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T cell Surveillance of Oncogene-induced Prostate Cancer is Impeded by T Cell-derived TGF-β1 Cytokine

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Summary

Tolerance induction in T cells takes place in most tumors and is thought to account for tumor evasion from immune eradication. Production of the cytokine TGF-β is implicated in immunosuppression, however the cellular mechanism by which TGF-β induces T cell dysfunction remains unclear. Using a transgenic model of prostate cancer, we showed that tumor development was not suppressed by the adaptive immune system, which was associated with heightened TGF-β signaling in T cells from the tumor-draining lymph nodes. Blockade of TGF-β signaling in T cells enhanced tumor antigen-specific T cell responses, and inhibited tumor development. Surprisingly, T cell- but not Treg cell-specific ablation of TGF-β1 was sufficient to augment T cell cytotoxic activity and blocked tumor growth and metastases. These findings reveal that T cell production of TGF-β1 is an essential requirement for tumors to evade immunosurveillance independent of TGF-β produced by tumors.

Introduction

Cancer develops as a result of intricate interactions between tumor cells and their environment. How the immune system responds to tumors is of interest not only for the understanding of disease mechanisms but also for cancer immunotherapy(Gattinoni et al., 2006; Pardoll, 2003). Studies in mice and humans have demonstrated that the host mounts antibody and T cell responses to tumor-associated antigens(Boon et al., 2006; Lee et al., 1999; Savage et al., 2008; Schietinger et al., 2008; Willimsky and Blankenstein, 2005). The outcomes of adaptive immune responses to cancer, however, appear to depend on tumor types and mechanisms of cell transformation. Experiments utilizing recombination-activating gene (Rag)-deficient mice have reported increased incidence of carcinogen-induced tumors(Shankaran et al., 2001). Antibody depletion of $CD4^+$ and $CD8^+$ T cells or

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neutralization of interferon-γ (IFN-γ) results in tumor outgrowth in wild-type mice(Koebel et al., 2007), suggesting that lymphocyte-mediated tumor rejection is dependent on type 1 T cell responses. In addition, tumors that develop under the conditions of immune deficiency are more immunogenic than tumors that develop in wild-type mice(Koebel et al., 2007; Shankaran et al., 2001). Although tumor antigen-specific T cell responses have yet to be demonstrated to suppress carcinogen-induced tumors, these findings provide compelling evidence that adaptive immunity can inhibit tumor growth and shape tumor immunogenicity, and thus support the cancer immunoeditting hypothesis(Dunn et al., 2002; Swann and Smyth, 2007).

Tumors are frequently associated with genetic alterations affecting oncogenes and tumor suppressor genes. In genetic models of mouse cancer, the functions of T cells in control of tumor development are incompletely understood. Mice heterozygous for the tumor suppressor p53 develop accelerated B cell lymphoma on a genetic background deficient in perforin(Smyth et al., 2000b), the pore-forming protein crucial for the cytolytic activity of $CD8^+$ T cells and NK cells. Lymphomas that develop in these mice are rejected upon transplantation into wild-type mice, but grow progressively in perforin-deficient mice or wild-type mice depleted of $CD8^+$ T cells(Smyth et al., 2000b). These observations imply a cytotoxic T lymphocyte-dependent surveillance mechanism of spontaneous B cell lymphomas. T cell-mediated rejection of tumors, however, is not observed in other strains of cancer-prone mice. In a transgenic model of spontaneous cancer, expression of the oncogene simian virus 40 T antigen (SV40 Tag) is triggered by stochastic events in diverse tissues(Willimsky and Blankenstein, 2005). Although tumors induce Tag-specific B cell and T cell responses, Tag-specific $CD8^+$ T cells are unable to kill target cells(Willimsky and Blankenstein, 2005; Willimsky et al., 2008). These findings reveal that sporadic tumors do not escape immune recognition, but induce T cell tolerance. We have recently identified a histone H4 peptide as a tumor-associated antigen for $CD8^+$ T cells in transgenic adenocarcinoma of mouse prostate (TRAMP) mice(Savage et al., 2008). Adoptive transfer of H4 antigen-reactive T cell-receptor transgenic (HRC) T cells into TRAMP shows that HRC T cells lack effector function(Savage et al., 2008). These observations suggest that defective effector T cell differentiation is likely a general phenomenon in models of oncogene-induced cancer.

The precise mechanisms of T cell tolerance to autochthonous tumors remain unknown. Many variables likely contribute to the T cell hypo-responsive phenotype in tumor-bearing mice(Blankenstein, 2007; Drake et al., 2006; Rabinovich et al., 2007). Transforming growth factor-β (TGF-β) is a regulatory cytokine with a well-documented role in inhibiting autoreactive T cell responses(Li and Flavell, 2008; Li et al., 2006; Marie et al., 2006; Ouyang et al., 2010). TGF-β elicits its biological activity by binding the serine or threonine kinases TGF-β type I (TGF-βRI) and type II (TGF-βRII) receptors leading to the phosphorylation and activation of Smad2 and Smad3 transcription factors(Li and Flavell, 2008). In studies of transplantable tumor cell lines, inhibition of TGF-β signaling in T cells through the expression of a dominant negative mutant of TGF-βRII, or the administration of blocking antibodies or soluble TGF-βRII results in enhancement of T cell responses and the inhibition of tumor growth(Gorelik and Flavell, 2001; Liu et al., 2007; Nam et al., 2008; Thomas and Massague, 2005; Zhang et al., 2005). However, important caveats are associated with transplanted tumor models such as potential microbe contamination of tumor cell lines and induction of tissue damage upon tumor cell inoculation, which may serve as adjuvants to stimulate T cell responses. Whether TGF-β signaling in T cells naturally suppresses tumor-reactive T cell responses to autochthonous tumors has not been investigated.

There are three family members of TGF-β in mammals, among which TGF-β1 is the most widely distributed cytokine expressed by tumor cells, as well as various subsets of leukocytes and stromal cells(Bierie and Moses, 2010; Flavell et al., 2010; Massague, 2008). An early study showed that over-expression of an active form of TGF-β1 in a highly immunogenic tumor cell line suppresses the anti-tumor immune responses(Torre-Amione et al., 1990), which led to the generally accepted assumption that tumor cell-produced TGF-β inhibits T cell responses and promotes tumor immune evasion. However, RNA interference-mediated knockdown of tumor-derived TGF-β1 does not enhance anti-tumor T cell responses in a transplantation model, whereas tumor expression of a soluble form of TGF-βRII, a decoy receptor for TGF-β produced by tumors and cells in the vicinity of tumors, enhances T cell cytolytic activity(Thomas and Massague, 2005). These findings suggest that TGF-β1 produced by host cells may play a more important role in inhibiting T cell responses to tumors. The precise *in vivo* source(s) of TGF-β1 mediating T cell tolerance to tumors remains to be determined.

In this report, we have utilized a transgenic mouse model of prostate cancer to investigate the role of TGF-β1 in control of T cell responses to spontaneous tumors. We found that the adaptive immune system did not naturally protect mice from tumor development, which was associated with enhanced TGF-β signaling in T cells from the tumor-draining lymph nodes. T cell-specific blockade of TGF-β signaling augmented tumor antigen-specific T cell proliferation and effector T cell differentiation, and inhibited tumor progression to advanced stages. In addition, T cells were found to be the major producers of TGF-β1 that conferred the enhanced TGF-β signaling in tumor-draining lymph node T cells. Consequently, T cell-specific deletion of the *Tgfb1* gene inhibited tumor growth and metastatic dissemination. These results reveal that the T cell-intrinsic TGF-β signaling pathway inhibits T cell surveillance of autochthonous prostate tumors.

Results

Adaptive immunity does not suppress tumor development in TRAMP mice

To assess immune response to autochthonous tumors, we used a model in which transgenic adenocarcinoma of mouse prostate (TRAMP) mice express the well characterized onocogene SV40 Tag under the control of a prostate-specific promoter(Greenberg et al., 1995). We analyzed by flow cytometry the immune cells infiltrating the prostate tumors of 8-month-old TRAMP mice. Among the CD45-positive cells found in the prostates, 57.7±4.4% expressed the T cell marker TCR-β, whereas 7.5±2.7% expressed the B cell marker B220 (Figure 1A). In the T cell compartment, 55.0±6.2% of the infiltrates were $CD8^+$ T cells, while 15.9±3.1% were CD4-positive (Figure 1B). Thus, T cells dominate the adaptive immune cell infiltrates in TRAMP mice.

A previous study demonstrated that deletion of NKG2D, a stimulatory receptor that is expressed by natural killer cells and activated T cells, results in increased incidence of large highly malignant early prostate carcinomas in TRAMP mice(Guerra et al., 2008), suggesting a critical role for NKG2D in tumor surveillance. However, because the aforementioned study employed total NKG2D deletion, it was not possible to distinguish the role of NKG2D in innate versus adaptive immune cells. To determine the importance of adaptive immunity in inhibiting tumor growth, we crossed TRAMP mice to mice deficient in the *Rag1* gene to obtain $Rag1^{-/-}$-TRAMP mice. Surprisingly, absence of the adaptive immune system did not affect tumor development in TRAMP mice in comparison to wild-type TRAMP mice (Figure 1C). These results suggest that in spite of profound T cell infiltration into the prostate tumors of TRAMP mice, T cells cannot control tumor development.

Tumor growth triggers enhanced TGF-β signaling in tumor-draining lymph node T cells

We wished to investigate the mechanisms underlying the T cell defects in TRAMP mice. Recent studies have demonstrated that T cell tolerance in tumor-bearing mice is associated with increased production of TGF-β1(Willimsky and Blankenstein, 2005; Willimsky et al., 2008). To determine whether TGF-β signaling in T cells is modulated during TRAMP tumor development, we evaluated Smad protein phosphorylation in 8-month-old TRAMP mice by flow cytometry. Smad2 and Smad3 phosphorylation was elevated in $CD4^+$ and $CD8^+$ T cells from tumor-draining periaortic lymph nodes compared to $CD4^+$ and $CD8^+$ T cells from other tissues (Figure 2A). Analysis of age-matched C57BL/6 mice revealed comparable Smad phosphorylation in T cells from periaortic lymph nodes and the other peripheral lymphoid organs (data not shown). Thus, tumor growth in TRAMP mice specifically triggers the enhanced Smad2 and Smad3 activation in tumor-draining lymph node T cells.

