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Full Length Research Paper

Regression of mouse-derived renal cancer by adoptive transfer of tumor-reactive RNAi-induced TGF-beta-insensitive CD8$^+$ T cells

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Transforming growth factor beta (TGF-beta) is a potent immunosuppressant. The present study was conducted to develop a treatment strategy through adoptive transfer of tumor-reactive RNAi-induced TGF-beta-insensitive CD8$^+$ T cells. BALB/c mice were primed with irradiated Renca cells. CD8$^+$ T cells were isolated from the spleen of primed animals, expanded *ex vivo* and were rendered TGF-beta-insensitive by infecting with a retrovirus containing shRNA to mouse TGF-beta type II receptor gene (MSCV-shRNA-T). Control CD8$^+$ T cells consist of those infected with retroviruses containing shRNA to non specific gene (MSCV-shRNA-N) and naive CD8$^+$ T cells. The effect of all groups of CD8$^+$ T cells on Renca cells were analyzed by semi-quantitative RT-PCR, Western-blot, *in vitro* and *in vivo* assay. MSCV-shRNA-T group of CD8$^+$ T cells were resistant to the antiproliferative effect of exogenous TGF-beta, while control groups were not. Results of Western blot showed the Smad pathway was disrupted in MSCV-shRNA-T group, which confirmed the blockade of the signal transduction pathway. *In vitro* cytotoxic assay revealed that these tumor-reactive, TGF-beta-insensitive CD8$^+$ T cells killed Renca cells specifically and strongly. Adoptive transfer of these MSCV-shRNA-T CD8$^+$ T cells to BALB/c tumor-bearing mice showed strong tumor-specific cytotoxic T lymphocyte responses and antitumor immunity against Renca renal cancer. Based on these results, we predict that adoptive transfer of tumor-reactive RNAi-induced TGF-beta-insensitive CD8$^+$ T cells may be effective to renal cancer therapy.

Key words: Transforming growth factor beta, adoptive transfer, RNA interference, renal cancer, renca cells, immunotherapy.

INTRODUCTION

In recent years, immune elements as diverse as cytokines, antibodies, vaccines and adoptive cell transfer regimens have emerged as promising cancer immunotherapy approaches with confirmed clinical efficacy (Rosenberg, 2001; Dudley and Rosenberg, 2003; Mehren et al., 2003; Smyth et al., 2004). However, clinical trials aimed at harnessing and enhancing the endogenous immune system against most cancers generally resulted in a very low frequency of durable complete responses (Rosenberg et al., 1986, 2004; Figlin et al., 1999). Analysis of immune parameters in the majority of patients following immunotherapy has demonstrated the infer-quent generation of endogenous T cells with activity against autologous tumors. The low success rate of immunotherapy may therefore be due to a deficiency of tumor-reactive T cells in the immune repertoire or their

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Table 1. Synthesized shRNA sequences.

Oligo name	Orientation	Sequence	Position	Length
MSCV-shRNA-T	Sense	5'-CCGTTCCCAAGTCGGTTAACTTCAAGAGAGTTAACCGACTT GGGAACGTTTTTG-3'	81 - 99	54
	Antisense	3'-CCGGGGCAAGGGTTCAGCCAATTGAAGTTCTCTCAATTGG CTGAACCCTTGCAAAAACTTAA-5'		62
MSCV-shRNA-N	Sense	5'-CTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTT CGGAGAATTTTTG-3'		54
	Antisense	3'-CCGGGAAGAGGCTTGCACAGTGCAAAGTTCTCTTGCACTG TGCAAGCCTCTT AAAAACTTAA-5'		62

non responsive state, rendering them unable to become activated and expand in response to antigens. In addition, tumor-reactive T cells may not traffic effectively to tumors and may be inhibited by tumor-derived factors such as TGF-ß (Gorelik and Flavell, 2002).

Transforming growth factor-beta (TGF-ß) is a 25-kDa multifunctional growth factor. It is implicated in many cellular processes, including growth and differentiation, angiogenesis, migration, deposition of extracellular matrix and immunosuppression (Massague, 1990; Moses et al., 1990; Sporn and Roberts, 1991; Huang et al., 1995; Barrack, 1997; Gordon and Blobe, 2008). TGF-ß exerts these effects through binding to specific cell-surface receptors that act cooperatively. Three types of TGF-ß receptors have been identified: type I (TßR-I), type II (TßR-II) and type III (TßR-III). TßR-III is a proteoglycan. The biological function of TßR-III may involve presentation of TGF-ß to target cells, and it does not directly participate in signal transduction (Sun and Chen, 1997). TßR-I and TßR-II, on the other hand, exhibit serine/threonine kinase activity in their intracellular domains (Wrana et al., 1992). The current understanding shows that TGF-ß first binds to TßR-II to form a complex. TßR-I is then recruited by this complex through phosphorylation and this leads to the phosphorylation of signaling pathway specific Smad-2 and Smad-3 molecules that oligomerize with the common mediator, Smad-4, resulting in their translocation to the nucleus (Massague, 1998; Jayaraman and Massague, 2000). Therefore, the type II receptor provides a suitable target for disruption of the signaling pathway. Targeted disruption of the TGF-ß signaling pathway has been effectively achieved by restricting the expression of a dominant negative type II TGF-ß receptor in immune cells such as the bone marrow cells and $CD8^+$ T cells (also called cytotoxic T Lymphocytes) or by silencing TßR-II via RNA interference in other tissue cells (Dudley et al., 2002; Shah et al., 2002a,b; Nakamura et al., 2004; Jazag et al., 2005; Mizuguchi et al., 2005; Zhang et al., 2005, 2006).

Historically, adoptive immunotherapy in cancer has shown promising clinical results by selection and amplification of autologous antigen-specific lymphocytes before reinjection into patients (Dudley et al., 2002; Mitchell et al., 2002; Yee et al., 2002). Although, renal carcinoma has no well-defined specific antigen, tumor-reactive $CD8^+$ T cells can be isolated from hosts suffering from renal cancer. In addition, renal carcinoma cells produce a large amount of TGF-ß to inhibit the function of $CD8^+$ T cells (Knoefel et al., 1997; Wunderlich et al., 1997, 1998; Kominsky et al., 2007), which leads to tumor escape from the host immunosurveillance and tumor progression.

