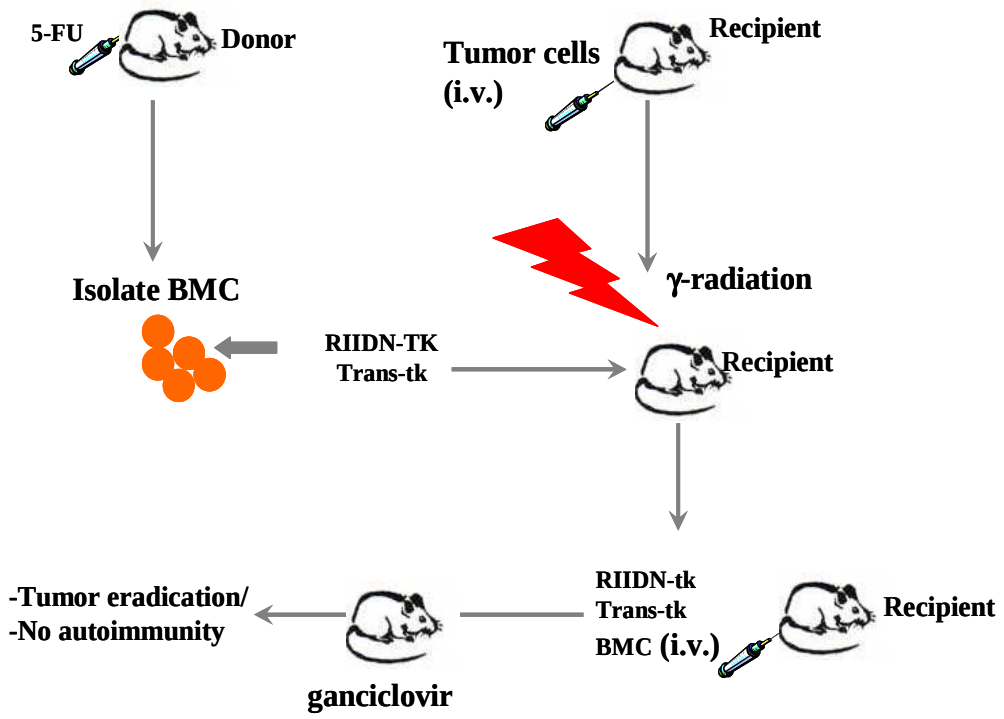

BMC
TβRIIDN-tk
GFP-tk
Trans-tk
HSV-tk
β-actin

Figure 43. Experimental design of *in vivo* studies using the TβRIIDN-tk/GCV suicide system. Donor mice will receive 5-FU treatment to enrich the stem cell population. Five days after the treatment, BMCs will be isolated from the donor mice and infected with TβRIIDN-tk or Trans-tk. Lethally irradiated recipient mice will be challenged with tumor cells (i.e TRAMP-C2) i.v. via tail vein 7 days prior to bone marrow transplant. After reconstitution with donor BMCs infected with TβRIIDN-tk or Trans-tk, tumor growth and autoimmunity development will be minored in the recipient mice. With this modified strategy employing a HSV-tk/GCV suicide system, recipient mice are expected to be able to eradicate already-established tumor cells without the development of autoimmunity.


5-FU
Donor
Tumor cells
(i.v.)
Recipient
Isolate BMC
γ-radiation
RIIDN-TK
Trans-tk
Recipient
-Tumor eradication/
-No autoimmunity
ganciclovir
RIIDN-tk
Trans-tk
BMC (i.v.)
Recipient

REFERENCES

1. Moses, H.L., E.L. Branum, J.A. Proper, and R.A. Robinson. 1981. Transforming growth factor production by chemically transformed cells. *Cancer Res* 41:2842-2848.
2. Roberts, A.B., M.A. Anzano, L.C. Lamb, J.M. Smith, and M.B. Sporn. 1981. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proc Natl Acad Sci U S A* 78:5339-5343.
3. Dennler, S., M.J. Goumans, and P. ten Dijke. 2002. Transforming growth factor beta signal transduction. *J Leukoc Biol* 71:731-740.
4. Moustakas, A., K. Pardali, A. Gaal, and C.H. Heldin. 2002. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunol Lett* 82:85-91.
5. Letterio, J.J., and A.B. Roberts. 1998. Regulation of immune responses by TGF-beta. *Annu Rev Immunol* 16:137-161.
6. de Martin, R., B. Haendler, R. Hofer-Warbinek, H. Gaugitsch, M. Wrann, H. Schlusener, J.M. Seifert, S. Bodmer, A. Fontana, and E. Hofer. 1987. Complementary DNA for human glioblastoma-derived T cell suppressor factor, a novel member of the transforming growth factor-beta gene family. *Embo J* 6:3673-3677.
7. Derynck, R., J.A. Jarrett, E.Y. Chen, D.H. Eaton, J.R. Bell, R.K. Assoian, A.B. Roberts, M.B. Sporn, and D.V. Goeddel. 1985. Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* 316:701-705.

8. ten Dijke, P., P. Hansen, K.K. Iwata, C. Pieler, and J.G. Foulkes. 1988. Identification of another member of the transforming growth factor type beta gene family. *Proc Natl Acad Sci U S A* 85:4715-4719.

9. ten Dijke, P., K.K. Iwata, M. Thorikay, J. Schwedes, A. Stewart, and C. Pieler. 1990. Molecular characterization of transforming growth factor type beta 3. *Ann N Y Acad Sci* 593:26-42.

10. Massague, J., S. Cheifetz, M. Laiho, D.A. Ralph, F.M. Weis, and A. Zentella. 1992. Transforming growth factor-beta. *Cancer Surv* 12:81-103.

11. Gleizes, P.E., J.S. Munger, I. Nunes, J.G. Harpel, R. Mazzieri, I. Noguera, and D.B. Rifkin. 1997. TGF-beta latency: biological significance and mechanisms of activation. *Stem Cells* 15:190-197.

12. Flaumenhaft, R., S. Kojima, M. Abe, and D.B. Rifkin. 1993. Activation of latent transforming growth factor beta. *Adv Pharmacol* 24:51-76.

13. Lyons, R.M., J. Keski-Oja, and H.L. Moses. 1988. Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. *J Cell Biol* 106:1659-1665.

14. Lyons, R.M., L.E. Gentry, A.F. Purchio, and H.L. Moses. 1990. Mechanism of activation of latent recombinant transforming growth factor beta 1 by plasmin. *J Cell Biol* 110:1361-1367.