To investigate whether the elevated Smad2 and Smad3 phosphorylation was due to TGF-β or other Smad activators such as activin proteins, we crossed TRAMP mice with a transgenic mouse line in which T cell TGF-β signaling was attenuated by the expression of a dominant negative form of TGF-βRII (DNR)(Gorelik and Flavell, 2000). Indeed, Smad2 and Smad3 phosphorylation in T cells from the tumor-draining lymph nodes of DNR-TRAMP mice was diminished compared to that of wild-type TRAMP mice (Figure 2B), whereas Smad protein phosphorylation in tumor-infiltrated T cells was comparable (Figure 2C). Thus, TGF-β signaling in T cells is regulated during oncogene-induced primary tumor development. Notably, these results indicate that the heightened T cell TGF-β signaling coincides with T cell priming in the tumor-draining lymph nodes.

Blockade of T cell TGF-β signaling inhibits tumor development in TRAMP mice

TRAMP mice develop prostate tumors after the onset of puberty and the tumors grow progressively and eventually kill the mice at 8–10 months of age(Greenberg et al., 1995). Furthermore, tumor growth in TRAMP mice profoundly induces tolerance in T cells (Anderson et al., 2007; Bai et al., 2008; Savage et al., 2008). To investigate the direct effect of TGF-β signaling in T cells on tumor immunosurveillance, we first examined tumor development in DNR-TRAMP mice. In contrast to wild-type TRAMP mice that had high tumor burden at 8 months of age, prostate tumor growth was inhibited in DNR-TRAMP mice (Figure 3A). Histopathologically, wild-type TRAMP mice had large prostatic glands that contained neoplastic polyps, but these polyps were absent in the prostates of DNR-TRAMP mice (Figure 3B). Instead, clusters of mononuclear cells heavily infiltrated the prostates of DNR-TRAMP mice (Figure 3B). When prostate tumors did appear in DNR-TRAMP mice, they were prevented from reaching advanced stages (histopathologic score 5 or 6) in comparison to wild-type TRAMP mice in which approximately 40% of prostate tumors evaluated had a score of 5 or higher (Figure 3C).

To determine the underlying mechanism of tumor protection in DNR-TRAMP mice, we first examined the numbers of T cells infiltrating the prostates. We found 3-fold more $CD4^+$ and $CD8^+$ T cells in the prostates of DNR-TRAMP mice than those in wild-type TRAMP mice (Figure 4A and B). By immunohistochemical staining, increased CD3 staining was observed in the prostates of DNR-TRAMP mice and these CD3-positive T cells localized to both the glands and stroma, whereas weak CD3 reactivity was mostly found in the tumor stroma of wild-type mice (Figure S1). Altogether, these observations demonstrate that TGF-β signaling in T cells inhibits T cell expansion and/or tumor infiltration, which prevents T cell tumor surveillance in TRAMP mice.

Recent studies showed that the killer cell cytotoxic pathway molecule perforin and the cytokine IFN-γ are required for the immunosurveillance of spontaneous malignancies as

well as methylcholanthrene-induced sarcomas(Koebel et al., 2007; Shankaran et al., 2001; Smyth et al., 2000b; Street et al., 2002; van den Broek et al., 1995). Furthermore, rejection of transplanted tumors in mice with T cell-specific inactivation of TGF-β signaling requires CTLs with enhanced expression of IFN-γ and GzmB(Gorelik and Flavell, 2001; Thomas and Massague, 2005). In line with these observations, $CD8^+$ T cells from tumor-draining lymph nodes of DNR-TRAMP mice expressed high amounts of IFN-γ and GzmB (Figure 4C). Enhanced IFN-γ was also detected in $CD4^+$ T cells from the draining lymph nodes of DNR-TRAMP mice (Figure 4C and data not shown). The expression of GzmB, but not IFN-γ, was further increased in tumor-infiltrated $CD8^+$ T cells from DNR-TRAMP mice (Figure 4C). Thus, blockade of TGF-β signaling in T cells results in T cell differentiation into effectors with augmented protective antitumor immunity.

Programmed death 1 (PD-1) is an inhibitory co-receptor that is expressed by activated T cells(Keir et al., 2008). PD-1 expression limits effector T-cell responses, and is critical for the induction and maintenance of T-cell dysfunction in tumors(Fourcade et al., 2010; Keir et al., 2008). Analysis of wild-type TRAMP mice showed that $CD8^+$ T cells expressed high amounts of PD-1 in the prostate but not in the tumor-draining lymph nodes (Figure 4C). Blockade of TGF-β signaling in T cells led to diminished PD-1 expression in tumor-infiltrating $CD8^+$ T cells (Figure 4C). In addition to inhibitory co-receptors, immune suppression by $CD4^+Foxp3^+$ regulatory T (Treg) cells has been implicated in restraining antitumor immune responses(Curiel et al., 2004; Turk et al., 2004; Yamaguchi and Sakaguchi, 2006). Our previous studies have revealed a dual function for TGF-β signaling in inhibiting Treg cell proliferation and in enhancing Treg cell survival(Li et al., 2006; Ouyang et al., 2010). Attenuated TGF-β signaling in T cells in TRAMP mice resulted in Treg cell expansion in the tumor-draining lymph nodes (Figure S2), whereas Treg cell frequencies decreased in the prostates (Figure S2). Thus, anti-tumor immunity in DNR-TRAMP mice is associated with reduced T-cell intrinsic and extrinsic inhibitory signals at the tumor site.

TGF-β suppression of antitumor immune responses is tumor antigen-specific

One caveat with spontaneous protective anti-tumor T cell responses as reported here is that it could be a result of secondary effects of generally enhanced inflammation in DNR mice(Gorelik and Flavell, 2000), although we kept DNR-TRAMP mice on a diet with antibiotics that prevented the prominent colitis phenotype that develops in these mice. Therefore, in the absence of defined tumor-associated antigens to track tumor-specific responses, interpretations of results could be complicated. In an earlier study, we identified a naturally arising $CD8^+$ T cell receptor (TCR) that is reactive to a histone H4 peptide uniquely recognized during tumor development in TRAMP mice(Savage et al., 2008). We have generated a strain of H4 peptide-reactive TCR transgenic (HRC) mice. In adoptive transfer experiments, HRC T cells are able to recognize the endogenous tumor-associated antigen, but are bereft of effector activity(Savage et al., 2008). Therefore, HRC T cells represent an ideal tool to analyze the molecular basis of tumor antigen-specific T cell tolerance. To this end, we crossed DNR mice with HRC mice onto a Rag1-deficient background and purified naïve wild-type HRC and DNR-HRC T cells and adoptively transferred these cells into tumor-bearing wild-type TRAMP mice (Figure 5A). Based on CFSE dilution, proliferation of wild-type HRC and DNR-HRC cells could be detected. Compared to wild-type HRC T cells, approximately twice as many DNR-HRC T cells proliferated in the tumor-draining lymph nodes (Figure 5B), supporting a critical role for TGF-β signaling in inhibiting tumor antigen-specific T cell priming. We also detected that three times more activated (CFSE-low) DNR-HRC T cells infiltrated prostate tumors (Figure 5B), among which a higher proportion acquired effector function as determined by GzmB production than wild-type HRC T cells (Figure 5B). These observations demonstrate

that TGF-β signaling regulates tumor antigen-specific T cell proliferation and effector T cell differentiation.

T cell-produced TGF-β1 controls tumor development in TRAMP mice

Studies using cell lines and tumor transplantation have suggested that immunosuppression is attributable to tumor-produced TGF-β(Friese et al., 2004; Kao et al., 2003; Liu et al., 2007; Torre-Amione et al., 1990). Nevertheless, shRNA knockdown of TGF-β1 in EL-4 cells revealed that the absence of tumor-derived TGF-β1 was not sufficient to confer protection against tumors(Thomas and Massague, 2005), which could be explained by redundant functions of TGF-β2 and TGF-β3. An alternative explanation is that TGF-β1 derived from host cells plays a more crucial role in T cell tolerance to tumors. We showed previously that TGF-β1 produced by T cells controls T cell differentiation and tolerance in autoimmunity settings(Li et al., 2007). To determine if TGF-β1 production by T cells might represent a previously unrecognized cellular mechanism of T cell tolerance to tumors, we first investigated whether T cells in tumor-bearing TRAMP mice secreted TGF-β1. By ELISA, $CD4^+$ and $CD8^+$ T cells from tumor-draining periaortic lymph nodes produced approximately 50% more TGF-β1 than T cells from non-draining lymph nodes (Figure 6A).

To determine the function of T cell-produced TGF-β1 in control of tumor development, we used mice with floxed/null alleles (*Tgfb1*$^{f/n}$) of TGF-β1 crossed with *Cd4-cre* transgenic mice(Li et al., 2007). *Tgfb1*$^{f/n}$ *Cd4-cre* mouse line was further crossed to TRAMP mice to obtain *Tgfb1*$^{f/n}$ *Cd4cre*-TRAMP mice. T cells in the tumor-draining lymph nodes of *Tgfb1*$^{f/n}$ *Cd4-cre*-TRAMP mice had attenuated Smad2 and Smad3 phosphorylation (Figure 6B), indicating that T cell-produced TGF-β1 accounted in part for the enhanced TGF-β signaling in T cells. To assess for specific causality between T cell-produced TGF-β1 and host susceptibility to tumors, we monitored *Tgfb1*$^{f/n}$ *CD4-cre*-TRAMP mice for tumor development. Similar to DNR-TRAMP mice, tumor development was inhibited in *Tgfb1*$^{f/n}$ *Cd4-cre*-TRAMP mice (Figure 6C). In the absence of T cell-derived TGF-β1, $CD4^+$ and $CD8^+$ T cells in TRAMP tumors acquired effector functions such as increased expression of IFN-γ in the draining lymph nodes and GzmB expression in $CD8^+$ T cells upon tumor infiltration (Figure 6D). In addition, $CD8^+$ T cells infiltrating the prostates of *Tgfb1*$^{f/n}$ *Cd4-cre*-TRAMP mice had diminished PD-1 expression (Figure 6C). Analogous to DNR-TRAMP mice, Treg cell frequencies increased in the tumor-draining lymph nodes but not in the prostates of *Tgfb1*$^{f/n}$ *Cd4-cre*-TRAMP mice (Figure S3). These results indicate that a deficit in T cell TGF-β1 production is sufficient to break T cell tolerance to spontaneous prostate tumors.

