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Blockade of TGF-β Signaling Greatly Enhances the Efficacy of TCR Gene Therapy of Cancer

Gavin M. Bendle,*1 Carsten Linnemann,*1 Laura Bies,* Ji-Ying Song, † and Ton N. M. Schumacher*

TCR gene therapy is a promising approach for the treatment of various human malignancies. However, the tumoricidal activity of TCR-modified T cells may be limited by local immunosuppressive mechanisms within the tumor environment. In particular, many malignancies induce T cell suppression in their microenvironment by TGF-β secretion. In this study, we evaluate whether blockade of TGF-β signaling in TCR-modified T cells enhances TCR gene therapy efficacy in an autologous mouse tumor model. Treatment of mice with advanced prostate cancer with T cells genetically engineered to express a tumor-reactive TCR and a dominant-negative TGF-β receptor II induces complete and sustained tumor regression, enhances survival, and leads to restored differentiation of prostate epithelium. These data demonstrate the potential to tailor the activity of TCR-modified T cells by additional genetic modification and provide a strong rationale for the clinical testing of TGF-β signaling blockade to enhance TCR gene therapy against advanced cancers. The Journal of Immunology, 2013, 191: 000–000.

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Abbreviations used in this article: ACT, adoptive cell transfer; dnTGF-βRII, dominant-negative TGF-β receptor II; TBI, total body irradiation; TRAMP, transgenic adenocarcinoma of mouse prostate.

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be insensitive to TGF-β signaling display enhanced antitumor ef-
cficacy in mouse transplantable tumor models (32–34) and inhibit tumor development in autochthonous mouse tumor models (35).
In contrast though, short-term experiments at a clinically more relevant time point in autochthonous mouse tumor models have not yielded encouraging data (36).
In this study we provide, to our knowledge, the first analysis of the long-term effects of joint genetic modification of T cells with a tumor-reactive TCR and a dominant-negative TGF-β receptor-II (dnTGFβRII) on invasive prostate carcinoma in an autochthonous mouse tumor model.

Materials and Methods
Mice
Transgenic adenocarcinoma of mouse prostate (TRAMP) mice (37) were obtained from the Experimental Animal Department of The Netherlands Cancer Institute. For all experiments, F1 offspring of B6 × TRAMP mice was used. All animal experiments were performed in accordance with institutional and national guidelines and were approved by the Experimental Animal Committee of The Netherlands Cancer Institute.

Retroviral constructs, retroviral transduction, and adoptive transfer of T cells
pMX-SV40ΔCys-β-P2A-SV40ΔCys-α (19) and pMX-dnTGFβRII-RES-GFP (19) retroviral vectors were used to transfect Phoenix-E packaging cells to generate retrovirus (38). Splenocytes from male TRAMP mice were modified by retroviral transduction with the indicated vectors, as described previously (3).
Six to 24 h prior to adoptive cell transfer, irradiation-induced host conditioning was achieved by 5 Gy total body irradiation (TBI) with a radiobiology constant potential x-ray unit (Pantak HF-320; Pantak). The following day, mice received an adoptive cell transfer of the activated and retrovirally modified splenocytes containing either 1 × 10^6 dnTGFβRII-transduced CD8+ T cells, 1 × 10^6 dnTGFβRII-RES-GFP transduced CD8+ T cells, 1 × 10^6 SV40ΔCys-αT-TCR-transduced CD8+ T cells, or equivalent numbers of nontransduced CD8+ T cells.