15. Miyazono, K., A. Olofsson, P. Colosetti, and C.H. Heldin. 1991. A role of the latent TGF-beta 1-binding protein in the assembly and secretion of TGF-beta 1. *Embo J* 10:1091-1101.

16. Odekon, L.E., F. Blasi, and D.B. Rifkin. 1994. Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-beta to TGF-beta. *J Cell Physiol* 158:398-407.

17. Sato, Y., and D.B. Rifkin. 1989. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture. *J Cell Biol* 109:309-315.

18. Sato, Y., R. Tsuboi, R. Lyons, H. Moses, and D.B. Rifkin. 1990. Characterization of the activation of latent TGF-beta by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. *J Cell Biol* 111:757-763.

19. Schultz-Cherry, S., J. Lawler, and J.E. Murphy-Ullrich. 1994. The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-beta. *J Biol Chem* 269:26783-26788.

20. Schultz-Cherry, S., S. Ribeiro, L. Gentry, and J.E. Murphy-Ullrich. 1994. Thrombospondin binds and activates the small and large forms of latent transforming growth factor-beta in a chemically defined system. *J Biol Chem* 269:26775-26782.

21. Engel, M.E., P.K. Datta, and H.L. Moses. 1998. Signal transduction by transforming growth factor-beta: a cooperative paradigm with extensive negative regulation. *J Cell Biochem Suppl* 30-31:111-122.

22. Massague, J. 2000. How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 1:169-178.

23. Franzen, P., C.H. Heldin, and K. Miyazono. 1995. The GS domain of the transforming growth factor-beta type I receptor is important in signal transduction. *Biochem Biophys Res Commun* 207:682-689.

24. Lastres, P., A. Letamendia, H. Zhang, C. Rius, N. Almendro, U. Raab, L.A. Lopez, C. Langa, A. Fabra, M. Letarte, and C. Bernabeu. 1996. Endoglin modulates cellular responses to TGF-beta 1. *J Cell Biol* 133:1109-1121.

25. Zhang, Q., X. Yang, M. Pins, B. Javonovic, T. Kuzel, S.J. Kim, L.V. Parijs, N.M. Greenberg, V. Liu, Y. Guo, and C. Lee. 2005. Adoptive transfer of tumor-reactive transforming growth factor-beta-insensitive CD8+ T cells: eradication of autologous mouse prostate cancer. *Cancer Res* 65:1761-1769.

26. Shah, A.H., W.B. Tabayoyong, S.D. Kundu, S.J. Kim, L. Van Parijs, V.C. Liu, E. Kwon, N.M. Greenberg, and C. Lee. 2002. Suppression of tumor metastasis by blockade of transforming growth factor beta signaling in bone marrow cells through a retroviral-mediated gene therapy in mice. *Cancer Res* 62:7135-7138.

27. Brabletz, T., I. Pfeuffer, E. Schorr, F. Siebelt, T. Wirth, and E. Serfling. 1993. Transforming growth factor beta and cyclosporin A inhibit the inducible activity of the interleukin-2 gene in T cells through a noncanonical octamer-binding site. *Mol Cell Biol* 13:1155-1162.

28. Swain, S.L., G. Huston, S. Tonkonogy, and A. Weinberg. 1991. Transforming growth factor-beta and IL-4 cause helper T cell precursors to develop into distinct effector helper cells that differ in lymphokine secretion pattern and cell surface phenotype. *J Immunol* 147:2991-3000.

29. Ranges, G.E., I.S. Figari, T. Espevik, and M.A. Palladino, Jr. 1987. Inhibition of cytotoxic T cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha. *J Exp Med* 166:991-998.

30. Inge, T.H., K.M. McCoy, B.M. Susskind, S.K. Barrett, G. Zhao, and H.D. Bear. 1992. Immunomodulatory effects of transforming growth factor-beta on T lymphocytes. Induction of CD8 expression in the CTLL-2 cell line and in normal thymocytes. *J Immunol* 148:3847-3856.

31. Smyth, M.J., S.L. Strobl, H.A. Young, J.R. Ortaldo, and A.C. Ochoa. 1991. Regulation of lymphokine-activated killer activity and pore-forming protein gene expression in human peripheral blood CD8+ T lymphocytes. Inhibition by transforming growth factor-beta. *J Immunol* 146:3289-3297.

32. Shull, M.M., I. Ormsby, A.B. Kier, S. Pawlowski, R.J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, and et al. 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 359:693-699.

33. Yaswen, L., A.B. Kulkarni, T. Fredrickson, B. Mittleman, R. Schiffman, S. Payne, G. Longenecker, E. Mozes, and S. Karlsson. 1996. Autoimmune manifestations in the transforming growth factor-beta 1 knockout mouse. *Blood* 87:1439-1445.

34. Kulkarni, A.B., C.G. Huh, D. Becker, A. Geiser, M. Lyght, K.C. Flanders, A.B. Roberts, M.B. Sporn, J.M. Ward, and S. Karlsson. 1993. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A* 90:770-774.

35. Dang, H., A.G. Geiser, J.J. Letterio, T. Nakabayashi, L. Kong, G. Fernandes, and N. Talal. 1995. SLE-like autoantibodies and Sjogren's syndrome-like lymphoproliferation in TGF-beta knockout mice. *J Immunol* 155:3205-3212.

36. Christ, M., N.L. McCartney-Francis, A.B. Kulkarni, J.M. Ward, D.E. Mizel, C.L. Mackall, R.E. Gress, K.L. Hines, H. Tian, S. Karlsson, and et al. 1994. Immune dysregulation in TGF-beta 1-deficient mice. *J Immunol* 153:1936-1946.

37. Letterio, J.J., A.G. Geiser, A.B. Kulkarni, H. Dang, L. Kong, T. Nakabayashi, C.L. Mackall, R.E. Gress, and A.B. Roberts. 1996. Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. *J Clin Invest* 98:2109-2119.

38. Kaartinen, V., J.W. Voncken, C. Shuler, D. Warburton, D. Bu, N. Heisterkamp, and J. Groffen. 1995. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* 11:415-421.

39. Proetzel, G., S.A. Pawlowski, M.V. Wiles, M. Yin, G.P. Boivin, P.N. Howles, J. Ding, M.W. Ferguson, and T. Doetschman. 1995. Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* 11:409-414.

40. Sanford, L.P., I. Ormsby, A.C. Gittenberger-de Groot, H. Sariola, R. Friedman, G.P. Boivin, E.L. Cardell, and T. Doetschman. 1997. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 124:2659-2670.