Treg cell-produced TGF-β1 is dispensable for the induction of tumor immune tolerance

Studies have shown that increased numbers of Treg cells predict unfavorable clinical outcomes(Curiel et al., 2004; Turk et al., 2004; Yamaguchi and Sakaguchi, 2006). Recently, we showed in a transfer model of colitis that Treg cell-produced TGF-β1 inhibits Th1 cell differentiation(Li et al., 2007), but it is dispensable for Th17 cell differentiation in a model of experimental autoimmune encephalomyelitis(Gutcher et al., 2011). To determine the function of Treg cell-produced TGF-β1 in tumor immune tolerance, we generated a Treg cell-specific TGF-β1-deficeint mouse strain by crossing *Tgfb1*$^{f/n}$ mice with *Foxp3-cre* mice(Gutcher et al., 2011). *Tgfb1*$^{f/n}$ *Foxp3-cre* mice were further crossed with TRAMP mice to obtain *Tgfb1*$^{f/n}$ *Foxp3-cre*-TRAMP mice. We found that concomitant with the lack of protection from tumor development, T cells from *Tgfb1*$^{f/n}$ *Foxp3cre*-TRAMP mice failed to differentiate into effectors (Figure S4A and S4B). Consistent with our recent finding that Treg cell-produced TGF-β1 inhibits Treg cell expansion(Gutcher et al., 2011), the numbers of Treg cells increased in the tumor-draining lymph nodes but not in the prostates of

Tgfb1$^{f/n}$ Foxp3cre-TRAMP mice (Figure S4C). Therefore Treg cell-produced TGF-β1 is not essential for the induction of T cell tolerance in TRAMP mice.

T cell-produced TGF-β1 promotes tumor metastatic growth

The major cause of mortality in cancer patients is metastastic dissemination. Given this evidence of a causal relationship between T cell TGF-β1 production and host failure to survey for malignancy, we asked whether T cell-produced TGF-β1 would also promote tumor metastasis. Because *Tgfb1*$^{f/n}$ *Cd4cre*-TRAMP mice were protected from prostate tumor growth, it was not possible to select mice with comparable primary tumor burdens to assess tumor metastasis. To circumvent this limitation and to test the broader applicability of our observations, we employed an experimental metastasis assay. We intravenously injected ovalbumin-expressing metastatic B16 melanoma cells (B16-OVA) into syngeneic *Tgfb1*$^{f/n}$ and *Tgfb1*$^{f/n}$ *Cd4cre* mice. Whereas a large number of B16-OVA cells colonized the lungs in *Tgfb1*$^{f/n}$ mice, pulmonary metastasis was greatly diminished in the absence of T cell-produced TGF-β1 (Figure S5A and S5B). Reduced lung colonization was associated with increased numbers of ovalbumin-specific CD8$^+$ T cells in *Tgfb1*$^{f/n}$ *Cd4cre* mice (Figure S5C) that produced high amounts of GzmB (Figure S5D). Collectively, these results corroborate our findings with the primary tumors that T cell-produced TGF-β1 inhibits the expansion and effector differentiation of tumor antigen-specific T cells, and further demonstrate that T cell-produced TGF-β1 promotes tumor metastasis.

To investigate the effects of T cell-produced TGF-β1 on tumor immune tolerance, *Tgfb1*$^{f/n}$ and *Tgfb1*$^{f/n}$ *Cd4cre* mice were also challenged intraperitoneally with EL-4, a murine tumor cell line that secretes large amounts of TGF-β1(Gorelik and Flavell, 2001). In accordance with the TRAMP and B16-OVA tumor data, deficiency of TGF-β1 in T cells also prevented EL-4 tumor establishment in the peritoneal cavity (data not shown), again supporting an essential role for T cell-derived TGF-β1 in fostering malignancy. Next we examined tumor-specific cytolytic activity using splenocytes to kill EL-4 target cells. Splenocytes from *Tgfb1*$^{f/n}$ *Cd4cre* mice showed significantly higher EL-4-specific cytolytic activity than splenocytes from *Tgfb1*$^{f/n}$ mice (Figure 7A). It has recently been demonstrated that rejection of transplanted tumors under conditions of abrogated T cell TGF-β signaling is mediated by the cytolytic activity of CD8$^+$ T cells although it requires the provision of CD4$^+$ T cell help(Gorelik and Flavell, 2001). In other experimental systems, CD4$^+$ T cells acquire sufficient cytotoxic activity to suppress tumor growth(Quezada et al., 2010; Xie et al., 2010). In line with the increased GzmB expression in CD8$^+$ T cells from *Tgfb1*$^{f/n}$ *Cd4cre* mice, the cytolytic activity was specifically abrogated when we depleted CD8$^+$ T cells prior to the addition of splenocytes to the target cells (Figure 7B). These findings imply that tumor protection in *Tgfb1*$^{f/n}$ *Cd4cre* mice is mediated by enhanced tumor-specific CTL responses.

Discussion

The adaptive immune system has evolved to mount robust responses to pathogens, and plays a critical role in inhibiting virus-induced tumors(Klein and Klein, 1977). However, the functions of lymphocytes in control of non-viral tumors remain incompletely understood. In this report, we used an oncogene-induced prostate tumor model to show that despite substantial lymphocyte infiltration into tumors, the adaptive immune system did not protect mice from tumor development. This failure of tumor immune protection was mediated by TGF-β suppression of tumor antigen-specific T cell responses. TGF-β inhibited T cell priming in the tumor-draining lymph nodes, and impeded T cell proliferation and effector T cell differentiation. Furthermore, we showed that T cells themselves provide the essential source of TGF-β1 for the blockade of T cell responses to tumors to promote tumor growth

and metastatic dissemination. These findings unveil a crucial immunosuppressive mechanism for tumor-associated T cell tolerance.

An important finding of this study is that the adaptive immune system did not naturally inhibit prostate tumor development in TRAMP mice. These observations, together with findings in a sporadic tumor model(Willimsky and Blankenstein, 2005; Willimsky et al., 2008), suggest that oncogene-induced spontaneous tumors do not induce protective adaptive immune responses. Yet, previous studies have provided strong evidence for T cell-dependent surveillance of carcinogen-induced tumors(Boesen et al., 2000; Girardi et al., 2001; Koebel et al., 2007; Shankaran et al., 2001; Smyth et al., 2000a; Svane et al., 1996). How tumors trigger differential T cell responses in these models remains to be determined. It is conceivable that extensive mutagenesis is induced during chemical carcinogenesis, which may generate high-affinity tumor-rejection antigens for T cells. In an SV40 Tag-induced tumor model, the immunogenicity of transformed cells causes systemic T cell unresponsiveness in tumor-bearing mice(Willimsky et al., 2008). Therefore, it is plausible that tolerance to SV40 Tag-induced tumors may be associated with the transforming oncogene that itself is a potent T cell antigen. Future studies of tumors induced by non-antigenic oncogenes can be used to test this hypothesis. In contrast to TRAMP mice on Rag1-deficient background, deficiency of the activating receptor NKG2D leads to enhanced tumor development in TRAMP mice(Guerra et al., 2008). NKG2D is expressed on effector T cells as well as on NK cells. Indeed, in addition to lymphocytes, high numbers of NK cells infiltrated the tumors in TRAMP mice (our unpublished observations). These observations suggest that NK cells may be responsible for NKG2D-dependent immunosurveillance in these mice. Thus, the effector functions of lymphocytes and innate immune cells are probably differentially regulated in response to spontaneous tumor development.

Multiple mechanisms have been proposed to explain the induction of T cell tolerance to tumors, such as the lack of high-affinity T cell antigens, weak co-stimulation and inflammation associated with tumor antigen presentation, and the immunosuppressive environment inside tumors(Blankenstein, 2007; Drake et al., 2006; Rabinovich et al., 2007). Using Smad2 and Smad3 phosphorylation as a readout, we found that tumor development triggers increased TGF-β signaling in T cells from the tumor-draining lymph nodes, but not in the tumor-infiltrated T cells. Blockade of TGF-β signaling in T cells resulted in enhanced T cell proliferation and effector T cell differentiation in the tumor-draining lymph nodes. Intriguingly, the expression of the cytolytic enzyme GzmB in $CD8^+$ T cells was further elevated in the tumor-infiltrated T cells. Although GzmB has been shown to be a direct target gene of Smad proteins(Thomas and Massague, 2005), we cannot exclude the possibility that there are Smad-independent pathways by which TGF-β suppresses GzmB expression upon T cell tumor infiltration. An alternative explanation is that T cell activation and differentiation in the tumor-draining lymph node has a long-lasting effect on GzmB expression in tumor-infiltrated T cells. Altogether, these observations imply a novel TGF-β-dependent suppressive mechanism that inhibits anti-tumor T cell responses at the stage of T cell priming. In contrast to enhanced GzmB expression, expression of the inhibitory co-receptor PD-1 in tumor-infiltrating $CD8^+$ T cells was diminished in mice with inhibited TGF-β signaling. TGF-β might directly promote PD-1 expression in tumor-infiltrating T cells. In addition, because PD-1 is expressed in exhausted T cells following chronic antigen stimulation, reduced PD-1 expression might be secondary to the tumor protection phenotype observed in these mice. The exact mechanism by which TGF-β regulates PD-1 expression is open for future investigation.