Considering this hypothesis, in the present study, we engineered tumor-reactive RNAi-induced TGF-ß-insensitive $CD8^+$ T cells and evaluated their antitumor effect *in vitro* and in tumor-bearing mice. Our results indicated that these $CD8^+$ T cells show strong tumor-specific cytotoxic T lymphocyte responses and antitumor immunity against Renca renal cancer, which provides proof that these $CD8^+$ T cells can be used in clinical renal carcinoma immunotherapy.

MATERIALS AND METHODS

Mice and Cells

Eight-week-old BALB/c mice obtained from the Laboratory Animal Research Center of the Fourth Military Medical University were used according to the approved guidelines of the Fourth Military Medical University. The mouse renal carcinoma cell line, Renca cells (ATCC, USA), were maintained in complete medium (CM) consisting of RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Construction of a retroviral vector for delivery of shRNA

MSCV-TßRIIDN-IRES-GFP vector and pEGFP/U6 vector were provided by Prof. Chung Lee (Zhang et al., 2005) and Prof. Yang Shi (Sui et al., 2002), respectively. One pair of complementary oligonucleotides encoding mouse TGF-ß type II receptor gene (GenBank accession No. S69114) shRNA with a loop motif (Mizuguchi et al., 2005) and negative control shRNA targeting none specific gene (Table 1) were synthesized, annealed and ligated into the linearized pEGFP/U6 vector between the *Apa* I and *Eco*R I linker sequences (Figure 1A). After construction of pEGFP/U6-shRNA, the U6-shRNA sequence was excised from it by *Bam*H I/*Eco*R I digestion and inserted into the MSCV-TßRIIDN-IRES-GFP vector, which was also linearized by *Bam*H I/*Eco*R I digestion

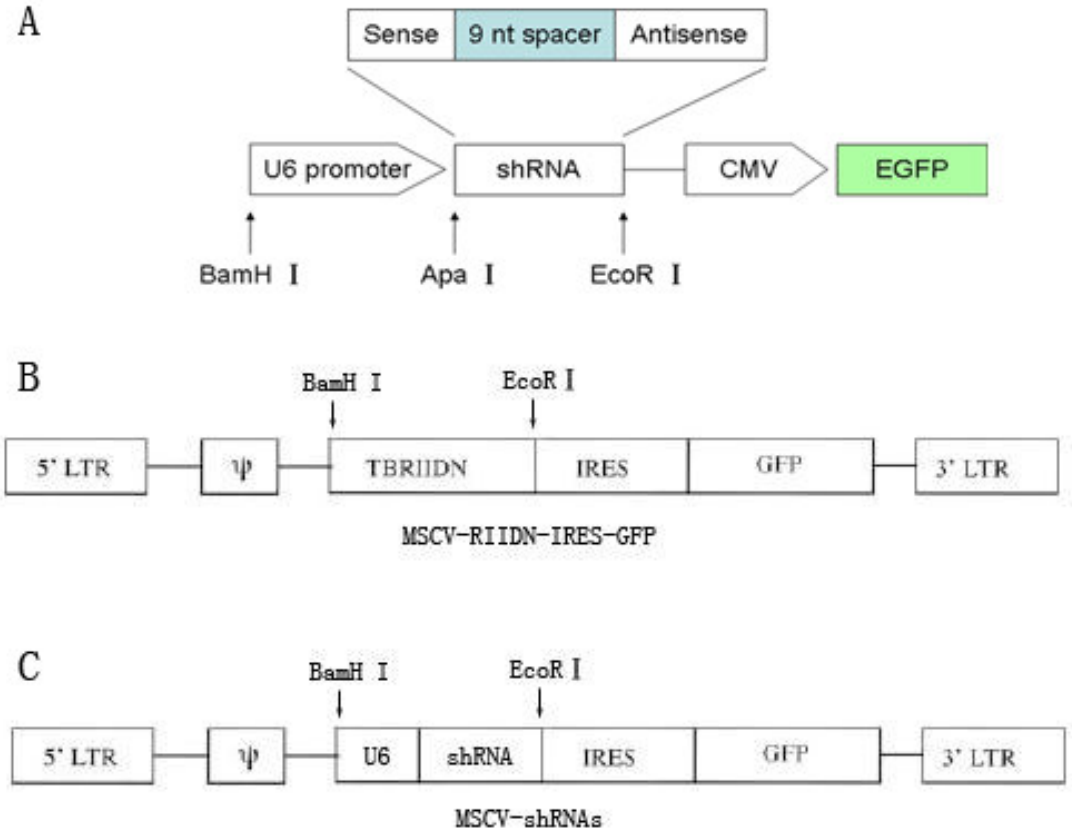

Figure 1. Construction of MSCV-shRNA retroviral vectors. A: shRNA was inserted into the pEGFP/U6 vector to construct the pEGFP/U6-shRNA vector; B: map of MSCV-TßRIIDN-IRES-GFP vector; C: U6-shRNA was excised and ligated into linearized MSCV-TßRIIDN-IRES-GFP to construct MSCV-shRNAs vector.

(Figures 1B and C). These clones were designated as MSCV-shRNA-T or MSCV-shRNA-N and screened by sequencing for correct orientation, sequence and insert number.

Isolation and expansion of tumor-reactive CD8$^+$ T cells

BALB/c mice were primed with irradiated Renca cells (5 × 10^6/mice at 20,000 ci) by subcutaneous injection every 10 days for a total of 3 inoculations. Two weeks following the last vaccination, splenic CD8$^+$ T cells were isolated by using a murine T cell CD8$^+$ subset column kit (R and D Systems, Minneapolis, MN) and expanded (10^5/ml) in the presence of Renca lysates (1 × 10^6) and irradiated autologous splenocytes (1 × 10^6/ml 3000 ci) in a medium containing RPMI-1640 with 10% FBS, IL-2 (50 U/ml), anti-CD3$^+$ monoclonal antibody (30 ng/ml, R and D), HEPE (25 mM), L-glutamine (4 mM), and 2-ME (25 mM). The medium was changed every 3 days.