41. Lucas, P.J., S.J. Kim, S.J. Melby, and R.E. Gress. 2000. Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. *J Exp Med* 191:1187-1196.

42. Gorelik, L., and R.A. Flavell. 2000. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 12:171-181.

43. Bogdan, C., and C. Nathan. 1993. Modulation of macrophage function by transforming growth factor beta, interleukin-4, and interleukin-10. *Ann N Y Acad Sci* 685:713-739.

44. Tsunawaki, S., M. Sporn, A. Ding, and C. Nathan. 1988. Deactivation of macrophages by transforming growth factor-beta. *Nature* 334:260-262.

45. Ding, A., C.F. Nathan, J. Graycar, R. Derynck, D.J. Stuehr, and S. Srimal. 1990. Macrophage deactivating factor and transforming growth factors-beta 1 -beta 2 and -beta 3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-gamma. *J Immunol* 145:940-944.

46. Silva, J.S., D.R. Twardzik, and S.G. Reed. 1991. Regulation of Trypanosoma cruzi infections in vitro and in vivo by transforming growth factor beta (TGF-beta). *J Exp Med* 174:539-545.

47. Mantovani, A., B. Bottazzi, F. Colotta, S. Sozzani, and L. Ruco. 1992. The origin and function of tumor-associated macrophages. *Immunol Today* 13:265-270.

48. van Ravenswaay Claasen, H.H., P.M. Kluin, and G.J. Fleuren. 1992. Tumor infiltrating cells in human cancer. On the possible role of CD16+ macrophages in antitumor cytotoxicity. *Lab Invest* 67:166-174.

49. Fidler, I.J., and A.J. Schroit. 1988. Recognition and destruction of neoplastic cells by activated macrophages: discrimination of altered self. *Biochim Biophys Acta* 948:151-173.

50. Bottazzi, B., S. Walter, D. Govoni, F. Colotta, and A. Mantovani. 1992. Monocyte chemotactic cytokine gene transfer modulates macrophage infiltration, growth, and susceptibility to IL-2 therapy of a murine melanoma. *J Immunol* 148:1280-1285.

51. Brunda, M.J., V. Sulich, R.B. Wright, and A.V. Palleroni. 1991. Tumoricidal activity and cytokine secretion by tumor-infiltrating macrophages. *Int J Cancer* 48:704-708.

52. Reiss, M. 1999. TGF-beta and cancer. *Microbes Infect* 1:1327-1347.

53. Derynck, R., R.J. Akhurst, and A. Balmain. 2001. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 29:117-129.

54. Pierce, D.F., Jr., A.E. Gorska, A. Chytil, K.S. Meise, D.L. Page, R.J. Coffey, Jr., and H.L. Moses. 1995. Mammary tumor suppression by transforming growth factor beta 1 transgene expression. *Proc Natl Acad Sci U S A* 92:4254-4258.

55. Glick, A.B., M.M. Lee, N. Darwiche, A.B. Kulkarni, S. Karlsson, and S.H. Yuspa. 1994. Targeted deletion of the TGF-beta 1 gene causes rapid progression to squamous cell carcinoma. *Genes Dev* 8:2429-2440.

56. Tang, B., E.P. Bottinger, S.B. Jakowlew, K.M. Bagnall, J. Mariano, M.R. Anver, J.J. Letterio, and L.M. Wakefield. 1998. Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency. *Nat Med* 4:802-807.

57. Shida, N., H. Ikeda, T. Yoshimoto, M. Oshima, M.M. Taketo, and I. Miyoshi. 1998. Estrogen-induced tumorigenesis in the pituitary gland of TGF-beta(+/-) knockout mice. *Biochim Biophys Acta* 1407:79-83.

58. Chen, T.C., D.R. Hinton, V.W. Yong, and F.M. Hofman. 1997. TGF-B2 and soluble p55 TNFR modulate VCAM-1 expression in glioma cells and brain derived endothelial cells. *J Neuroimmunol* 73:155-161.

59. Xu, J., A. Ahmad, J.F. Jones, R. Dolcetti, E. Vaccher, U. Prasad, and J. Menezes. 2000. Elevated serum transforming growth factor beta1 levels in Epstein-Barr virus-associated

diseases and their correlation with virus-specific immunoglobulin A (IgA) and IgM. *J Virol* 74:2443-2446.

60. Kong, F.M., M.K. Washington, R.L. Jirtle, and M.S. Anscher. 1996. Plasma transforming growth factor-beta 1 reflects disease status in patients with lung cancer after radiotherapy: a possible tumor marker. *Lung Cancer* 16:47-59.

61. Abou-Shady, M., H.U. Baer, H. Friess, P. Berberat, A. Zimmermann, H. Graber, L.I. Gold, M. Korc, and M.W. Buchler. 1999. Transforming growth factor betas and their signaling receptors in human hepatocellular carcinoma. *Am J Surg* 177:209-215.

62. Wikstrom, P., A. Bergh, and J.E. Damber. 2000. Transforming growth factor-beta1 and prostate cancer. *Scand J Urol Nephrol* 34:85-94.

63. Whiteside, T.L. 2006. Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention. *Semin Cancer Biol* 16:3-15.

64. Ahmad, M., R.C. Rees, and S.A. Ali. 2004. Escape from immunotherapy: possible mechanisms that influence tumor regression/progression. *Cancer Immunol Immunother* 53:844-854.

65. Algarra, I., A. Garcia-Lora, T. Cabrera, F. Ruiz-Cabello, and F. Garrido. 2004. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. *Cancer Immunol Immunother* 53:904-910.

66. Garcia-Lora, A., M. Martinez, I. Algarra, J.J. Gaforio, and F. Garrido. 2003. MHC class I-deficient metastatic tumor variants immunoselected by T lymphocytes originate from the coordinated downregulation of APM components. *Int J Cancer* 106:521-527.

67. Doherty, P.C., B.B. Knowles, and P.J. Wettstein. 1984. Immunological surveillance of tumors in the context of major histocompatibility complex restriction of T cell function. *Adv Cancer Res* 42:1-65.

68. Ferrone, S., and F.M. Marincola. 1995. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunol Today* 16:487-494.

69. Seliger, B., M.J. Maeurer, and S. Ferrone. 1997. TAP off--tumors on. *Immunol Today* 18:292-299.

70. Seliger, B., U. Ritz, R. Abele, M. Bock, R. Tampe, G. Sutter, I. Drexler, C. Huber, and S. Ferrone. 2001. Immune escape of melanoma: first evidence of structural alterations in two distinct components of the MHC class I antigen processing pathway. *Cancer Res* 61:8647-8650.