Tumors and other host cells secrete TGF-β, and it has been unclear which specific cell type-produced TGF-β mediates the suppression of T cell responses to tumors. Earlier, our work demonstrated an essential role for T cell-produced TGF-β1 in the control of Th1 and Th17

cell differentiation, and the inflammatory diseases inflicted by the effector $CD4^+$ T cells(Li et al., 2007). Here in this report, we found that T cell-produced TGF-β1 is also crucial for the inhibition of $CD8^+$ T cell differentiation to cytolytic T cells and for the control of tumor development. The absence of TGF-β1 in T cells resulted in diminished T cell TGF-β signaling in the tumor-draining lymph node, providing a cellular mechanism for TGF-β suppression of T cell priming. Tumor growth likely releases high amounts of tumor-associated antigens. Thus, it is conceivable that increased T cell TGF-β1 secretion is triggered by chronic antigen stimulation of T cells in the tumor-draining lymph nodes. It is important to note that TGF-β1 is secreted as an inactive form that needs to be liberated from the constraints of the latency-associated protein(Annes et al., 2003). Recent studies have revealed that dendritic cell-expressed αvβ8 integrin is required for the activation of latent TGF-β1 and for the regulation of T cell responses(Lacy-Hulbert et al., 2007; Travis et al., 2007). Therefore, the selectively enhanced TGF-β signaling in tumor-draining lymph node T cells may also be due to the specific requirement of dendritic cells to prime naïve T cells. However, the exact TGF-β1-producing T cell subset required for the control of tumor immune tolerance remains to be determined. Abrogation of TGF-β1 from $CD4^+Foxp3^+$ regulatory T cells was insufficient to inhibit tumor growth, suggesting that autocrine TGF-β1 might be involved in inhibiting effector T cell responses to tumors. It is also possible that Treg cell- and effector T cell-produced TGF-β1 might be redundant in promoting tumor T cell tolerance. Future studies using T cell subset-specific TGF-β1-deficient mouse models can be used to differentiate these possibilities.

Blockade of TGF-β signaling in T cells or T cell-specific deletion of *Tgfb1* gene inhibits tumor development in TRAMP mice. These findings provide strong evidence that in the absence of self-directed TGF-β signaling in T cells, a spontaneous non-virus-induced tumor can induce and sustain tumor antigen-specific T cell responses to protect the host from tumor development. Increased TGF-β1 has been detected in another sporadic tumor model(Willimsky and Blankenstein, 2005; Willimsky et al., 2008). Importantly, high TGF-β1 levels are specifically associated with the induction of general cytotoxic T lymphocyte unresponsiveness in tumor-bearing mice(Willimsky et al., 2008). Although the precise function of increased TGF-β1 remains to be determined in that model, these observations suggest that TGF-β-dependent T cell suppression may provide a general mechanism for defective tumor immunity in mice. Tumor-specific T cell responses have also been detected in cancer patients(Boon et al., 2006; Lee et al., 1999); yet, similar to experimental models, tumors grow progressively as a possible consequence of failed T cell surveillance. It will therefore be of great interest to determine whether the self-directed TGF-β pathway also controls T cell tolerance to primary tumors in humans.

Disseminated metastases are the primary cause of mortality in cancer patients. Cancer has very complex manifestations; tumor suppressor mechanisms can affect primary tumor development without any significant impact on tumor metastatic growth and vice versa. This dichotomy indicates that mechanisms controlling primary tumor growth and metastasis could be independent. We have shown here that, besides fostering primary tumor development, T-cell production of TGF-β1 facilitates tumor metastatic growth as deletion of the *Tgfb1* gene from T cells also inhibited the colonization of lungs by the aggressive B16 tumors as well as the establishment of the peritoneal cavity by EL-4 tumors. These findings uncovered surprisingly comprehensive effects of T cell TGF-β1 production on tumor development and dissemination, and singularly revealed that absence of TGF-β1 from T cells is sufficient to break tolerance to tumors.

Our findings showed enhancement of anti-tumor immune responses in the absence of T cell-produced TGF-β1 independent of tumor-produced TGF-β1. In contrast, work published by several groups has shown that tumor-produced TGF-β promotes tumor escape from immune

surveillance(Friese et al., 2004; Kao et al., 2003; Liu et al., 2007; Torre-Amione et al., 1990). One explanation for these different findings is that most of these studies based their conclusions on observations of specific cell lines or transplanted tumors. It is plausible that mechanisms of TGF-β-mediated T cell tolerance differ between primary and transplanted models. The TGF-β receptors are widely expressed in tumor cells and multiple lineages of tumor stromal cells(Massague, 2008). Tumor cells and other cells in the immediate vicinity of the tumor will most likely consume TGF-β secreted by tumors before it reaches the draining lymph nodes to regulate T cell differentiation. Thus, tumor-derived TGF-β1 probably makes only a minor contribution to the observed TGF-β-mediated T cell tolerance, which we demonstrated is initiated at the stage of T cell priming. Nevertheless, the definitive function of tumor cell-produced TGF-β1 in control of tumor immunosurveillance awaits the generation of tumor cell-specific TGF-β1-deficient mice.

In conclusion, we have revealed in this report a TGF-β1-dependent mechanism that subverts T cell anti-tumor responses resulting in ineffective control of tumor growth and metastasis. These findings may have profound implications for targeting TGF-β for cancer immunotherapy. A systemic TGF-β blocking strategy will probably neutralize all the TGF-β, and hosts lose the benefits of the cytostatic effects of TGF-β on tumor cells(Pu et al., 2009). Targeted blockade of the self-directed TGF-β signaling pathway in T cells will likely provide a better strategy to specifically awaken anti-tumor immunity and eradicate cancer.

Experimental Procedures

Mice

TRAMP, DNR and HRC TCR transgenic mice and mice with floxed and null alleles of *Tgfb1* gene have previously been described(Gorelik and Flavell, 2000; Greenberg et al., 1995; Li et al., 2007; Savage et al., 2008). Wild-type TRAMP mice were crossed with DNR mice or mice with null alleles of *Rag-1* gene to generate DNR-TRAMP or Rag1KO-TRAMP mice respectively. Crossing mice with floxed and null alleles of *Tgfb1* gene (f/n) with CD4-Cre or Foxp3-Cre mice generated $Tgfb1^{f/n}$ *Cd4-cre* (TKO) or $Tgfb1^{f/n}$ *Foxp3-cre* (TregKO) mice. TKO-TRAMP or TregKO-TRAMP mice were produced by crossing $Tgfb1^{f/n}$ *Cd4-cre* or $Tgfb1^{f/n}$ *Foxp3-cre* mice to wild-type TRAMP mice. All mice were maintained under specific pathogen-free conditions, and animal experimentation was conducted in accordance with institutional guidelines.

Tumor cell lines and injections

Ovalbumin-expressing B16 melanoma cells and EL-4 cells were cultured *in vitro* in DMEM and RPMI-1640 media respectively supplemented with 10% fetal calf serum, 0.1 mM glutamine and 10 U/ml penicillin. B16 melanoma tumor cells were collected by incubation in 0.25% trypsin. Tumor cells were washed two times in endotoxin-free PBS, and 1.0×10^5 or 1.0×10^6 cells injected via tail vein (B16) or intraperitoneally (EL-4) respectively in a volume of 0.2 ml PBS. Cell viability was at least 90% as determined by trypan blue exclusion.

Isolation of tumor-infiltrating lymphocytes

Tumor tissues from sacrificed mice were prepared by mechanical disruption followed by 1-hr treatment with 0.5 mg/ml collagenase Type D at 37°C in a shaker. Digested tissues were mashed between glass slides, layered on a percoll gradient and centrifuged at 3000 rpm for 30 min. The separated tumor-infiltrating lymphocyte (TIL) fraction was washed two times in PBS before use.

Flow cytometry

Fluorescently labeled antibodies against B220, CD4, CD8, TCR-β, CD45, GzmB, Foxp3, PD-1 and IFN-γ markers were purchased from eBiosciences. Fluorescently labeled anti-goat IgG was obtained from Jackson Immunoresearch. Phospho-Smad2/3 (Ser 423/425, clone sc-11769) antibody was obtained from Santa Cruz Biotechnology, Inc. Splenocytes and lymph node cells were depleted of erythrocytes by hypotonic lysis. Splenocytes, lymph node cells and TILs were incubated with specific antibodies for 20 min on ice in the presence of 2.4G2 mAb to block FcγR binding. p-Smad2/3, IFN-γ, and GzmB staining were carried out with the nuclear protein or the intracellular cytokine staining kits from eBiosciences and BD Biosciences. For intracellular cytokine staining, cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma), 1 μM ionomycin (Sigma) and GolgiStop (BD Biosciences) for 4–5 hr. After stimulation, cells were stained with cell surface marker antibodies, fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences). All samples were acquired using LSR II flow cytometer (Becton Dickinson) and analyzed with FlowJo software (Tree Star).

Enzyme-linked immunosorbent assay (ELISA)

A mixture of MicroBeads (Miltenyi Biotec)-purified lymph node $CD4^+$ and $CD8^+$ T cells from 8-month-old tumor-bearing TRAMP mice was cultured in serum-free Nutridoma medium (Roche). After 24 hr, TGF-β1 concentrations in cell supernatants, normalized to the number of live cells, were determined. Latent TGF-β1 in the culture supernatant was activated by acid treatment and assayed with antibody pairs from R&D Systems (BAF240 and MAB1835).

T cell transfer

Congenically marked HRC (CD45.1/CD45.1) and DNR-HRC (CD45.1/CD45.2) $CD8^+$ T cells were purified from TCR transgenic mice that had been crossed to Rag1-deficient background with MicroBeads (Miltenyi Biotec). HRC and DNR-HRC $CD8^+$ T cells were mixed at a ratio of 1:1 and labeled with CFSE. A total of 2×10^5 cells were injected via tail vein into 8-month-old tumor-bearing CD45.2/CD45.2 TRAMP mice. Recipient mice were analyzed 12 days after the transfer.