Production of infectious MSCV-shRNA retrovirus

Pantropic GP293 retroviral packaging cells (Clontech, San Diego, CA) were seeded at a density of 2.5 × 10^6 cells in collagen-I-coated T-25 flasks (BIOCOAT, BD Biosciences) 24 h before plasmid transfection in antibiotic-free 10% DMEM, such that the cells were 70 - 90% confluent at the time of transfection. At this point, the cells were rinsed with PBS to remove residual serum. A mixture of 2 µg retroviral plasmid and 2 µg VSV-G envelope plasmid was co-transfected in serum-free DMEM using Lipofectamine-Plus (Invitrogen, USA) according to the manufacturer's protocols with some modifications. Briefly, cells were transfected for 12 h followed by the addition of an equivalent volume of 10% DMEM and reincubation for an additional 12 h. After the reincubation, the supe-rnatant was aspirated, the cells were rinsed gently in PBS, and 3 ml of fresh 10% DMEM was added into each flask. Virus-containing super-natant was collected 24 h later and used to infect the target cells or stored at -80℃.

Infection of CD8$^+$ T cells with retrovirus containing shRNA-T or shRNA-N

CD8$^+$ T cells were infected with MSCV-shRNA-T and MSCV-shRNA-N. The infection efficiency was 93.9% for the MSCV-shRNA-T vector and 92.8% for the MSCV-shRNA-N control vector (Figure 2A). Three types of CD8$^+$ T cells were established in each time group. The first type was tumor-reactive RNAi-induced TGF-ß-insensitive CD8$^+$ T cells (tumor-reactive CD8$^+$ T cells infected with the MSCV-shRNA-T virus). The second type was tumor-reactive CD8$^+$ T cells infected with the MSCV-shRNA-N virus. The third type was naive CD8$^+$ T cells, which were freshly isolated from the

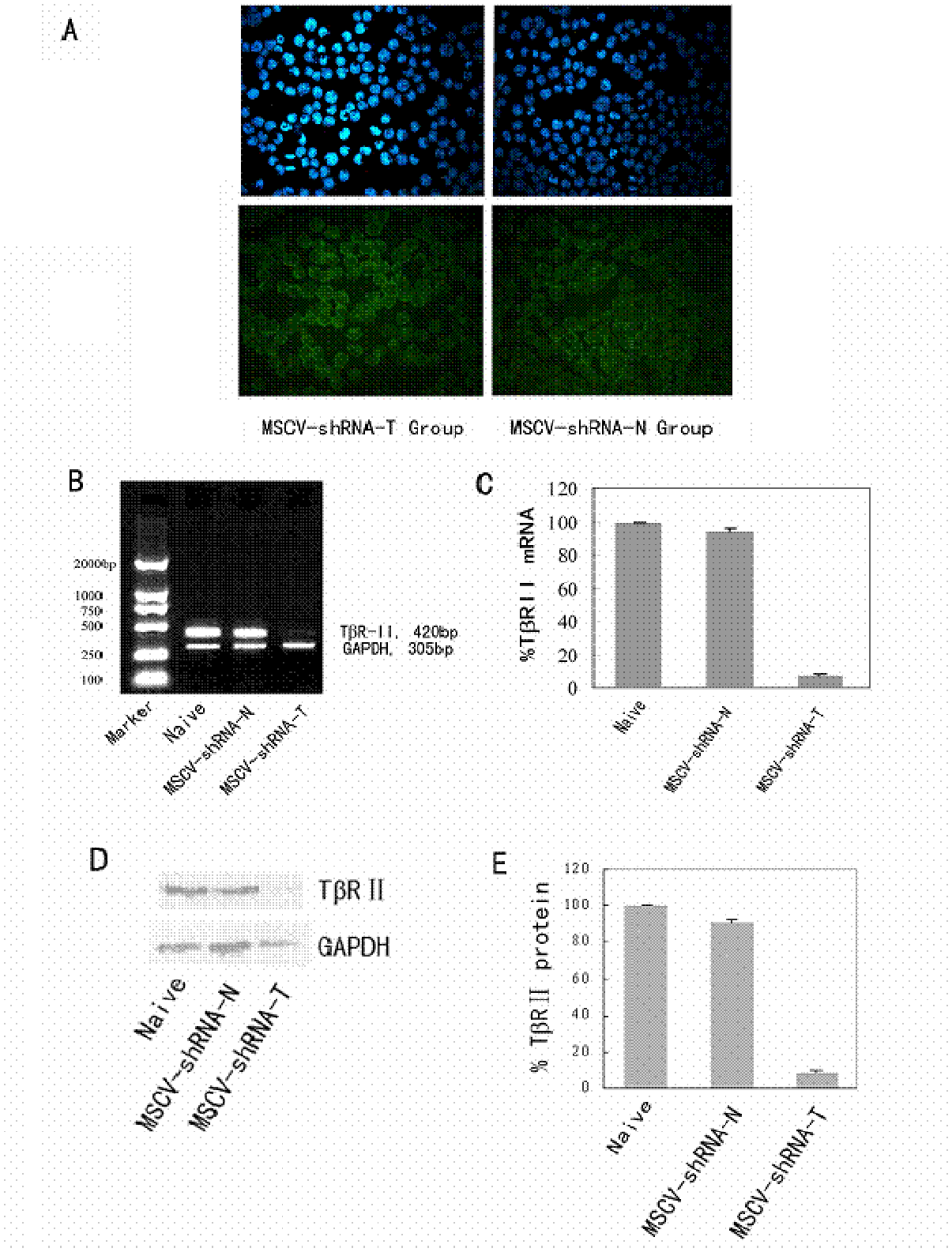

Figure 2. Efficient Inhibition of TGF-ß Type II Receptor by MSCV-shRNA-T Retrovirus. A: Most $CD8^+$ T cells appeared green under a fluorescent microscope, which indicated that the efficiency of the infection was high and the reconstruction of MSCV-shRNAs was successful, B and C: compared with control groups, the level of TßRII mRNA was down-regulated in the MSCV-shRNA-T-infected group ($P < 0.05$), Correspondingly, D and E: show that the protein level of TßRII in the MSCV-shRNA-T group was also reduced($P < 0.05$). There were 3 samples in every group when the semi-quantitative RT-PCR and Western-blot were performed.

spleen of naive donor animals without any treatment.