Flow cytometry
Cell surface TCR expression and expression of the dnTGFβRII gene were measured 24 h posttransduction by flow cytometry. Cells were stained with biotin anti-TCR Vβ9 mAb (eBiosciences), or with K+-SV40ΔCys, or multimers, in combination with PE- or allophycocyanin-conjugated anti-CD8α mAb (BD Biosciences). To measure dnTGFβRII expression, GFP expression was used as a surrogate marker as the dnTGFβRII was followed by a two-step immunoenzymatic procedure, involving biotin-labeled goat anti-mouse IgG (1:500, 1 h; DAKO) being applied, followed by HRP-labeled avidin-biotin complex (1 h; DAKO). The 3-amin-9-ethylcarbazole (Sigma-Aldrich) was used as a substrate-chromagen, and slides were counterstained with hematoxylin. Images were acquired using an Axioscam HR digital camera and processed with Axiovision 4 software (Carl Zeiss Vision). The extent of expression of SV40 large T Ag, Ki67, TGF-β1, and probasin in the prostate was determined semiquantitatively as no expression (grade 0), expressed in <25% of cells (grade 1), expressed in >25% and <50% of cells (grade 2), expressed in >50% and <75% of cells (grade 3), expressed in >75% and <100% of cells (grade 4), and expressed in 100% of cells (grade 5).

Statistical analysis
Histopathological scores were compared using a nonpaired two-tailed t test in Microsoft Excel software. Survival curves were analyzed using a log-rank (Mantel–Cox) test in GraphPad Prism software. The p values < 0.05 were considered significant.

Results
Expression of TGF-β in advanced, invasive prostate tumors in TRAMP mice
To develop an autochthonous tumor model in which the effect of manipulation of T cell sensitivity to tumor-derived TGF-β could be analyzed, we established prostate tumors of TRAMP mice (37). TRAMP mice express the transforming protein SV40 large T Ag (SV40 large T) under the control of the rat probasin promoter, and prostate epithelial cells in these mice undergo pathological changes that lead to the development of invasive prostate carcinoma by 24 wk of age (37). Histopathological analysis of advanced TRAMP tumors shows that these tumors are characterized by increased cellularity within the prostate glands, loss of gland architecture, and invasion of the tumor cells into the stroma. The neoplastically transformed cells display an increased nucleus/cytoplasm ratio, enhanced intensity of nuclear staining, and increased mitotic activity (Fig. 1 and data not shown). Furthermore, as documented previously (40), development of prostate carcinoma is characterized by impaired differentiation of the secretory prostate epithelium, as demonstrated by the loss of probasin expression in TRAMP mice with invasive carcinoma of the prostate (Fig. 1, Supplemental Fig. 1A). Notably, in line with the findings from previous studies (41, 42), the development of prostate carcinoma in the TRAMP mice is also accompanied by increased expression of TGF-β1 in the prostate (Fig. 1, Supplemental Fig. 1B), with TGF-β1 usually detected at both the apical and lateral domains of cell membranes.

In addition to the expression of TGF-β in advanced tumors, the autochthonous tumor model used in this study bears other important similarities to the clinical setting in which TCR gene therapy of cancer is being tested. First, analogous to the situation for most human tumor-associated self-Ags targeted with TCR gene therapy, the endogenous T cell repertoire of TRAMP mice has been shown to be tolerant toward SV40Ag (43, 44). Therefore, in the context of the TRAMP model, the SV40 large T Ag should be viewed as a tumor-associated self-Ag rather than a foreign Ag. Second, lymphodepleting preconditioning regimens are routinely used prior to adoptive cell transfer in the clinical setting (5–7), and the mice in this study also receive a lymphodepleting preconditioning regimen prior to the adoptive transfer of T cells. Third, analogous to the clinical use of high-affinity TCRs isolated from nontolerant T cell repertoires (6, 7), a high-affinity TCR isolated from a nontolerant T cell repertoire is used in this study.
Combination of TCR gene therapy and blockade of TGF-β signaling can lead to complete regression of advanced, invasive prostate tumors in TRAMP mice