Histopathology, immunohistochemistry, and pulmonary nodule enumeration

Dissected prostates and lungs from sacrificed animals were fixed in 10% buffered neutral formalin and embedded in paraffin. For macroscopic pulmonary nodule analysis, lungs were fixed in Bouin's fixative and the number of metastases counted with a dissecting microscope. For histopathological and imunohistochemical analyses, 4–5 μm tissue sections were routinely stained with haematoxylin and eosin and microscopically examined for the stages of tumor development. For immunohistochemical analyses, tissues sections were stained with anti-CD3 antibody. Tumor stage classification followed the criteria as previously described(Hurwitz et al., 2001).

Antitumor cytolytic assay

Freshly isolated splenocytes were depleted of erythrocytes by hypotonic lysis and evaluated for their anti-EL-4 lytic activity as described(Gorelik and Flavell, 2001). Chromium-51-labeled target EL-4 cells were incubated with effector splenocytes at different effector:target ratios for 4 hr. The release of radioactive Cr was measured using a γ-counter (Perkin Elmer) and the percentage of specific Cr-51 release was calculated by the formula: $[(CPM^{experimental}-CPM^{spontaneous})/(CPM^{maximum}-CPM^{spontaneous})] \times 100\%$; where $CPM^{experimental}$ is Cr-51 release by target cells incubated with effector cells, $CPM^{spontaneous}$

is Cr-51 release by equivalent number of targets without effector cells, and $CPM^{maximum}$ is total Cr-51 release after addition of 2% Triton X-100 to an equivalent number of target cells.

Statistical analysis

Student's t-test was used to calculate statistical significance for difference in a particular measurement between groups. The Wilcoxon rank-sum test was used to assess differences between samples that did not meet the assumptions of normality. A p value of < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Anderson MJ, Shafer-Weaver K, Greenberg NM, Hurwitz AA. Tolerization of tumor-specific T cells despite efficient initial priming in a primary murine model of prostate cancer. J Immunol. 2007; 178:1268–1276. [PubMed: 17237372]

Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. J Cell Sci. 2003; 116:217–224. [PubMed: 12482908]

Bai A, Higham E, Eisen HN, Wittrup KD, Chen J. Rapid tolerization of virusactivated tumor-specific CD8+ T cells in prostate tumors of TRAMP mice. Proc Natl Acad Sci U S A. 2008; 105:13003–13008. [PubMed: 18723683]

Bierie B, Moses HL. Transforming growth factor beta (TGF-beta) and inflammation in cancer. Cytokine Growth Factor Rev. 2010; 21:49–59. [PubMed: 20018551]

Blankenstein T. Do autochthonous tumors interfere with effector T cell responses? Semin Cancer Biol. 2007; 17:267–274. [PubMed: 17659881]

Boesen M, Svane IM, Engel AM, Rygaard J, Thomsen AR, Werdelin O. CD8+ T cells are crucial for the ability of congenic normal mice to reject highly immunogenic sarcomas induced in nude mice with 3-methylcholanthrene. Clin Exp Immunol. 2000; 121:210–215. [PubMed: 10931133]

Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P. Human T cell responses against melanoma. Annu Rev Immunol. 2006; 24:175–208. [PubMed: 16551247]

Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004; 10:942–949. [PubMed: 15322536]

Drake CG, Jaffee E, Pardoll DM. Mechanisms of immune evasion by tumors. Adv Immunol. 2006; 90:51–81. [PubMed: 16730261]

Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002; 3:991–998. [PubMed: 12407406]

Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limon P. The polarization of immune cells in the tumour environment by TGFbeta. Nat Rev Immunol. 2010; 10:554–567. [PubMed: 20616810]

Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, Kirkwood JM, Kuchroo V, Zarour HM. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. J Exp Med. 2010; 207:2175–2186. [PubMed: 20819923]

Friese MA, Wischhusen J, Wick W, Weiler M, Eisele G, Steinle A, Weller M. RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antiglioma immune

response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity in vivo. Cancer Res. 2004; 64:7596–7603. [PubMed: 15492287]

Gattinoni L, Powell DJ Jr, Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol. 2006; 6:383–393. [PubMed: 16622476]

Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, Hobby P, Sutton B, Tigelaar RE, Hayday AC. Regulation of cutaneous malignancy by gammadelta T cells. Science. 2001; 294:605–609. [PubMed: 11567106]

Gorelik L, Flavell RA. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity. 2000; 12:171–181. [PubMed: 10714683]

Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. Nat Med. 2001; 7:1118–1122. [PubMed: 11590434]

Greenberg NM, DeMayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ, Rosen JM. Prostate cancer in a transgenic mouse. Proc Natl Acad Sci U S A. 1995; 92:3439–3443. [PubMed: 7724580]

Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, Knoblaugh S, Cado D, Greenberg NM, Raulet DH. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity. 2008; 28:571–580. [PubMed: 18394936]

Gutcher I, Donkor MK, Ma Q, Rudensky AY, Flavell RA, Li MO. Autocrine Transforming Growth Factor-beta1 Promotes In Vivo Th17 Cell Differentiation. Immunity. 2011; 34:396–408. [PubMed: 21435587]

Hurwitz AA, Foster BA, Allison JP, Greenberg NM, Kwon ED. The TRAMP mouse as a model for prostate cancer Chapter 20. Curr Protoc Immunol. 2001 Unit 20 25.

Kao JY, Gong Y, Chen CM, Zheng QD, Chen JJ. Tumor-derived TGF-beta reduces the efficacy of dendritic cell/tumor fusion vaccine. J Immunol. 2003; 170:3806–3811. [PubMed: 12646647]

Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol. 2008; 26:677–704. [PubMed: 18173375]

Klein G, Klein E. Immune surveillance against virus-induced tumors and nonrejectability of spontaneous tumors: contrasting consequences of host versus tumor evolution. Proc Natl Acad Sci U S A. 1977; 74:2121–2125. [PubMed: 194247]

Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, Smyth MJ, Schreiber RD. Adaptive immunity maintains occult cancer in an equilibrium state. Nature. 2007; 450:903–907. [PubMed: 18026089]

Lacy-Hulbert A, Smith AM, Tissire H, Barry M, Crowley D, Bronson RT, Roes JT, Savill JS, Hynes RO. Ulcerative colitis and autoimmunity induced by loss of myeloid alphav integrins. Proc Natl Acad Sci U S A. 2007; 104:15823–15828. [PubMed: 17895374]

Lee PP, Yee C, Savage PA, Fong L, Brockstedt D, Weber JS, Johnson D, Swetter S, Thompson J, Greenberg PD, et al. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat Med. 1999; 5:677–685. [PubMed: 10371507]

Li MO, Flavell RA. TGF-beta: a master of all T cell trades. Cell. 2008; 134:392–404. [PubMed: 18692464]

Li MO, Sanjabi S, Flavell RA. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and - independent mechanisms. Immunity. 2006; 25:455–471. [PubMed: 16973386]

Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factorbeta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity. 2007; 26:579–591. [PubMed: 17481928]

Liu VC, Wong LY, Jang T, Shah AH, Park I, Yang X, Zhang Q, Lonning S, Teicher BA, Lee C. Tumor evasion of the immune system by converting CD4+CD25- T cells into CD4+CD25+ T regulatory cells: role of tumor-derived TGF-beta. J Immunol. 2007; 178:2883–2892. [PubMed: 17312132]

Marie JC, Liggitt D, Rudensky AY. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. Immunity. 2006; 25:441–454. [PubMed: 16973387]

Massague J. TGFbeta in Cancer. Cell. 2008; 134:215–230. [PubMed: 18662538]

Nam JS, Terabe M, Mamura M, Kang MJ, Chae H, Stuelten C, Kohn E, Tang B, Sabzevari H, Anver MR, et al. An anti-transforming growth factor beta antibody suppresses metastasis via cooperative effects on multiple cell compartments. Cancer Res. 2008; 68:3835–3843. [PubMed: 18483268]

Ouyang W, Beckett O, Ma Q, Li MO. Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development. Immunity. 2010; 32:642–653. [PubMed: 20471291]

Pardoll D. Does the immune system see tumors as foreign or self? Annu Rev Immunol. 2003; 21:807–839. [PubMed: 12615893]

Pu H, Collazo J, Jones E, Gayheart D, Sakamoto S, Vogt A, Mitchell B, Kyprianou N. Dysfunctional transforming growth factor-beta receptor II accelerates prostate tumorigenesis in the TRAMP mouse model. Cancer Res. 2009; 69:7366–7374. [PubMed: 19738062]

Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, Blasberg R, Yagita H, Muranski P, Antony PA, et al. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. J Exp Med. 2010; 207:637–650. [PubMed: 20156971]

Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol. 2007; 25:267–296. [PubMed: 17134371]

Savage PA, Vosseller K, Kang C, Larimore K, Riedel E, Wojnoonski K, Jungbluth AA, Allison JP. Recognition of a ubiquitous self antigen by prostate cancerinfiltrating CD8+ T lymphocytes. Science. 2008; 319:215–220. [PubMed: 18187659]

Schietinger A, Philip M, Schreiber H. Specificity in cancer immunotherapy. Semin Immunol. 2008; 20:276–285. [PubMed: 18684640]

Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001; 410:1107–1111. [PubMed: 11323675]

Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, Kawano T, Pelikan SB, Crowe NY, Godfrey DI. Differential tumor surveillance by natural killer (NK) and NKT cells. J Exp Med. 2000a; 191:661–668. [PubMed: 10684858]

Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani JA. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J Exp Med. 2000b; 192:755–760. [PubMed: 10974040]

Street SE, Trapani JA, MacGregor D, Smyth MJ. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. J Exp Med. 2002; 196:129–134. [PubMed: 12093877]

Svane IM, Engel AM, Nielsen MB, Ljunggren HG, Rygaard J, Werdelin O. Chemically induced sarcomas from nude mice are more immunogenic than similar sarcomas from congenic normal mice. Eur J Immunol. 1996; 26:1844–1850. [PubMed: 8765030]

Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Invest. 2007; 117:1137–1146. [PubMed: 17476343]