Reverse transcription-PCR analysis for TGF-ß type II receptor mRNA

Total RNA of $CD8^+$ T cells infected with two shRNA expression vectors after 48 h was extracted by using Trizol reagent according to the manufacturer's protocol (Invitrogen) and quantified at a 1/100 dilution of the RNA on a spectrophotometer prior to use. cDNA synthesis was performed at 50°C for 30 min (SuperScript™ preamplification system, Invitrogen) and the PCR conditions were as follows: 1 cycle of pre-denaturation (94°C, 5 min), 30 cycles of denaturation (94°C, 15 s), annealing (52°C, 30 s), extension (72°C, 40 s), and 1 cycle of final extension (72°C, 7 min). Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as an internal control. The primers used were as follows: TßRII-forward, 5'-ttcgccgaggtctacaag-3'; TßRII-reverse, 5'-gacttgacctgttgcctgt-3'. GAPDH-forward, 5'-tcggagtcaacggatttggtcgta-3'; GAPDH-reverse, 5'-agccttctccatggtggtgaaga-3'. Then, quantitate mRNA levels of TßRII and GAPDH were analyzed. PCR products were separated on a 1% agarose gel in 0.5% TBE. A semi-quantitative PCR method was used to determine the percentage of inhibition of TßRII, along with the use of the National Institutes of Health (NIH) ImageJ v1.34 software to scan the PCR amplified products directly from the images of the agarose gel bands. Thus, a relative quantification of the TßRII products was obtained in comparison with the expression level of TßRII in naïve $CD8^+$ T cells. The relative values were expressed in pixels as integrated densities.

Western blot analysis for TßRII protein and SMAD-2 phosphorylation

After 48 h of infection, $CD8^+$ T cells were treated with 10 ng/ml of TGF-ß1 for 16 h. Then, $CD8^+$ T cells were harvested and total proteins were extracted by adding an RIPA buffer (Zhang et al., 2005) to cell pellets. Approximately 30 µg of total protein extract was subjected to electrophoresis (Novex/10% acrylamide gel) and blotted onto a polyvinylidene difluoride membrane. Blots were probed using anti-TßRII (Jackson ImmunoResearch), anti-Smad2 (Upstate Biotechnology, Lake Placid, NY), antiphospho-Smad2 (Upstate Biotechnology) or anti-GAPDH (Chemicon, Temecula, CA) mAb. Proteins of interest were detected using an enhanced chemi-luminescence system (ECL Kit, Pierce) followed by exposure to a Kodak X-OMAT AR film. The National Institutes of Health (NIH) ImageJ v1.34 software was used to scan the bands.

Thymidine incorporation assay

$CD8^+$ T cells (3×10^4 cells/24-well) were treated with or without 10 ng/ml murine TGF-ß1 (R and D Systems, USA) for 16 h. A medium containing [^{3}H]-thymidine (0.5 µCi/ml; Amersham Pharmacia Biotech) was introduced and cells were cultured for an additional 5 h. The experiment was terminated by washing with warm serum-free medium and the samples were harvested for determining the radioactivity. The antiproliferative effect of TGF-ß1 on these $CD8^+$ T cells was expressed as follows: $100 \times (R_1 - R_2)/R_1$, where R_1 represents the count of radioactivity in groups without TGF-ß1 and R_2, that with TGF-ß1.

In vitro cytotoxic assay

The above three types of $CD8^+$ T cells were used as effector cells against ^{51}Cr-Renca cells or ^{51}Cr-B16-F10 cells (mouse melanoma cell line, an irrelevant cancer cell line). The standard ^{51}Cr-release assay was performed as described elsewhere (Shah et al., 2002b). Briefly, target cells were labeled with 0.1 mCi of ^{51}Cr per 10^6 cells for 4 h at 37°C, followed by 5 washes in PBS(–) and they were seeded in 96 well U-bottom plates (5,000 cells/well). $CD8^+$ T-cells were added at different effector/target ratios (1:1 to 100:1) for 5 h. The supernatants were collected and the released radioactivity was measured using a gamma counter. The percentage of specific lysis was determined as follows: 100 × ([Experimental ^{51}Cr Release – Spontaneous ^{51}Cr Release]/ [Maximum ^{51}Cr Release – Spontaneous ^{51}Cr Release]). The maximum release was determined by adding 2% sodium dodecyl sulfate to the target cells.

In vivo anti-tumor assay

Renca cells (3×10^5 cells per mouse) were subcutaneously (s.c.) implanted into BALB/c mice (n = 10/group) in the right flank (day 0). Tumors that were approximately 2 - 3 mm in diameter developed ten days later. After the tumors developed, the adoptive transfer of $CD8^+$ T cells (2×10^6) through the tail vein was performed on either day 2, 9, or 20. Tumor growth and mouse survival were monitored weekly post-inoculation. Forty days after adoptive transfer, all animals were sacrificed, although, some animals died earlier due to poor health conditions. Splenic $CD8^+$ T cells were isolated and the percentage of GFP-positive $CD8^+$ T cells in each spleen was calculated following analysis by flow cytometry. All the animals' subcutaneous tumors were excised for observation. The diameters of the tumors were measured using a digital caliper and the tumor volumes were calculated by the formula: $v = a \times b^2/2$ mm^3, where a is the long diameter and b, the short diameter.

Statistical analysis

Statistical significance was determined by unpaired Student's t-test, ANOVA and Mantel-Haenszel log-rank test using commercially available software (Stat-200, BIOSOFT, Cambridge, UK). $P < 0.05$ was considered as significant.