We have previously reported that TCR gene therapy using T cells modified with a SV40 epitope IV–specific TCR (SV40IV-TCR) at 10 wk of age leads to a marked delay in tumor development in TRAMP mice (43). However, the same TCR gene therapy regimen fails to show a substantial benefit when T cell infusion is performed at 24 wk of age when neoplastic lesions are fully developed (G. Bendle, unpublished observations; see also below). Furthermore, as described above, high levels of TGF-β expression are observed in 24-wk-old TRAMP mice (Supplemental Fig. 1B). On the basis of these data, we hypothesized that the elevated levels of TGF-β expression observed in advanced prostate tumors may suppress the antitumor reactivity of TCR-modified T cells within the prostate. To test this hypothesis, we generated a retroviral vector that encodes a dnTGFβRII (19). Subsequently, the ability to control advanced prostate cancer of T cells that had been modified with only the dnTGFβRII, only the SV40IV-TCR, or the combination of the two transgenes was compared. The 24-wk-old TRAMP mice received a preconditioning regimen of nonmyeloablative TBI (5 Gy), followed by the adoptive transfer of either $1 \times 10^6$ SV40IV-TCR–transduced CD8$^+$ T cells, $1 \times 10^6$ dnTGFβRII-transduced CD8$^+$ T cells, or $1 \times 10^6$ SV40IV-TCR–transduced CD8$^+$ T cells that had been cotransduced with the dnTGFβRII. As a control, a cohort of mice received equivalent numbers of nonmodified CD8$^+$ T cells (Supplemental Fig. 2A, 2B). Four weeks after adoptive cell transfer (ACT), mice were sacrificed to analyze the short-term effects of the different gene-modified T cell populations on tumor development by histopathology. In all experiments, histopathological analysis was carried out by an animal pathologist who was blinded with respect to the treatment group to which samples belonged. In 100% of mice that received either nontransduced T cells or T cells transduced with the dnTGFβRII alone, large invasive prostate carcinomas were found. In line with this, widespread expression of the SV40 large T oncogene, the Ki67 proliferation marker, and TGF-β was observed in the prostate of these mice (Figs. 1, 2A). In mice receiving T cells modified with only the SV40IV-TCR, areas of local regression were sporadically encountered in the prostate tumors. Such areas were characterized by reduced cellularity of epithelial cells, morphological changes in epithelial cells (cuboidal shape and low nucleus/cytoplasm ratio), the local elimination of SV40 expression, and reduced Ki67 and TGF-β expression (Figs. 1, 2A). Nevertheless, many clusters of transformed cells were invariably found, and only

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FIGURE 1. Immunohistochemical analysis of the prostate in TRAMP mice 4 wk after treatment with a combination of TCR gene therapy and blockade of TGF-β signaling. H&E staining and expression of SV40 large T, Ki67, TGF-β1, and probasin in representative prostate sections from recipients of non-Td T cells, dnTGFβRII-transduced T cells, SV40IV-TCR–transduced T cells, or SV40IV-TCR/dnTGFβRII–cotransduced T cells. The partial regression of neoplastic lesions in recipients of SV40IV-TCR–transduced T cells is indicated by the arrows. Scale bar, 50 μM in all micrographs.
partial regression of the invasive prostate carcinoma was observed in all mice analyzed (Fig. 2B). In contrast, mice treated with T cells that were modified with both the SV40 IV-TCR and the dnTGFβRII displayed decreased cellularity and changes in the cellular morphology in all of their prostate neoplastic lesions (Fig. 1). In line with these histological changes, SV40 large T and Ki67 expression was found to be minimal or fully absent (~10% of cells) (Figs. 1, 2A). Likewise, the expression of TGF-β was found to be minimal or fully absent (~10% of cells) (Figs. 1, 2A).

FIGURE 2. Combination of TCR gene therapy and blockade of TGF-β signaling leads to complete regression of invasive prostate carcinoma in TRAMP mice. (A) Immunohistochemistry scoring showing reduced SV40 large T, Ki67, and TGF-β1 expression, but increased probasin expression in the prostate of recipients of SV40 IV-TCR/dnTGFβRII–cotransduced T cells. Symbols represent individual mice; bars indicate group averages. *p < 0.05, **p < 0.01, ***p < 0.001. (B) Grade of tumor regression in 28-wk-old TRAMP mice that were recipients of either non-Td T cells, dnTGFβRII–transduced T cells, SV40 IV-TCR–transduced T cells, or SV40 IV-TCR/dnTGFβRII–cotransduced T cells. Symbols represent individual mice.