71. Hodson, I., M. Bock, U. Ritz, W. Brenner, C. Huber, and B. Seliger. 2003. Analysis of the structural integrity of the TAP2 gene in renal cell carcinoma. *Int J Oncol* 23:991-999.

72. Chen, H.L., D. Gabrilovich, R. Tampe, K.R. Girgis, S. Nadaf, and D.P. Carbone. 1996. A functionally defective allele of TAP1 results in loss of MHC class I antigen presentation in a human lung cancer. *Nat Genet* 13:210-213.

73. de Vries, T.J., A. Fourkour, T. Wobbes, G. Verkroost, D.J. Ruiter, and G.N. van Muijen. 1997. Heterogeneous expression of immunotherapy candidate proteins gp100, MART-1, and tyrosinase in human melanoma cell lines and in human melanocytic lesions. *Cancer Res* 57:3223-3229.

74. Hofbauer, G.F., J. Kamarashev, R. Geertsen, R. Boni, and R. Dummer. 1998. Melan A/MART-1 immunoreactivity in formalin-fixed paraffin-embedded primary and metastatic melanoma: frequency and distribution. *Melanoma Res* 8:337-343.

75. Tada, T., S. Ohzeki, K. Utsumi, H. Takiuchi, M. Muramatsu, X.F. Li, J. Shimizu, H. Fujiwara, and T. Hamaoka. 1991. Transforming growth factor-beta-induced inhibition of T cell function. Susceptibility difference in T cells of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state. *J Immunol* 146:1077-1082.

76. Hellstrom, K.E., I. Hellstrom, and L. Chen. 1995. Can co-stimulated tumor immunity be therapeutically efficacious? *Immunol Rev* 145:123-145.

77. Gimmi, C.D., G.J. Freeman, J.G. Gribben, G. Gray, and L.M. Nadler. 1993. Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. *Proc Natl Acad Sci U S A* 90:6586-6590.

78. Gastman, B.R., Y. Atarshi, T.E. Reichert, T. Saito, L. Balkir, H. Rabinowich, and T.L. Whiteside. 1999. Fas ligand is expressed on human squamous cell carcinomas of the head and neck, and it promotes apoptosis of T lymphocytes. *Cancer Res* 59:5356-5364.

79. Strand, S., W.J. Hofmann, H. Hug, M. Muller, G. Otto, D. Strand, S.M. Mariani, W. Stremmel, P.H. Krammer, and P.R. Galle. 1996. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells--a mechanism of immune evasion? *Nat Med* 2:1361-1366.

80. Curiel, T.J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J.R. Conejo-Garcia, L. Zhang, M. Burow, Y. Zhu, S. Wei, I. Kryczek, B. Daniel, A.

Gordon, L. Myers, A. Lackner, M.L. Disis, K.L. Knutson, L. Chen, and W. Zou. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10:942-949.

81. Shevach, E.M. 2002. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2:389-400.

82. Shevach, E.M. 2000. Regulatory T cells in autoimmmunity*. *Annu Rev Immunol* 18:423-449.

83. Gershon, R.K., P. Cohen, R. Hencin, and S.A. Liebhaber. 1972. Suppressor T cells. *J Immunol* 108:586-590.

84. Seddon, B., and D. Mason. 1999. Regulatory T cells in the control of autoimmunity: the essential role of transforming growth factor beta and interleukin 4 in the prevention of autoimmune thyroiditis in rats by peripheral CD4(+)CD45RC- cells and CD4(+)CD8(-) thymocytes. *J Exp Med* 189:279-288.

85. Asseman, C., S. Mauze, M.W. Leach, R.L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 190:995-1004.

86. Suri-Payer, E., and H. Cantor. 2001. Differential cytokine requirements for regulation of autoimmune gastritis and colitis by CD4(+)CD25(+) T cells. *J Autoimmun* 16:115-123.

87. Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 184:387-396.

88. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151-1164.

89. Shimizu, J., S. Yamazaki, and S. Sakaguchi. 1999. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 163:5211-5218.

90. Golgher, D., E. Jones, F. Powrie, T. Elliott, and A. Gallimore. 2002. Depletion of CD25+ regulatory cells uncovers immune responses to shared murine tumor rejection antigens. *Eur J Immunol* 32:3267-3275.

91. Tanaka, H., J. Tanaka, J. Kjaergaard, and S. Shu. 2002. Depletion of CD4+ CD25+ regulatory cells augments the generation of specific immune T cells in tumor-draining lymph nodes. *J Immunother* 25:207-217.

92. Akbar, A.N., L.S. Taams, M. Salmon, and M. Vukmanovic-Stejic. 2003. The peripheral generation of CD4+ CD25+ regulatory T cells. *Immunology* 109:319-325.

93. Burnet, F.M. 1970. The concept of immunological surveillance. *Prog Exp Tumor Res* 13:1-27.

94. Yee, C., J.A. Thompson, D. Byrd, S.R. Riddell, P. Roche, E. Celis, and P.D. Greenberg. 2002. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci U S A* 99:16168-16173.

95. Rosenberg, S.A. 2001. Progress in the development of immunotherapy for the treatment of patients with cancer. *J Intern Med* 250:462-475.

96. Rosenberg, S.A., J.R. Yannelli, J.C. Yang, S.L. Topalian, D.J. Schwartzentruber, J.S. Weber, D.R. Parkinson, C.A. Seipp, J.H. Einhorn, and D.E. White. 1994. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J Natl Cancer Inst* 86:1159-1166.

97. Boon, T., and P. van der Bruggen. 1996. Human tumor antigens recognized by T lymphocytes. *J Exp Med* 183:725-729.

98. Dudley, M.E., J.R. Wunderlich, P.F. Robbins, J.C. Yang, P. Hwu, D.J. Schwartzentruber, S.L. Topalian, R. Sherry, N.P. Restifo, A.M. Hubicki, M.R. Robinson, M. Raffeld, P. Duray, C.A. Seipp, L. Rogers-Freezer, K.E. Morton, S.A. Mavroukakis, D.E. White, and S.A. Rosenberg. 2002. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 298:850-854.

99. Zhang, L., J.R. Conejo-Garcia, D. Katsaros, P.A. Gimotty, M. Massobrio, G. Regnani, A. Makrigiannakis, H. Gray, K. Schlienger, M.N. Liebman, S.C. Rubin, and G. Coukos. 2003. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 348:203-213.