RESULTS

Efficient inhibition of TGF-ß Type II receptor by MSCV-shRNA-T retrovirus

Tumor-reactive $CD8^+$ T cells were infected with MSCV-shRNA-T or MSCV-shRNA-N retrovirus. Twenty-four hours after infection, we observed the $CD8^+$ T cells using a Nikon TE2000-U fluorescent microscope (Nikon Corp., Tokyo, Japan). Most cells were green (Figure 2A), which indicated that insertion of the U6 promoter has no noticeable effects on virus packaging or infectivity. Forty-eight hours after infection, the infected or naïve $CD8^+$ T cells were harvested for RT-PCR and Western blot analysis of TßRII. Compared with control groups, the level of TßRII mRNA was down-regulated in the MSCV-shRNA-T-infected group (Figures 2B and C). Correspondingly, the results of Western blot analysis showed that the protein level of TßRII in MSCV-shRNA-T group was also reduced (Figures 2D and E). These results revealed that the MSCV-shRNA-T retrovirus efficiently inhibited the expression of TßRII in tumor-reactive $CD8^+$ T cells.

Functional status of TGF-ß signaling in RNAi-induced TGF-ß-insensitive CD8$^+$ T cells

Under normal conditions, CD8$^+$ T cells express a high level of TßRII and they are highly sensitive to TGF-ß. When CD8$^+$ T cells were infected with the retrovirus containing shRNA-T, they became insensitive to TGF-ß, as shown by the following tests. In our study, all three types of CD8$^+$ T cells were treated with or without 10 ng/ml TGF-ß1. Western blot analysis demonstrated the presence of Smad-2 in all the CD8$^+$ T cell groups. However, phosphorylated Smad-2 was only detected in MSCV-shRNA-N-infected CD8$^+$ T cells and naïve CD8$^+$ T cells treated with TGF-ß1. In contrast, phosphorylated Smad-2 was not detected in MSCV-shRNA-T-infected CD8$^+$ T cells after the addition of TGF-ß1, confirming that TGF-ß signal transduction was blocked by the presence of shRNA to TßRII (Figure 3A).

To confirm whether the MSCV-shRNA-T-infected CD8$^+$ T cells could overcome the antiproliferative effects of TGF-ß1, thymidine incorporation assay was performed. TGF-ß1 showed a dramatic antiproliferative effect on the established MSCV-shRNA-N-infected CD8$^+$ T cells and naive CD8$^+$ T cells, inhibiting uptake by a mean of 62.5% (range, 47 - 80%) and 65.5% (range, 49 - 84%), respectively (Figure 3B). On the other hand, the mean inhibition of thymidine uptake by MSCV-shRNA-T-infected CD8$^+$ T cells was 15% (range, 0 - 28%) (Figure 3B). The resistance to antiproliferative effects in MSCV-shRNA-T-infected CD8$^+$ T cells was statistically significant when compared with that in MSCV-shRNA-T-infected CD8$^+$ T cells ($P = 0.02$) or naive CD8$^+$ T cells ($P = 0.02$).

In vitro anti-tumor activity of tumor-reactive RNAi-induced TGF-ß-insensitive CD8$^+$ T cells

Tumor-reactive RNAi induced TGF-ß-insensitive CD8$^+$ T cells exhibited potent specific lysis against Renca cells (Figure 3C). These cells showed a 4 fold greater tumor-killing activity than their TGF-ß-sensitive counterparts and 20 fold greater activities over that of naive CD8$^+$ T cells. Both the TGF-ß-sensitive and TGF-ß-insensitive tumor-reactive CD8$^+$ T cells exhibited no apparent lysis when incubated with an irrelevant cell line, mouse B16-F10 melanoma cells (Figure 3D).

Increased survival and suppressed growth of renca renal cancer in RNAi-induced TGF-ß-insensitive CD8$^+$ T cells adoptive transferred mice

In the absence of any intervention, on day 10, following the s.c. injection of tumor cells, the subcutaneous tumor burden was evident. Animals that received an adoptive transfer of tumor-reactive RNAi induced TGF-ß-insensitive CD8$^+$ T cells showed the least degree of tumor burden (Figures 4A and B). There was no evidence of subcutaneous tumors in the group of mice that received an adoptive transfer 2 days after the formation of tumor burden. Four animals out of ten in the 9 day group were found to have small subcutaneous tumors at the time of sacrifice. Two animals out of 10 in the 20 day group had to be sacrificed earlier due to poor health conditions and they were found to have obvious subcutaneous tumors. All the remaining 8 animals in the 20 day group were found to have a tumor burden with the volume range of 183.5-387.5 mm^3. Animals that received adoptive transfer of MSCV-shRNA-N-infected CD8$^+$ T cells showed an intermediate degree of tumor burden, while those that received an adoptive transfer of naive CD8$^+$ T cells were ineffective in inhibiting tumor progression. Analysis of Kaplan-Meier survival cure showed highly significant differences among the three treatment groups (Figure 4C).

Furthermore, we detected the fraction of GFP-positive CD8$^+$ T cells in the spleens of tumor-burdened mice. After sacrifice of the tumor-burdened mice, the CD8$^+$ T cells were isolated for flow cytometry analysis. Adoptively transferred tumor-reactive RNAi-induced TGF-ß-insensitive CD8$^+$ T cells were detected with a percentage of 2.05 ± 0.31%, suggesting that these CD8$^+$ T cells were able to persist in recipient hosts at least at the time of sacrifice, which occurred 40 days after the initial adoptive transfer. In contrast, in animals that received the MSCV-shRNA-N infected tumor-reactive CD8$^+$ T cells or naive CD8$^+$ T cells, only occasional CD8$^+$ T cells were detected with a percentage of 0.13 ± 0.02% (Figure 4D). These results indicated that RNAi-induced blockade of TGF-ß signaling in tumor-reactive CD8$^+$ T cells efficiently activated the antitumor effect on Renca renal cancer.