Complete tumor regression of TRAMP prostate tumors induced by combination of TCR gene therapy and blockade of TGF-β signaling is sustained long-term

Next, we addressed whether the observed tumor regression in TRAMP mice 4 wk after treatment with TCR-modified T cells that are cotransduced with the dnTGFβRII was sustained long-term. To this end, 24-wk-old TRAMP mice received TBI (5 Gy) and ACT with either 1 × 10^6 SV40 IV-TCR–transduced CD8^+ T cells, 1 × 10^6 SV40 IV-TCR–transduced CD8^+ T cells that had been cotransduced with the dnTGFβRII, or equivalent numbers of nontransduced CD8^+ T cells (Supplemental Fig. 2C, 2D). At 40 wk of age (16 wk after ACT), mice were sacrificed to analyze tumor development within the prostate. TRAMP mice treated with nontransduced T cells or that received no T cell infusion (but that did receive the preconditioning regimen) all exhibited large, invasive prostate carcinomas characterized by abnormalities in cell morphology and architecture, as described above. Furthermore, analysis by immunohistochemistry demonstrated that most cells within the prostate expressed SV40 large T, Ki67, and TGF-β (Figs. 3, 4A). The majority of mice treated with SV40 IV-TCR–transduced T cells showed some locally restricted signs of tumor regression (5 of 6 mice; 83%; Figs. 3, 4B), indicating some antitumor activity of the T cells that were solely modified with the tumor-reactive TCR. However, the total level of SV40 large T expression in neoplastic lesions of the prostate did not differ to any appreciable extent from that observed in mock-treated mice (Fig. 4A). Likewise, as revealed by Ki67 staining, overall a similarly high level of proliferation in neoplastic lesions was observed in mock-treated and SV40 IV-TCR–treated mice (Fig. 4A), indicating that tumor control by the TCR-modified cell population was very limited (Fig. 4B). Strikingly, the majority of mice that were treated with T cells that were cotransduced with the SV40 IV-TCR and dnTGFβRII displayed complete regression of their prostate neoplastic lesions even at this late time point posttherapy (6 of 8 mice; 75%; Fig. 4B). Furthermore, at this late time point, prostate glands in these mice still only expressed minimal levels of SV40 large T, Ki67, and TGF-β (Figs. 3, 4A). Together, these data indicate that T cell therapy with SV40 IV-TCR/dnTGFβRII double-modified T cells leads to a sustained regression of advanced prostate tumors in TRAMP mice.

To assess whether this sustained tumor regression observed following TCR gene therapy in the presence of TGF-β blockade would lead to prolonged survival of TRAMP mice, we treated 24-wk-old preconditioned mice with either 1 × 10^6 SV40 IV-TCR–transduced CD8^+ T cells, 1 × 10^6 SV40 IV-TCR–transduced CD8^+ T cells that had been cotransduced with the dnTGFβRII, or equivalent numbers of nontransduced CD8^+ T cells (Fig. 5A). TRAMP mice infused with nontransduced T cells had a median survival of 32 wk post-ACT before mice had to be euthanized (7 of 7 mice; with 1 censored event due to lymphoma in the absence of regression of TRAMP lesions) (Fig. 5B). Histopathological analysis of the prostate in these mice after sacrifice revealed large tumor masses, macroscopically apparent especially in the seminal vesicles (data not shown). As expected, no signs of regression of TRAMP lesions in the prostate glands were observed (data not shown). TRAMP mice treated with SV40 IV-TCR–transduced T cells had a comparable median survival of 31 wk post-ACT. From this cohort, 100% (4 of 4 mice; with 1 censored event due to hepatocarcinoma in the absence of regression of TRAMP lesions) of animals had to be euthanized as a result of progressive prostate carcinoma tumor growth (Fig. 5B), and survival within this group was not significantly increased compared with mice receiving nontransduced T cells (p = 0.98).