100. Clemente, C.G., M.C. Mihm, Jr., R. Bufalino, S. Zurrida, P. Collini, and N. Cascinelli. 1996. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* 77:1303-1310.

101. Clark, W.H., Jr., D.E. Elder, D.t. Guerry, L.E. Braitman, B.J. Trock, D. Schultz, M. Synnestvedt, and A.C. Halpern. 1989. Model predicting survival in stage I melanoma based on tumor progression. *J Natl Cancer Inst* 81:1893-1904.

102. Nakano, O., M. Sato, Y. Naito, K. Suzuki, S. Orikasa, M. Aizawa, Y. Suzuki, I. Shintaku, H. Nagura, and H. Ohtani. 2001. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer Res* 61:5132-5136.

103. Schumacher, K., W. Haensch, C. Roefzaad, and P.M. Schlag. 2001. Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. *Cancer Res* 61:3932-3936.

104. Sasada, T., M. Kimura, Y. Yoshida, M. Kanai, and A. Takabayashi. 2003. CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. *Cancer* 98:1089-1099.

105. Marshall, N.A., L.E. Christie, L.R. Munro, D.J. Culligan, P.W. Johnston, R.N. Barker, and M.A. Vickers. 2004. Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood* 103:1755-1762.

106. Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 162:5317-5326.

107. Liyanage, U.K., T.T. Moore, H.G. Joo, Y. Tanaka, V. Herrmann, G. Doherty, J.A. Drebin, S.M. Strasberg, T.J. Eberlein, P.S. Goedegebuure, and D.C. Linehan. 2002.

Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol* 169:2756-2761.

108. Murakami, M., A. Sakamoto, J. Bender, J. Kappler, and P. Marrack. 2002. CD25+CD4+ T cells contribute to the control of memory CD8+ T cells. *Proc Natl Acad Sci U S A* 99:8832-8837.

109. Shimizu, J., S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi. 2002. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 3:135-142.

110. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 192:295-302.

111. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.

112. Wahl, S.M., J. Swisher, N. McCartney-Francis, and W. Chen. 2004. TGF-beta: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. *J Leukoc Biol* 76:15-24.

113. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.

114. Bluestone, J.A., and A.K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 3:253-257.

115. Woo, E.Y., H. Yeh, C.S. Chu, K. Schlienger, R.G. Carroll, J.L. Riley, L.R. Kaiser, and C.H. June. 2002. Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol* 168:4272-4276.

116. Van den Eynde, B.J., and P. van der Bruggen. 1997. T cell defined tumor antigens. *Curr Opin Immunol* 9:684-693.

117. Marchand, M., N. van Baren, P. Weynants, V. Brichard, B. Dreno, M.H. Tessier, E. Rankin, G. Parmiani, F. Arienti, Y. Humblet, A. Bourlond, R. Vanwijck, D. Lienard, M. Beauduin, P.Y. Dietrich, V. Russo, J. Kerger, G. Masucci, E. Jager, J. De Greve, J. Atzpodien, F. Brasseur, P.G. Coulie, P. van der Bruggen, and T. Boon. 1999. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int J Cancer* 80:219-230.

118. Tjoa, B.A., P.A. Lodge, M.L. Salgaller, A.L. Boynton, and G.P. Murphy. 1999. Dendritic cell-based immunotherapy for prostate cancer. *CA Cancer J Clin* 49:117-128, 165.

119. Condon, C., S.C. Watkins, C.M. Celluzzi, K. Thompson, and L.D. Falo, Jr. 1996. DNA-based immunization by in vivo transfection of dendritic cells. *Nat Med* 2:1122-1128.

120. Yannelli, J.R., C. Hyatt, S. McConnell, K. Hines, L. Jacknin, L. Parker, M. Sanders, and S.A. Rosenberg. 1996. Growth of tumor-infiltrating lymphocytes from human solid cancers: summary of a 5-year experience. *Int J Cancer* 65:413-421.

121. Matthews, E., T. Yang, L. Janulis, S. Goodwin, S.D. Kundu, W.J. Karpus, and C. Lee. 2000. Down-regulation of TGF-beta1 production restores immunogenicity in prostate cancer cells. *Br J Cancer* 83:519-525.

122. Shah, A.H., W.B. Tabayoyong, S.Y. Kimm, S.J. Kim, L. Van Parijs, and C. Lee. 2002. Reconstitution of lethally irradiated adult mice with dominant negative TGF-beta type II receptor-transduced bone marrow leads to myeloid expansion and inflammatory disease. *J Immunol* 169:3485-3491.

123. Moolten, F.L. 1986. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res* 46:5276-5281.

124. Moolten, F.L., and J.M. Wells. 1990. Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. *J Natl Cancer Inst* 82:297-300.

125. Nagy, H.J., Y. Panis, M. Fabre, D. Klatzmann, D. Houssin, and O. Soubrane. 2000. Suicide gene therapy of ovarian cancer: an experimental study in rats using retroviral-mediated transfer of herpes simplex virus thymidine kinase gene. *Anticancer Res* 20:4633-4638.

126. Obaru, K., S. Fujii, S. Matsushita, T. Shimada, and K. Takatsuki. 1996. Gene therapy for adult T cell leukemia using human immunodeficiency virus vector carrying the thymidine kinase gene of herpes simplex virus type 1. *Hum Gene Ther* 7:2203-2208.

127. Moolten, F.L., J.M. Wells, R.A. Heyman, and R.M. Evans. 1990. Lymphoma regression induced by ganciclovir in mice bearing a herpes thymidine kinase transgene. *Hum Gene Ther* 1:125-134.

128. Faulds, D., and R.C. Heel. 1990. Ganciclovir. A review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy in cytomegalovirus infections. *Drugs* 39:597-638.

129. Fillat, C., M. Carrio, A. Cascante, and B. Sangro. 2003. Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application. *Curr Gene Ther* 3:13-26.

130. Jones, E., M. Dahm-Vicker, A.K. Simon, A. Green, F. Powrie, V. Cerundolo, and A. Gallimore. 2002. Depletion of CD25+ regulatory cells results in suppression of melanoma growth and induction of autoreactivity in mice. *Cancer Immun* 2:1.

131. Nicholas, T.W., S.B. Read, F.J. Burrows, and C.A. Kruse. 2003. Suicide gene therapy with Herpes simplex virus thymidine kinase and ganciclovir is enhanced with connexins to improve gap junctions and bystander effects. *Histol Histopathol* 18:495-507.