DISCUSSION

Secretion of TGF-ß is a strategy commonly used by tumors to thwart cellular immune responses. The secretion may interfere with several CD8$^+$ T cell functions, including down-regulation of cell surface MHC antigens or molecules, as well the inhibition of CD8$^+$ T cell proliferation (Gorelik and Flavell, 2002). This may severely affect the tumor-killing activity of CD8$^+$ T cells. Tumor cells themselves may be sensitive to TGF-ß-induced differentiation and apoptosis but can avoid this if they also possess mutant receptors for the cytokine (Wunderlich et al., 1997, 1998; Engel et al., 1999; Kominsky et al., 2007). We now show that such mutants can be adapted for the protection of CD8$^+$ T cells. RNAi to TGF-ß type II receptor may provide an approach to protect CD8$^+$ T cells from the inhibitory effects of TGF-ß signaling (Nakamura et al., 2004; Jazag et al., 2005; Mizuguchi et al., 2005). This tumor-derived defense may have clinical values if TGF-ß-insensitive CD8$^+$ T cells were used for the treatment of TGF-ß-secreting tumors,

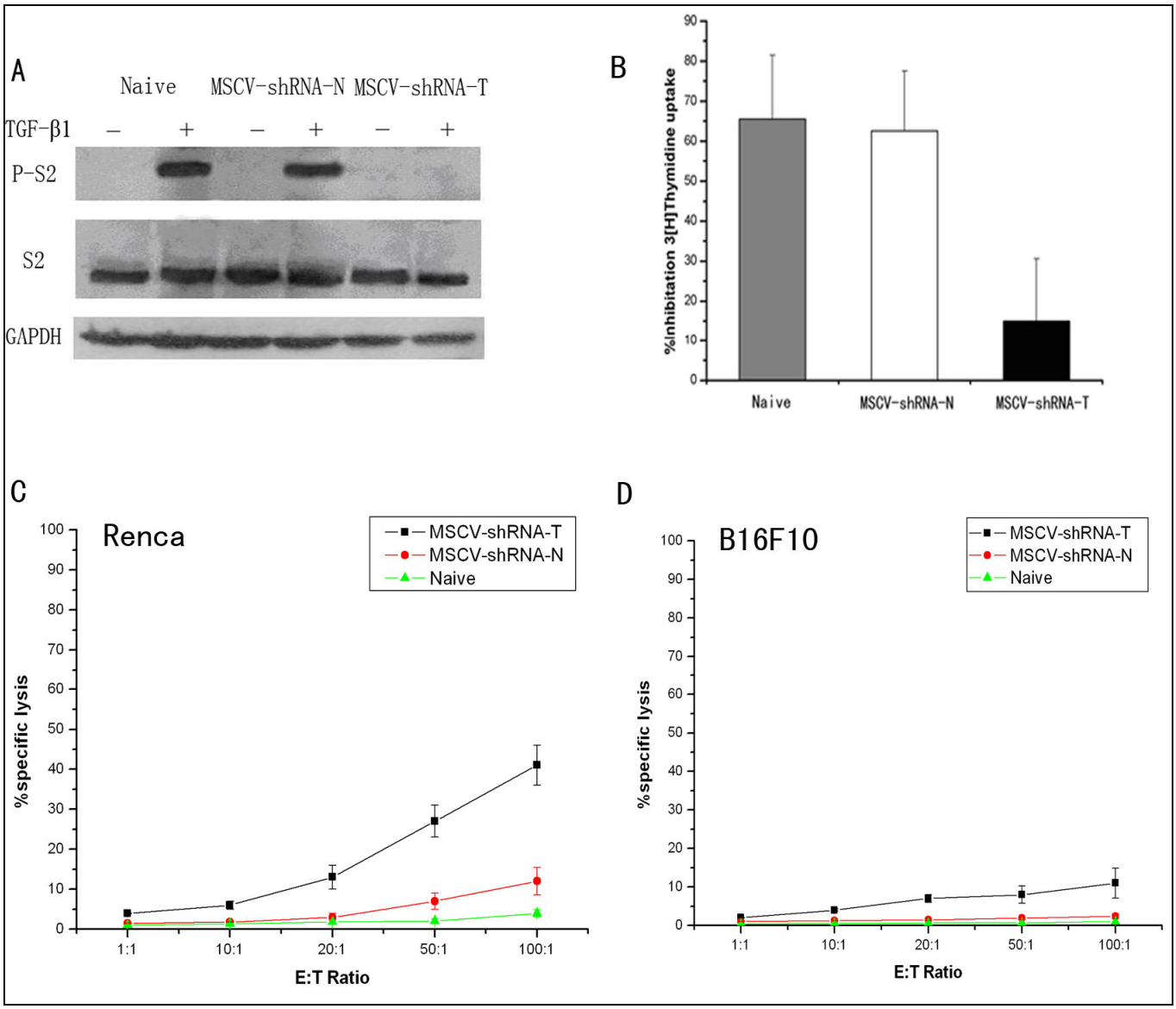

Figure 3. Western blot analysis for phosphorylated Smad2, thymidine incorporation assay for the proliferation of $CD8^+$ T Cells and *in vitro* cytotoxicity assay**. A:** All three types of $CD8^+$ T cells were treated with or without 10 ng/ml TGF-ß1. The presence or absence of Smad2 (S2) and phosphorylated Smad2 (P-S2) was detected by Western blot using anti-Smad2 and antiphospho-Smad2 antibodies, respectively. In contrast to the fact that phosphorylated Smad-2 was detected in the other two groups with TGF-ß1, we did not detect P-S2 in MSCV-shRNA-T-infected $CD8^+$ T cells after the addition of TGF-ß1. This confirmed that TGF-ß signal transduction was blocked by the presence of the shRNA to TßRII; B: proliferative responses of $CD8^+$ T cells were measured with the ^{3}[H] thymidine. TGF-ß1 showed a dramatic antiproliferative effect on the established MSCV-shRNA-N-infected $CD8^+$ T cells and naive $CD8^+$ T cells, whereas the mean inhibition of thymidine uptake by MSCV-shRNA-T-infected $CD8^+$ T cells was lower. It was statistically significant between the MSCV-shRNA-T-infected group and the other two control groups ($P < 0.05$). *In Vitro* cytotoxicity assay was also performed; C: Renca mouse-derived renal cancer cells were used as the targets. Tumor-reactive RNAi induced TGF-ß-insensitive $CD8^+$ T cells exhibited potent specific lysis against Renca cells compared with other two groups ($P < 0.05$); D: B16-F10 mouse-derived melanoma cells were used as targets. Both the TGF-ß-sensitive and TGF-ß-insensitive tumor-reactive $CD8^+$ T cells exhibited no apparent lysis ($P > 0.05$). Each point represents the mean of triplicate cultures. For thymidine incorporation assay, we had taken 3 wells of cultured cells as targets in every group. For *in vitro* cytotoxicity assay, at each effector/target ratio, 5 wells of cultured cells were targeted in every group. One representative experiment is shown.

including renal cancer (Knoefel et al., 1997; Wunderlich et al., 1997, 1998). Immunotherapy using adoptive transfer of immune cells is a promising approach for treating cancer patients. However, currently available therapies have not achieved a significant number of complete responders. A success-sful adoptive therapy for cancer should be the development of robust effector cells with specific anti-tumor efficacy. At the same time, the treatment