At a median follow-up of 57 wk post-ACT/81 wk of age TRAMP mice receiving SV40 IV-TCR/dnTGFβRII double-modified T cells were sacrificed and examined histologically (3 of 5 censored events due to lymphoma). As at 40 wk of age, we again observed that the majority of treated mice displayed complete regression of their prostate neoplastic lesions (4 of 5 mice; the fifth mouse displayed regression in >75% of its prostatic neoplastic lesions) (Fig. 5B). Importantly, contrary to mice treated with SV40 IV-TCR–modified T cells, the survival of mice receiving SV40 IV-TCR-dnTGFβRII double-modified T cells was significantly higher than that of both mice receiving nontransduced T cells (p = 0.001) and mice treated with the SV40 IV-TCR single-modified T cells (p = 0.025).

Probasin is a marker of differentiated secretory prostate epithelium in mice, and it has previously been demonstrated that the expression of this differentiation marker is lost in TRAMP mice.
with advanced prostate carcinoma (40). To evaluate whether successful TCR gene therapy could lead to restored differentiation of the prostate epithelium, we analyzed prostate tissue of the different treatment groups at various time points posttherapy. Strikingly, we observed clear expression of probasin within the prostate epithelial cells in the majority of mice following treatment with SV40IV-TCR and dnTGFβRII-cotransduced T cells at both 4 wk (28 wk of age) (Figs. 1, 2A) and 16 wk (40 wk of age) (Figs. 3, 4A) after ACT. In contrast, no expression of probasin was observed in TRAMP mice in which no tumor regression was observed. Thus, treatment of mice with advanced prostate cancer with TCR-modified cells that have been rendered insensitive to TGF-β signaling not only leads to long-term cancer regression, but also to the recovery of normal prostate gland function in TRAMP mice.

**Discussion**

In this study, we demonstrate that the blockade of TGF-β signaling can greatly enhance the efficacy of TCR gene therapy in an autochthonous mouse model of prostate carcinoma. This combination treatment not only leads to the sustained regression of advanced and invasive prostate carcinoma, but can also be shown to result in prolonged survival of treated mice. To our knowledge, this is the first example of long-term tumor control by TCR-modified T cells in an autochthonous tumor model. Strikingly, the complete tumor regression observed in these mice is accompanied by the restoration of probasin expression in prostate epithelial cells. Probasin is a marker of differentiated secretory prostate epithelium in rodents (40), and our data therefore suggest that treatment with dual-engineered T cells also leads to the recovery of normal prostate gland function. The molecular mechanism responsible for the recovery of normal prostate gland function following treatment with dual-engineered T cells has not been elucidated. However, the adult murine prostate is known to contain a population of stem cells capable of mediating the regeneration of a functional prostate following repeated cycles of androgen deprivation and replacement therapy (45–47). Therefore, it seems feasible that the prostate epithelial cells expressing probasin we observe following treatment with dual-engineered T cells are derived from this population of prostatic stem cells.