132. Melcher, A., S. Todryk, N. Hardwick, M. Ford, M. Jacobson, and R.G. Vile. 1998. Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression. *Nat Med* 4:581-587.

133. Hamel, W., L. Magnelli, V.P. Chiarugi, and M.A. Israel. 1996. Herpes simplex virus thymidine kinase/ganciclovir-mediated apoptotic death of bystander cells. *Cancer Res* 56:2697-2702.

134. Culver, K.W., Z. Ram, S. Wallbridge, H. Ishii, E.H. Oldfield, and R.M. Blaese. 1992. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* 256:1550-1552.

135. Takamiya, Y., M.P. Short, F.L. Moolten, C. Fleet, T. Mineta, X.O. Breakefield, and R.L. Martuza. 1993. An experimental model of retrovirus gene therapy for malignant brain tumors. *J Neurosurg* 79:104-110.

136. Freeman, S.M., C.N. Abboud, K.A. Whartenby, C.H. Packman, D.S. Koeplin, F.L. Moolten, and G.N. Abraham. 1993. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res* 53:5274-5283.

137. Fick, J., F.G. Barker, 2nd, P. Dazin, E.M. Westphale, E.C. Beyer, and M.A. Israel. 1995. The extent of heterocellular communication mediated by gap junctions is predictive of bystander tumor cytotoxicity in vitro. *Proc Natl Acad Sci U S A* 92:11071-11075.

138. Rettig, M.P., J.K. Ritchey, J.L. Prior, J.S. Haug, D. Piwnica-Worms, and J.F. DiPersio. 2004. Kinetics of in vivo elimination of suicide gene-expressing T cells affects engraftment, graft-versus-host disease, and graft-versus-leukemia after allogeneic bone marrow transplantation. *J Immunol* 173:3620-3630.

139. Minasi, L.E., Y. Kamogawa, S. Carding, K. Bottomly, and R.A. Flavell. 1993. The selective ablation of interleukin 2-producing cells isolated from transgenic mice. *J Exp Med* 177:1451-1459.

140. Lechanteur, C., F. Princen, S. Lo Bue, B. Detroz, G. Fillet, J. Gielen, V. Bours, and M.P. Merville. 1997. HSV-1 thymidine kinase gene therapy for colorectal adenocarcinoma-derived peritoneal carcinomatosis. *Gene Ther* 4:1189-1194.

141. Tollefsbol, T.O. 2004. Methods of epigenetic analysis. *Methods Mol Biol* 287:1-8.

142. Woo, E.Y., C.S. Chu, T.J. Goletz, K. Schlienger, H. Yeh, G. Coukos, S.C. Rubin, L.R. Kaiser, and C.H. June. 2001. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 61:4766-4772.

143. Ormandy, L.A., T. Hillemann, H. Wedemeyer, M.P. Manns, T.F. Greten, and F. Korangy. 2005. Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. *Cancer Res* 65:2457-2464.

144. Wolf, A.M., D. Wolf, M. Steurer, G. Gastl, E. Gunsilius, and B. Grubeck-Loebenstein. 2003. Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin Cancer Res* 9:606-612.

145. Chen, W., W. Jin, N. Hardegen, K.J. Lei, L. Li, N. Marinos, G. McGrady, and S.M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198:1875-1886.

146. Zheng, S.G., J.D. Gray, K. Ohtsuka, S. Yamagiwa, and D.A. Horwitz. 2002. Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25- precursors. *J Immunol* 169:4183-4189.

147. Valzasina, B., S. Piconese, C. Guiducci, and M.P. Colombo. 2006. Tumor-induced expansion of regulatory T cells by conversion of CD4+CD25- lymphocytes is thymus and proliferation independent. *Cancer Res* 66:4488-4495.

148. Ghiringhelli, F., P.E. Puig, S. Roux, A. Parcellier, E. Schmitt, E. Solary, G. Kroemer, F. Martin, B. Chauffert, and L. Zitvogel. 2005. Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. *J Exp Med* 202:919-929.

149. Zou, W. 2006. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 6:295-307.

150. Fantini, M.C., C. Becker, G. Monteleone, F. Pallone, P.R. Galle, and M.F. Neurath. 2004. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 172:5149-5153.

151. Yamagiwa, S., J.D. Gray, S. Hashimoto, and D.A. Horwitz. 2001. A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. *J Immunol* 166:7282-7289.

152. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 194:629-644.

153. Peng, Y., Y. Laouar, M.O. Li, E.A. Green, and R.A. Flavell. 2004. TGF-beta regulates in vivo expansion of Foxp3-expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci U S A* 101:4572-4577.

154. Dasch, J.R., D.R. Pace, W. Waegell, D. Inenaga, and L. Ellingsworth. 1989. Monoclonal antibodies recognizing transforming growth factor-beta. Bioactivity neutralization and transforming growth factor beta 2 affinity purification. *J Immunol* 142:1536-1541.

155. Danielpour, D. 1999. Transdifferentiation of NRP-152 rat prostatic basal epithelial cells toward a luminal phenotype: regulation by glucocorticoid, insulin-like growth factor-I and transforming growth factor-beta. *J Cell Sci* 112 (Pt 2):169-179.

156. Fu, S., N. Zhang, A.C. Yopp, D. Chen, M. Mao, H. Zhang, Y. Ding, and J.S. Bromberg. 2004. TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors. *Am J Transplant* 4:1614-1627.

157. Huber, S., and C. Schramm. 2006. TGF-beta and CD4+CD25+ regulatory T cells. *Front Biosci* 11:1014-1023.

158. Turker, M.S. 2002. Gene silencing in mammalian cells and the spread of DNA methylation. *Oncogene* 21:5388-5393.

159. Bird, A. 2002. DNA methylation patterns and epigenetic memory. *Genes Dev* 16:6-21.

160. Cooper, D.N., and M. Krawczak. 1989. Cytosine methylation and the fate of CpG dinucleotides in vertebrate genomes. *Hum Genet* 83:181-188.

161. Robertson, K.D., and P.A. Jones. 2000. DNA methylation: past, present and future directions. *Carcinogenesis* 21:461-467.