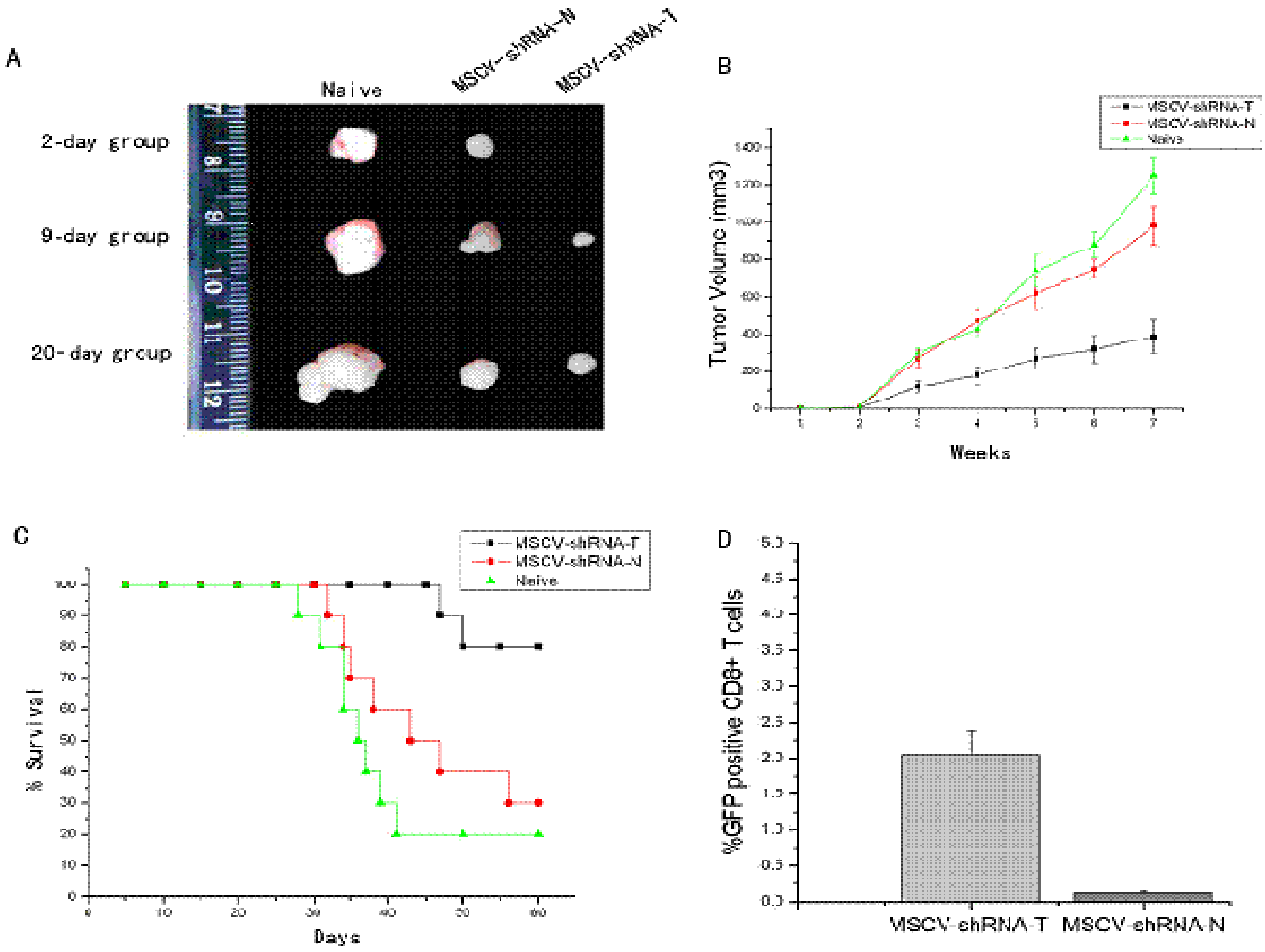

Figure 4. *In vivo* anti-tumor assays. Recipient mice received a single subcutaneous injection of Renca cells (5×10^5). At 2, 9, or 20 days following the tumor-burden formation, the adoptive transfer of $CD8^+$ T cells was carried out. Total animals (n = 10) were sacrificed 40 days after the adoptive transfer or sooner due to poor health conditions. A: Representative subcutaneous tumors from tumor-bearing mice 40 days after the administration of adoptive transfer; B: volume of tumors. MSCV-shRNA-T-treated mice in the 20-day group were found to have the smallest and lightest tumor burden, $P < 0.05$; C: Survival was defined as 'still be alive'. Kaplan-Meier survival curve of tumor-bearing mice received adoptive transfer of naïve $CD8^+$ T cells, MSCV-shRNA-N-infected $CD8^+$, and MSCV-shRNA-T-infected $CD8^+$ T cells. $P < 0.05$ according to the log-rank test for the MSCV-shRNA-T group versus the naive or MSCV-shRNA-N group. B and C show the results for the 20-day group; D: %GFP-positive $CD8^+$ T cells in the spleen of tumor-bearing animals (20-day group). A total of 2×10^6 $CD8^+$ T cells were tail-vein-injected into tumor-bearing BALB/c mice. %GFP-positive $CD8^+$ T cells in the spleen was analyzed by fluorescent-activated cell sorting. Adoptively transferred tumor-reactive RNAi-induced TGF-ß-insensitive $CD8^+$ T cells were detected with a percentage of 2.05 ± 0.31%, while in animals that received the MSCV-shRNA-N-infected tumor-reactive $CD8^+$ T cells or naïve $CD8^+$ T cells, $CD8^+$ T cells were detected only occasional with a percentage of 0.13 ± 0.02%, $P < 0.05$.

should overcome the tumor-derived immunosuppressive effect.

In our study, we found that the adoptive transfer of tumor-reactive RNAi-induced TGF-ß-insensitive $CD8^+$ T cells to BALB/c tumor-bearing mice could obviously increase the survival and suppress the growth of renca renal cancer. Furthermore, complete tumor regression occurred in the 2 day group of tumor-bearing mice. In contrast, the MSCV-shRNA-N-infected $CD8^+$ T cells or the naïve $CD8^+$ T cells did not show such therapeutic effects. We also found GFP-positive $CD8^+$ T cells isolated in sacrificed tumor-burdened mice. These results suggest that these tumor-reactive RNAi-induced TGF-ß-insensitive $CD8^+$ T cells may overcome the tumor-derived immunosuppressive effect to reduce tumor burden effectively and may be maintained in tumor-burdened mice for at least 40 days.

Inhibition of the TGF-ß signal transduction pathway in the MSCV-shRNA-T infected $CD8^+$ T cells was demonstrated by an absence of detectable phosphorylated Smad-2, as indicated by the Western blot, in the presence of TGF-ß1 and a resistance to the antiproliferative effects

of TGF-ß1, as found in ^{3}[H]thymidine incorporation assays. In the present study, the complete absence of phosphorylated Smad-2 in the MSCV-shRNA-T infected $CD8^+$ T cells and the 15% mean inhibition of thymidine uptake by these cells suggests the efficient blockade of the TGF-ß signaling pathway.