**FIGURE 3.** Immunohistochemical analysis of the prostate in TRAMP mice 16 wk after treatment with a combination of TCR gene therapy and blockade of TGF-β signaling. H&E staining and expression of SV40 large T, Ki67, TGF-β1, and probasin in representative prostate sections from recipients of non-Td T cells, SV40IV-TCR–transduced T cells, and SV40IV-TCR/dnTGFβRII–cotransduced T cells. The partial regression of neoplastic lesions in recipients of SV40IV-TCR–transduced T cells is indicated by the arrows. Scale bar, 50 μM in all micrographs.
cancer. One clear difference between these studies is that in our study a cancer-driving Ag was the target of adoptively transferred T cells, whereas Chou et al. (36) targeted an Ag that did not play a role in tumor development or the maintenance of the malignant phenotype. Therefore, the likelihood of the emergence of tumor Ag loss variants would have been greater in the study by Chou et al. (36), but whether tumor Ag loss variants occurred in their study was not directly addressed. Another major difference between these studies is that the mice in our study received a lymphodepleting preconditioning regimen prior to the adoptive transfer of T cells, whereas no such regimen was used in the study by Chou et al. (36). Lymphodepleting preconditioning regimens are routinely used prior to adoptive cell transfer in the clinical setting (5–7), and studies in mice have demonstrated the crucial role this process plays in promoting the in vivo function of adoptively transferred T cells (48–50). In line with this, we have previously shown that, in the absence of the in vivo T cell activation provided by either lymphodepleting preconditioning or concomitant viral vaccination, adoptively transferred SV40 TCR-transduced T cells fail to prevent tumor development in 10-wk-old treated TRAMP mice (43). Based on these data, we suggest that the marked effect of TGF-β blockade observed in this study can only manifest itself under conditions in which the TCR-modified T cells are activated in vivo, either by vaccination, or by infusion into a lymphodepleted host.

A recent clinical study using TCR gene therapy targeting the cancer germline Ag NY-ESO-1 to treat patients with metastatic melanoma and synovial cell carcinoma has reported very encouraging clinical response rates (7). However, the majority of these clinical responses were partial and not durable. It is tempting to speculate that, among other factors, the in vivo efficacy of TCR-modified T cells in these patients was hampered by suppression within the tumor microenvironment. In support of this notion are studies demonstrating that immune-suppressive mechanisms can be operational in melanoma (51, 52) as well as the findings reported in this work that demonstrate that tumor regression of advanced, invasive prostate tumors is transient and does not prolong survival for the majority of TRAMP mice when treated with TCR-modified T cells in the absence of TGF-β blockade.

The TGF-β signaling pathway in T cells (and tumor cells) is amenable to pharmacological blockade using small molecule inhibitors (53). However, given the central role of TGF-β in many biological processes, including immune homeostasis (21, 22), systemic blockade of TGF-β signaling may potentially lead to a variety of side effects. Therefore, the specific blockade of TGF-β signaling in TCR-transduced T cells, that is, with a dnTGFRII, seems preferable. This study shows the feasibility, safety, and efficacy of such a T cell–targeted approach and demonstrates that it can be used to promote successful TCR gene therapy of advanced, immunosuppressive cancers. Given the observation that a variety of human malignancies has been reported to express elevated levels of TGF-β (25, 26), blockade of TGF-β signaling in TCR-modified T cells can be viewed as a generally applicable approach to enhance the therapeutic efficacy of TCR gene therapy. However, one note of caution is that in transgenic mouse models in which a
dominant-negative TGF-β receptor transgene is expressed under control of the CD2 or CD4 promoter, development of CD8+ T cell lymphomas has been observed (54–56). Although in these models expression of the dominant-negative TGF-β receptor already initiates during T cell development, on the basis of these data it may be prudent to design clinically applicable dominant-negative TGF-β receptor formats in which the duration of suppression of TGF-β signaling can be controlled.

On a more general note, the targeted manipulation of the activity of TCR-modified T cells by additional genetic modification appears an attractive strategy to promote the efficacy of TCR gene therapy. First, such additional modifications can easily be accommodated in current clinical protocols (57). Second, such modifications may be tailored allowing, for example, the control of gene expression using NFAT-promoter elements (57, 58), the use of small interfering RNA instead of genes (59), or the use of gene-editing strategies to permanently silence specific genes (20). Third, and probably most importantly, such a targeted approach is likely to avoid deleterious side effects associated with systemic adjuvant therapies such as cytokine supplementation (60, 61) or blockade of T cell checkpoints (62). Thus, the further exploration of combined genetic modifications to enhance the therapeutic efficacy of TCR gene therapy for the treatment of advanced cancers seems warranted.

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Disclosures

The authors have no financial conflicts of interest.

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