162. Venter, J.C., M.D. Adams, E.W. Myers, P.W. Li, R.J. Mural, G.G. Sutton, H.O. Smith, M. Yandell, C.A. Evans, R.A. Holt, J.D. Gocayne, P. Amanatides, R.M. Ballew, D.H. Huson, J.R. Wortman, Q. Zhang, C.D. Kodira, X.H. Zheng, L. Chen, M. Skupski, G. Subramanian, P.D. Thomas, J. Zhang, G.L. Gabor Miklos, C. Nelson, S. Broder, A.G. Clark, J. Nadeau, V.A. McKusick, N. Zinder, A.J. Levine, R.J. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. Deng, V. Di Francesco, P. Dunn, K. Eilbeck, C. Evangelista, A.E. Gabrielian, W. Gan, W. Ge, F. Gong, Z. Gu, P. Guan, T.J. Heiman, M.E. Higgins, R.R. Ji, Z. Ke, K.A. Ketchum, Z. Lai, Y. Lei, Z. Li, J. Li, Y. Liang, X. Lin, F. Lu, G.V. Merkulov, N. Milshina, H.M. Moore, A.K. Naik, V.A. Narayan, B. Neelam, D. Nusskern, D.B. Rusch, S. Salzberg, W. Shao, B. Shue, J. Sun, Z.

Wang, A. Wang, X. Wang, J. Wang, M. Wei, R. Wides, C. Xiao, C. Yan, A. Yao, J. Ye, M. Zhan, W. Zhang, H. Zhang, Q. Zhao, L. Zheng, F. Zhong, W. Zhong, S. Zhu, S. Zhao, D. Gilbert, S. Baumhueter, G. Spier, C. Carter, A. Cravchik, T. Woodage, F. Ali, H. An, A. Awe, D. Baldwin, H. Baden, M. Barnstead, I. Barrow, K. Beeson, D. Busam, A. Carver, A. Center, M.L. Cheng, L. Curry, S. Danaher, L. Davenport, R. Desilets, S. Dietz, K. Dodson, L. Doup, S. Ferriera, N. Garg, A. Gluecksmann, B. Hart, J. Haynes, C. Haynes, C. Heiner, S. Hladun, D. Hostin, J. Houck, T. Howland, C. Ibegwam, J. Johnson, F. Kalush, L. Kline, S. Koduru, A. Love, F. Mann, D. May, S. McCawley, T. McIntosh, I. McMullen, M. Moy, L. Moy, B. Murphy, K. Nelson, C. Pfannkoch, E. Pratts, V. Puri, H. Qureshi, M. Reardon, R. Rodriguez, Y.H. Rogers, D. Romblad, B. Ruhfel, R. Scott, C. Sitter, M. Smallwood, E. Stewart, R. Strong, E. Suh, R. Thomas, N.N. Tint, S. Tse, C. Vech, G. Wang, J. Wetter, S. Williams, M. Williams, S. Windsor, E. Winn-Deen, K. Wolfe, J. Zaveri, K. Zaveri, J.F. Abril, R. Guigo, M.J. Campbell, K.V. Sjolander, B. Karlak, A. Kejariwal, H. Mi, B. Lazareva, T. Hatton, A. Narechania, K. Diemer, A. Muruganujan, N. Guo, S. Sato, V. Bafna, S. Istrail, R. Lippert, R. Schwartz, B. Walenz, S. Yooseph, D. Allen, A. Basu, J. Baxendale, L. Blick, M. Caminha, J. Carnes-Stine, P. Caulk, Y.H. Chiang, M. Coyne, C. Dahlke, A. Mays, M. Dombroski, M. Donnelly, D. Ely, S. Esparham, C. Fosler, H. Gire, S. Glanowski, K. Glasser, A. Glodek, M. Gorokhov, K. Graham, B. Gropman, M. Harris, J. Heil, S. Henderson, J. Hoover, D. Jennings, C. Jordan, J. Jordan, J. Kasha, L. Kagan, C. Kraft, A. Levitsky, M. Lewis, X. Liu, J. Lopez, D. Ma, W. Majoros, J. McDaniel, S. Murphy, M. Newman, T. Nguyen, N. Nguyen, M. Nodell, S. Pan, J. Peck, M. Peterson, W. Rowe, R. Sanders, J. Scott, M.

Simpson, T. Smith, A. Sprague, T. Stockwell, R. Turner, E. Venter, M. Wang, M. Wen, D. Wu, M. Wu, A. Xia, A. Zandieh, and X. Zhu. 2001. The sequence of the human genome. *Science* 291:1304-1351.

163. Lander, E.S., L.M. Linton, B. Birren, C. Nusbaum, M.C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczky, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J.P. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, N. Stange-Thomann, N. Stojanovic, A. Subramanian, D. Wyman, J. Rogers, J. Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J.C. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R.H. Waterston, R.K. Wilson, L.W. Hillier, J.D. McPherson, M.A. Marra, E.R. Mardis, L.A. Fulton, A.T. Chinwalla, K.H. Pepin, W.R. Gish, S.L. Chissoe, M.C. Wendl, K.D. Delehaunty, T.L. Miner, A. Delehaunty, J.B. Kramer, L.L. Cook, R.S. Fulton, D.L. Johnson, P.J. Minx, S.W. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J.F. Cheng, A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier, R.A. Gibbs, D.M. Muzny, S.E. Scherer, J.B. Bouck, E.J. Sodergren, K.C. Worley, C.M. Rives, J.H. Gorrell, M.L. Metzker, S.L. Naylor, R.S. Kucherlapati, D.L. Nelson, G.M. Weinstock, Y. Sakaki, A. Fujiyama, M. Hattori, T. Yada, A. Toyoda, T. Itoh, C. Kawagoe, H. Watanabe, Y. Totoki, T. Taylor, J. Weissenbach, R. Heilig, W. Saurin, F. Artiguenave, P. Brottier, T. Bruls, E. Pelletier, C.