In our study, we chose mouse renca renal cancer to investigate this strategy because the tumor cells represent an aggressive line of malignant cells that secrete large amounts of TGF-ß (Knoefel et al., 1997; Wunderlich et al., 1997, 1998). The results of *in vitro* cytotoxic assay demonstrated that tumor-reactive RNAi-induced TGF-ß-insensitive $CD8^+$ T cells represent potent specific lysis against renca cells (Figure 3C). These cells showed a 4 fold greater tumor-killing activity than their TGF-ß-sensitive counterparts and a 20 fold greater activity over naïve $CD8^+$ T cells. Both TGF-ß-sensitive and TGF-ß-insensitive tumor-reactive $CD8^+$ T cells showed no apparent lysis when incubated with an irrelevant cell line, mouse B16-F10 melanoma cells (Figure 3D). Based on these results, we concluded that tumor-reactive RNAi-induced TGF-ß-insensitive $CD8^+$ T cells showed strong tumor-specific cytotoxic T lymphocyte responses and antitumor immunity against renca renal cancer. To our knowledge, the present study is the first report in which murine stem cell virus (MSCV)-induced RNAi to TGF-ß type II receptor was applied to evaluate adoptive transfer immunotherapy of tumor-reactive RNAi-induced TGF-ß-insensitive $CD8^+$ T cells to renca renal cancer. Though the infection efficacy of MSCV retrovirus *in vitro* was satisfied, the infection efficacy *in vivo* is still unknown. We still need more investigations to show the infection efficacy of MSCV retrovirus *in vivo*. Our present results showed that adoptive transfer with tumor-reactive RNAi-induced TGF-ß-insensitive $CD8^+$ T cells to tumor-bearing mice was able to increase survival and suppress growth of autologous tumors. These tumor-reactive RNAi-induced TGF-ß-insensitive $CD8^+$ T cells have two exclusive characteristics. One is that they are specifically reactive against tumor tissues and the other is that they are insensitive to TGF-ß. These two properties endowed these $CD8^+$ T cells with the ability of strong tumor-specific cytotoxic T lymphocyte responses and antitumor immunity against tumor cells. We found no evidence that even long-term expression of the shRNA to TßRII had any deleterious effects on the $CD8^+$ T cells. In particular, their phenotype and cytotoxic specificity were unmodified. Our preliminary observations seem to suggest an apparent absence of the development of autoimmune disease in these animals. Further studies are required to verify this conjecture.

In summary, this present study demonstrated a potential therapeutic approach to eliminate immunosup-pressive tumor-derived factors and improve the effectiveness of $CD8^+$ T cells. Based on these results, we predict that adoptive transfer of tumor-reactive RNAi-induced TGF-ß-insensitive $CD8^+$ T cells may be effective in renal cancer therapy.

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Abbreviations

CM, Complete medium; **CTL,** cytotoxic thymus lymphocyte; **FBS,** fetal bovine serum; **GFP,** green fluorescent protein; **TGF-ß,** transforming growth factor beta; **Renca,** mouse-derived renal cancer; **TßRI,** type I TGF-ß receptor; **TßRII,** type II TGF-ß receptor; **TßRIII,** type III TGF-ß receptor; **RNAi,** RNA interference; **GAPDH,** glyceraldehyde-3-phosphate-dehydrogenase; **MHC,** major histocompatibility complex; **MSCV,** murine stem cell virus; **DMEM,** dulbecco's modification of eagle's medium; **RT-PCR,** reverse transcription-polymerase chain reaction.

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