Thomas DA, Massague J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell. 2005; 8:369–380. [PubMed: 16286245]

Torre-Amione G, Beauchamp RD, Koeppen H, Park BH, Schreiber H, Moses HL, Rowley DA. A highly immunogenic tumor transfected with a murine transforming growth factor type beta 1 cDNA escapes immune surveillance. Proc Natl Acad Sci U S A. 1990; 87:1486–1490. [PubMed: 2137615]

Travis MA, Reizis B, Melton AC, Masteller E, Tang Q, Proctor JM, Wang Y, Bernstein X, Huang X, Reichardt LF, et al. Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. Nature. 2007; 449:361–365. [PubMed: 17694047]

Turk MJ, Guevara-Patino JA, Rizzuto GA, Engelhorn ME, Sakaguchi S, Houghton AN. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. J Exp Med. 2004; 200:771–782. [PubMed: 15381730]

van den Broek MF, Kagi D, Zinkernagel RM, Hengartner H. Perforin dependence of natural killer cell-mediated tumor control in vivo. Eur J Immunol. 1995; 25:3514–3516. [PubMed: 8566046]

Willimsky G, Blankenstein T. Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. Nature. 2005; 437:141–146. [PubMed: 16136144]

Willimsky G, Czeh M, Loddenkemper C, Gellermann J, Schmidt K, Wust P, Stein H, Blankenstein T. Immunogenicity of premalignant lesions is the primary cause of general cytotoxic T lymphocyte unresponsiveness. J Exp Med. 2008; 205:1687–1700. [PubMed: 18573907]

Xie Y, Akpinarli A, Maris C, Hipkiss EL, Lane M, Kwon EK, Muranski P, Restifo NP, Antony PA. Naive tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma. J Exp Med. 2010; 207:651–667. [PubMed: 20156973]

Yamaguchi T, Sakaguchi S. Regulatory T cells in immune surveillance and treatment of cancer. Semin Cancer Biol. 2006; 16:115–123. [PubMed: 16376102]

Zhang Q, Yang X, Pins M, Javonovic B, Kuzel T, Kim SJ, Parijs LV, Greenberg NM, Liu V, Guo Y, Lee C. Adoptive transfer of tumor-reactive transforming growth factor-beta-insensitive CD8+ T cells: eradication of autologous mouse prostate cancer. Cancer Res. 2005; 65:1761–1769. [PubMed: 15753372]

Highlights

- The adaptive immunity does not protect against TRAMP tumor development.
- Lack of protective immunity is associated with enhanced TGF-β signaling in T cells.
- Blockade of TGF-β signaling in T cells protects TRAMP mice from tumor development.
- T cell-specific deletion of TGF-β1 is sufficient to break tumor T-cell tolerance.

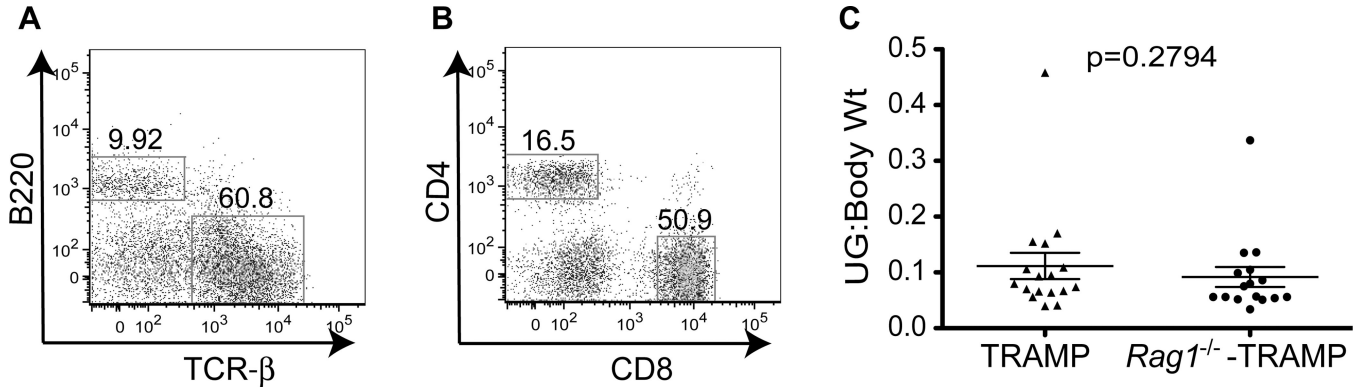


Figure 1. Absence of adaptive immunity does not increase the susceptibility of TRAMP mice to tumor development

(**A, B**) Flow cytometric analysis of lymphocytes infiltrating the prostates of 8-month-old TRAMP mice. The percentage of B220- and TCR-β-expressing cells among $CD45^+$ cells (**A**) and the percentage of $CD4^+$ and $CD8^+$ T cells in TCR-β-positive cells **(B)** are shown. (**C**) Littermates of wild-type TRAMP and *Rag1*$^{-/-}$ TRAMP mice were evaluated for tumor development at 8 months of age. The weights (Wt) of urogenital tracts (UG) normalized to body Wt ± s.e.m of wild type TRAMP (n=17) and *Rag1*$^{-/-}$ -TRAMP (n=16) mice are shown. The p value between two groups of tumor burden is shown (Wilcoxon rank-sum test).

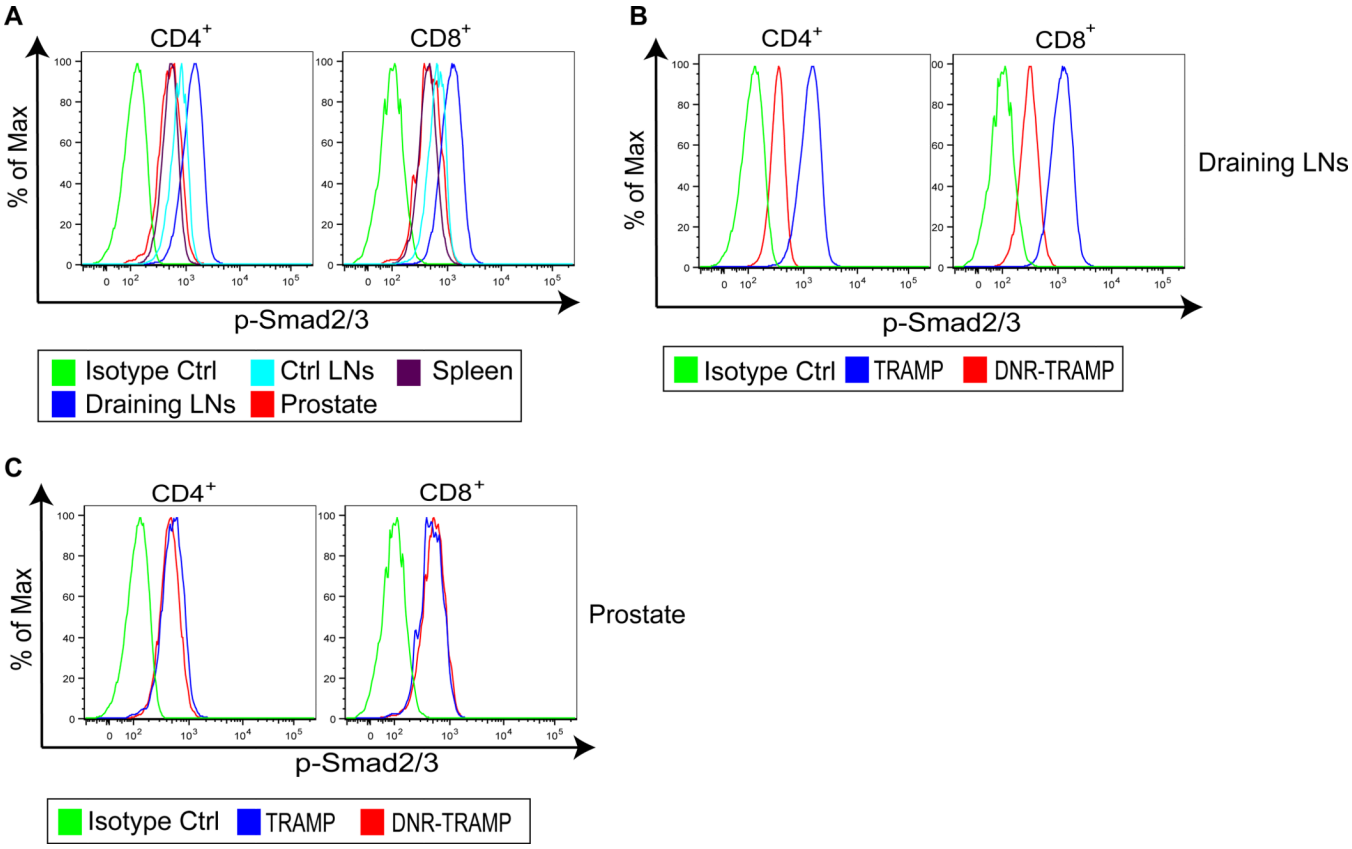
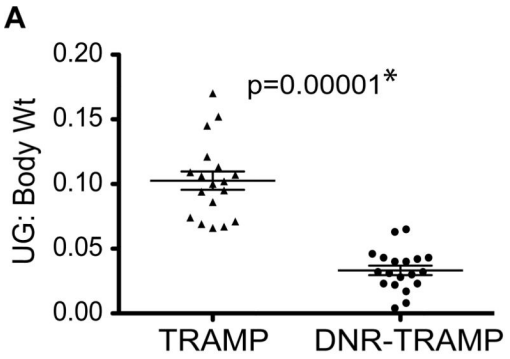
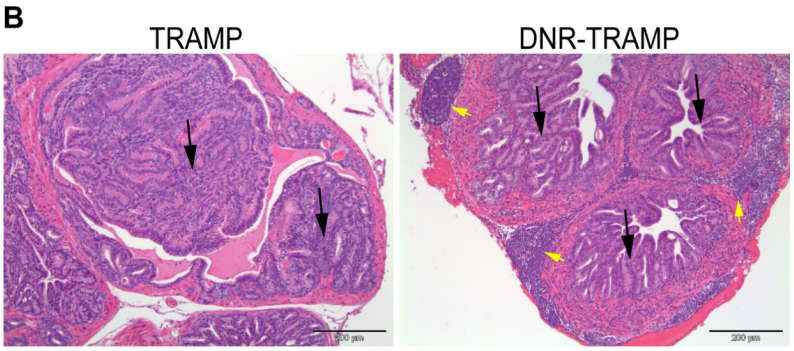


Figure 2. Enhanced TGF-β signaling in T cells from tumor-draining lymph nodes of TRAMP mice

(A) Flow cytometric analysis of Smad2 and Smad3 phosphorylation in $CD4^+$ and $CD8^+$ T cells from 8-month-old wild-type prostate tumor-bearing TRAMP mice. Draining LNs represents lymph nodes draining prostate, Ctrl LNs represents control axillary and brachial lymph nodes, and Isotype Ctrl represents isotype control antibody staining. **(B, C)** Smad2 and Smad3 phophorylation in $CD4^+$ and $CD8^+$ T cells from prostate tumor-draining lymph nodes **(B)** and prostates **(C)** of TRAMP and DNR-TRAMP mice as determined by flow cytometry. Representatives of five independent experiments are shown **(A–C)**.