Robert, P. Wincker, D.R. Smith, L. Doucette-Stamm, M. Rubenfield, K. Weinstock, H.M. Lee, J. Dubois, A. Rosenthal, M. Platzer, G. Nyakatura, S. Taudien, A. Rump, H. Yang, J. Yu, J. Wang, G. Huang, J. Gu, L. Hood, L. Rowen, A. Madan, S. Qin, R.W. Davis, N.A. Federspiel, A.P. Abola, M.J. Proctor, R.M. Myers, J. Schmutz, M. Dickson, J. Grimwood, D.R. Cox, M.V. Olson, R. Kaul, N. Shimizu, K. Kawasaki, S. Minoshima, G.A. Evans, M. Athanasiou, R. Schultz, B.A. Roe, F. Chen, H. Pan, J. Ramser, H. Lehrach, R. Reinhardt, W.R. McCombie, M. de la Bastide, N. Dedhia, H. Blocker, K. Hornischer, G. Nordsiek, R. Agarwala, L. Aravind, J.A. Bailey, A. Bateman, S. Batzoglou, E. Birney, P. Bork, D.G. Brown, C.B. Burge, L. Cerutti, H.C. Chen, D. Church, M. Clamp, R.R. Copley, T. Doerks, S.R. Eddy, E.E. Eichler, T.S. Furey, J. Galagan, J.G. Gilbert, C. Harmon, Y. Hayashizaki, D. Haussler, H. Hermjakob, K. Hokamp, W. Jang, L.S. Johnson, T.A. Jones, S. Kasif, A. Kaspryzk, S. Kennedy, W.J. Kent, P. Kitts, E.V. Koonin, I. Korf, D. Kulp, D. Lancet, T.M. Lowe, A. McLysaght, T. Mikkelsen, J.V. Moran, N. Mulder, V.J. Pollara, C.P. Ponting, G. Schuler, J. Schultz, G. Slater, A.F. Smit, E. Stupka, J. Szustakowski, D. Thierry-Mieg, J. Thierry-Mieg, L. Wagner, J. Wallis, R. Wheeler, A. Williams, Y.I. Wolf, K.H. Wolfe, S.P. Yang, R.F. Yeh, F. Collins, M.S. Guyer, J. Peterson, A. Felsenfeld, K.A. Wetterstrand, A. Patrinos, M.J. Morgan, P. de Jong, J.J. Catanese, K. Osoegawa, H. Shizuya, S. Choi, and Y.J. Chen. 2001. Initial sequencing and analysis of the human genome. *Nature* 409:860-921.

164. Li, E., T.H. Bestor, and R. Jaenisch. 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69:915-926.

165. Reik, W., W. Dean, and J. Walter. 2001. Epigenetic reprogramming in mammalian development. *Science* 293:1089-1093.

166. Li, E., C. Beard, and R. Jaenisch. 1993. Role for DNA methylation in genomic imprinting. *Nature* 366:362-365.

167. Panning, B., and R. Jaenisch. 1998. RNA and the epigenetic regulation of X chromosome inactivation. *Cell* 93:305-308.

168. Walsh, C.P., J.R. Chaillet, and T.H. Bestor. 1998. Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nat Genet* 20:116-117.

169. Li, L.C., and R. Dahiya. 2002. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* 18:1427-1431.

170. DeSilva, D.R., E.A. Jones, W.S. Feeser, E.J. Manos, and P.A. Scherle. 1997. The p38 mitogen-activated protein kinase pathway in activated and anergic Th1 cells. *Cell Immunol* 180:116-123.

171. Su, B., E. Jacinto, M. Hibi, T. Kallunki, M. Karin, and Y. Ben-Neriah. 1994. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* 77:727-736.

172. Schiott, A., H.O. Sjogren, and M. Lindvall. 2000. Association of decreased phosphorylation of ERK-2 with costimulation of rat T cell activation by MEK-1 inhibitors and TGF-beta1. *Immunol Lett* 72:183-190.

173. Deng, C., M.J. Kaplan, J. Yang, D. Ray, Z. Zhang, W.J. McCune, S.M. Hanash, and B.C. Richardson. 2001. Decreased Ras-mitogen-activated protein kinase signaling may cause DNA hypomethylation in T lymphocytes from lupus patients. *Arthritis Rheum* 44:397-407.

174. Deng, C., J. Yang, J. Scott, S. Hanash, and B.C. Richardson. 1998. Role of the ras-MAPK signaling pathway in the DNA methyltransferase response to DNA hypomethylation. *Biol Chem* 379:1113-1120.

175. MacLeod, A.R., J. Rouleau, and M. Szyf. 1995. Regulation of DNA methylation by the Ras signaling pathway. *J Biol Chem* 270:11327-11337.

176. Robertson, K.D. 2002. DNA methylation and chromatin - unraveling the tangled web. *Oncogene* 21:5361-5379.

177. Bestor, T.H. 2000. The DNA methyltransferases of mammals. *Hum Mol Genet* 9:2395-2402.

178. Leonhardt, H., A.W. Page, H.U. Weier, and T.H. Bestor. 1992. A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* 71:865-873.

179. Bestor, T.H., and B. Tycko. 1996. Creation of genomic methylation patterns. *Nat Genet* 12:363-367.

180. Hermann, A., S. Schmitt, and A. Jeltsch. 2003. The human Dnmt2 has residual DNA-(cytosine-C5) methyltransferase activity. *J Biol Chem* 278:31717-31721.

181. Okano, M., S. Xie, and E. Li. 1998. Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. *Nucleic Acids Res* 26:2536-2540.

182. Yoder, J.A., and T.H. Bestor. 1998. A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast. *Hum Mol Genet* 7:279-284.

183. Okano, M., D.W. Bell, D.A. Haber, and E. Li. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99:247-257.

184. Yokochi, T., and K.D. Robertson. 2002. Preferential methylation of unmethylated DNA by Mammalian de novo DNA methyltransferase Dnmt3a. *J Biol Chem* 277:11735-11745.

185. Prud'homme, G.J., and C.A. Piccirillo. 2000. The inhibitory effects of transforming growth factor-beta-1 (TGF-beta1) in autoimmune diseases. *J Autoimmun* 14:23-42.

186. Steitz, J., J. Bruck, J. Lenz, J. Knop, and T. Tuting. 2001. Depletion of CD25(+) CD4(+) T cells and treatment with tyrosinase-related protein 2-transduced dendritic cells enhance the interferon alpha-induced, CD8(+) T-cell-dependent immune defense of B16 melanoma. *Cancer Res* 61:8643-8646.

187. Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson, K. Velinzon, K. Inaba, and R.M. Steinman. 2003. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* 198:235-247.

188. Wing, K., Z. Fehervari, and S. Sakaguchi. 2006. Emerging possibilities in the development and function of regulatory T cells. *Int Immunol* 18:991-1000.

189. Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M.C. Nussenzweig, and H. von Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6:1219-1227.

190. Thomas, D.A., and J. Massague. 2005. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* 8:369-380.

191. Liang, S., P. Alard, Y. Zhao, S. Parnell, S.L. Clark, and M.M. Kosiewicz. 2005. Conversion of CD4+ CD25- cells into CD4+ CD25+ regulatory T cells in vivo requires B7 costimulation, but not the thymus. *J Exp Med* 201:127-137.