C

Histopathology Score	TRAMP % (n=23)	DNR-TRAMP % (n=21)
1	4.3	4.8
2	8.7	9.5
3	4.3	47.6
4	43.5	38.1
5	34.8	0.0
6	4.3	0.0

Figure 3. Blockade of TGF-β signaling in T cells protects TRAMP mice from tumor development

(A) The weights (Wt) of urogenital tracts (UG) normalized to body Wt ± s.e.m of wild type TRAMP (n=18) and DNR-TRAMP (n=19) mice at 8 months of age. The p value between two groups of tumor burden is shown (Wilcoxon rank-sum test). * depicts statistically significant difference. **(B)** Histological analysis of sections of the prostates of 8-month-old TRAMP and DNR-TRAMP mice. Sections of representative prostates from TRAMP and DNR-TRAMP mice were stained with hematoxylin and eosin. **(C)** Pathologic scoring for stages of tumor development in the prostates of littermates of TRAMP and DNR-TRAMP mice. 1= Normal; 2= Low-grade intraepithelial neoplasia; 3=High-grade intraepithelial neoplasia; 4=Well-differentiated prostate adenocarcinoma; 5= Moderately differentiated prostate adenocarcinoma; 6= Poorly differentiated prostate adenocarcinoma.

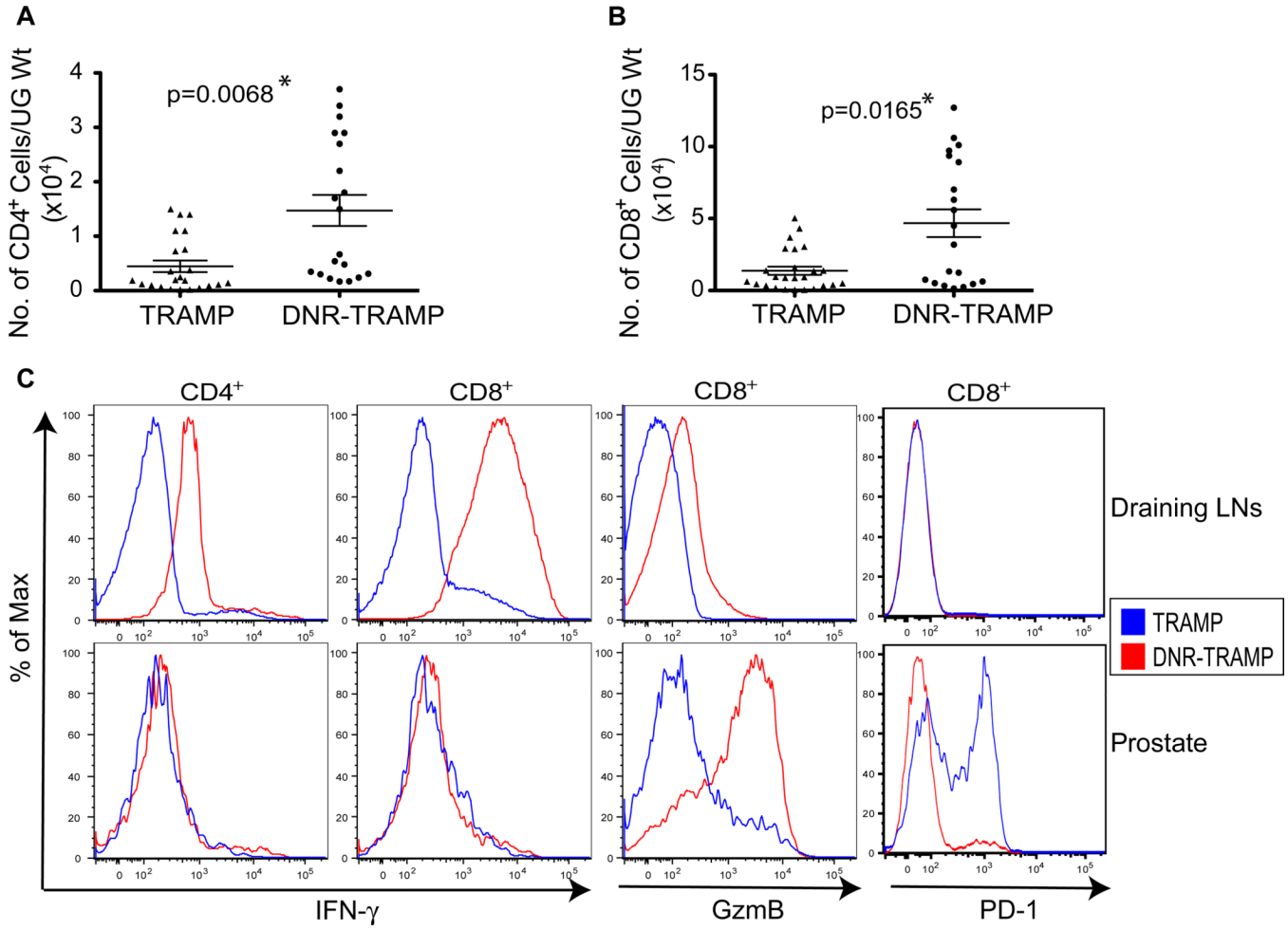


Figure 4. TGF-β signaling regulates T cell responses to tumors

(A, B) Cell counts of T lymphocytes isolated from the prostates of 8-month-old mice. Results shown are the absolute numbers normalized to weights (Wt) of urogenital tracts (UG) ± s.e.m of $CD4^+$ (a) and $CD8^+$ (**B**) T cells infiltrating the prostates of TRAMP (n=20) and DNR-TRAMP (n=20) mice. The p values between the two groups of T cell numbers are shown (Wilcoxon rank-sum test). * depicts statistically significant difference. **(C)** Flow cytometric analysis examining the expression of interferon gamma (IFN-γ, granzyme B (GzmB) and PD-1 proteins in $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes and prostates of TRAMP and DNR-TRAMP mice. For IFN-γ expression, T cells were re-stimulated *in vitro* for 5 hr with phorbol 12-myristate 13-acetate (PMA), ionomycin and GolgiStop. Representatives of five independent experiments are shown.

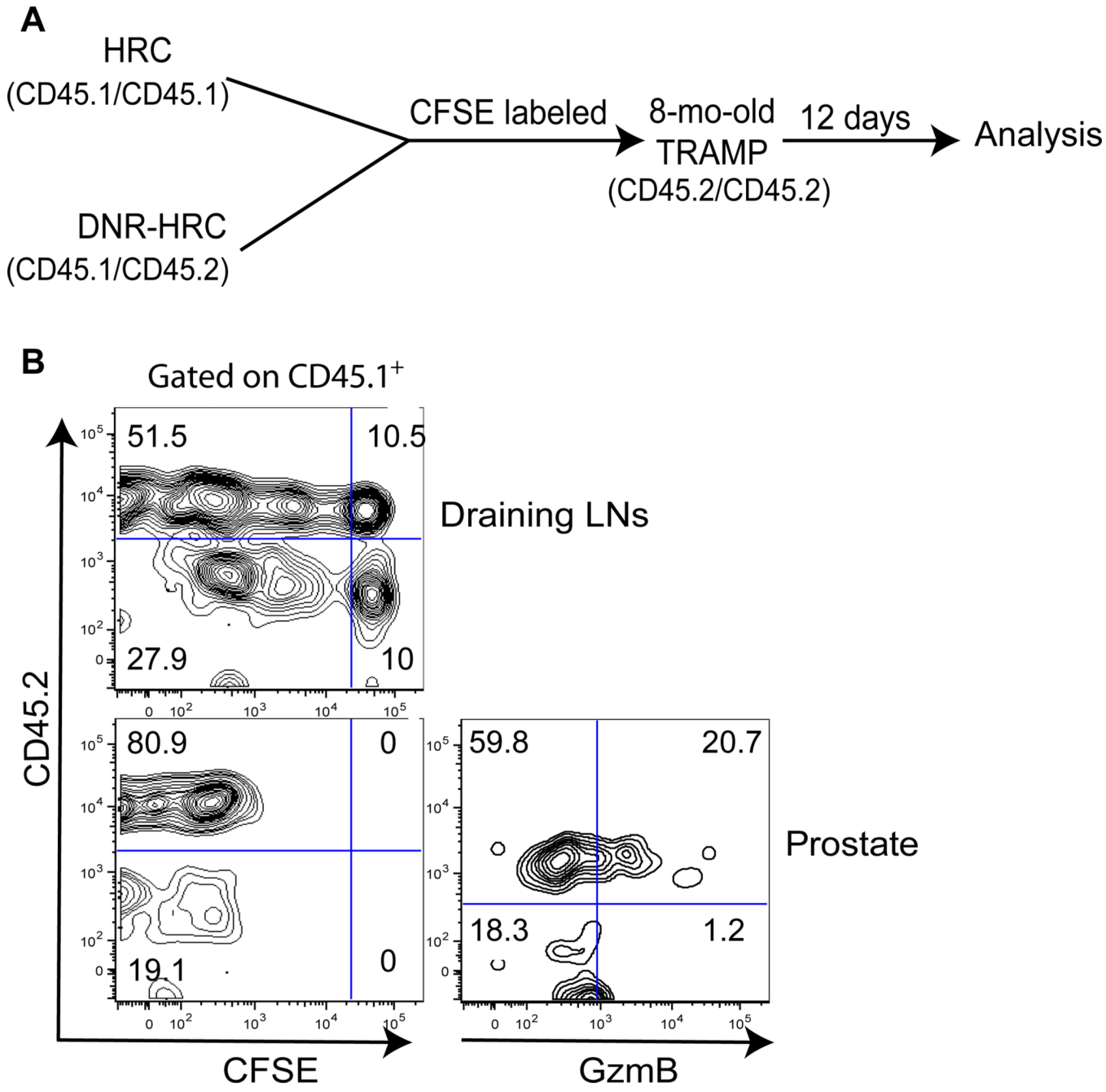


Figure 5. TGF-β signaling inhibits tumor antigen-specific T cell proliferation and effector T cell differentiation

(**A, B**) Tumor antigen-specific T-cell response assessment in TRAMP mice **(A)** Schematic of T cell transfer into tumor-bearing TRAMP mice. **(B)** Donor T cell proliferation and GzmB expression as determined by flow cytometry. A representative of three independent experiments is shown.

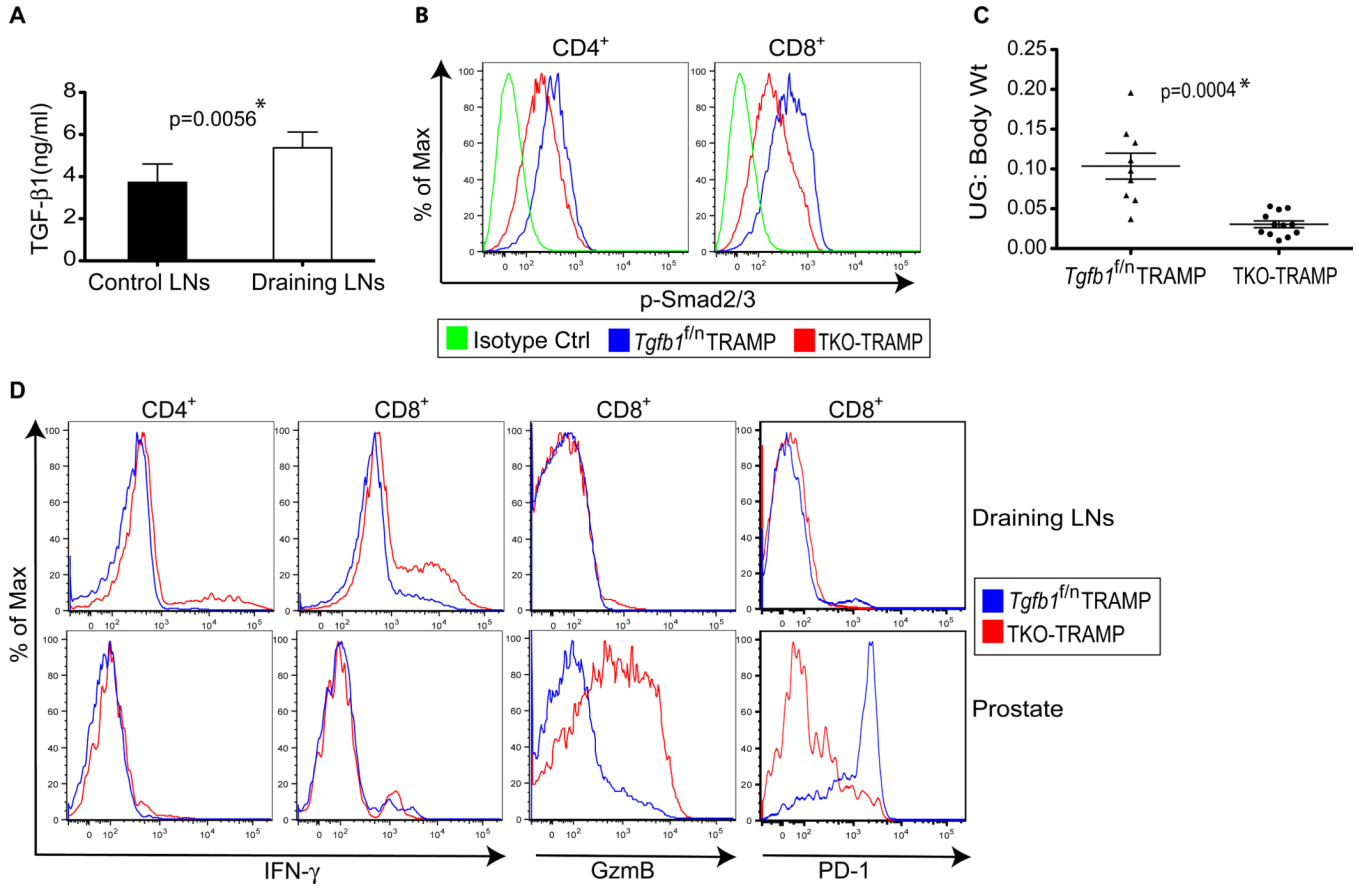


Figure 6. T cell-specific ablation of TGF-β1 enhances anti-tumor T cell responses, and inhibits tumor development

(A) TGF-β1 production by purified CD4$^+$ and CD8$^+$ T cells from the tumor-draining periaortic lymph nodes, and control axillary and brachial lymph nodes of 8-month-old TRAMP mice. Data shown are mean ± s.e.m (n=3 per group). The p value between two groups of TGF-β1 concentrations is shown (Student's t-test). * depicts statistically significant difference.(**B**) Representative flow cytometric analysis of Smad2 and Smad3 phosphorylation in T cells from lymph nodes draining prostates of 8-month-old *Tgfb1$^{f/n}$* TRAMP and *Tgfb1$^{f/n}$ Cd4-Cre* (TKO)-TRAMP mice. A representative of three independent experiments is shown. **(C)** Evaluation of the weights (Wt) of urogenital tracts (UG) normalized to body Wt ± s.e.m of *Tgfb1$^{f/n}$* TRAMP (n=9) and TKO-TRAMP (n=11) mice at 8 months of age. The p value between the two groups of tumor burden is shown (Wilcoxon rank-sum test). * depicts statistically significant difference. **(D)** Expression of IFN-γ, GzmB and PD-1 in CD4$^+$ and CD8$^+$ T cells in *Tgfb1$^{f/n}$* TRAMP and TKO-TRAMP mice. Data are representative of three independent experiments.

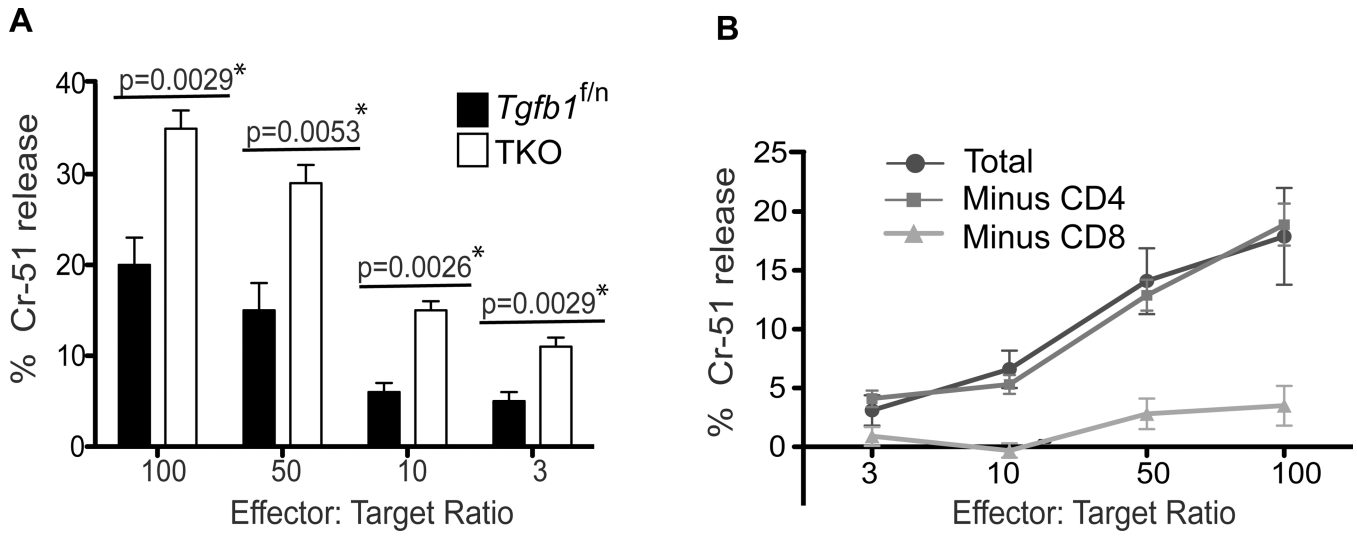


Figure 7. Enhanced tumor-specific cytolytic CD8$^+$ T cell activity in the absence of T cell-produced TGF-β1

(**A, B**) *Tgfb1$^{f/n}$* or *Tgfb1$^{f/n}$ Cd4-Cre* (TKO) mice were challenged intraperitoneally with 1×10^6 El-4 tumor cells. (**A**) Cr-51 release assay 10 days after tumor challenge. Splenocytes were used as effectors against EL-4 targets. The p values between groups of Cr-51 release at a given effector:target ratio are shown (Wilcoxon rank-sum test). * denotes statistically significant difference. (**B**) Total, CD4$^+$-depleted or CD8$^+$-depleted splenocytes from TKO mice were used as effectors against EL-4 targets at the indicated effector:target ratios.