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Immunotherapy for Human Renal Cell Carcinoma by Adoptive Transfer of Autologous Transforming Growth Factor β–Insensitive CD8+ T Cells

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Abstract

Purpose: Transforming growth factor-β (TGF-β) is a potent immunosuppressor that has been associated with tumor evasion from the host immune surveillance and, thus, tumor progression. We tested a novel immunotherapy for human renal cell cancer (RCC) using a technique that involves the adoptive transfer of autologous tumor–reactive, TGF-β–insensitive CD8+ T cells into human RCC–challenged immunodeficient mice to identify its potent antitumor responses.

Experimental Design: The present study was conducted using a one-to-one adoptive transfer strategy to treat tumor-bearing severe combined immunodeficient (SCID/beige) mouse. The SCID/beige mice were humanized with peripheral blood mononuclear cells from patients with RCC (Hu-PBMC-SCID) before adoptive transfer. Autologous CD8+ T cells were expanded *ex vivo* using autologous patient's dendritic cells pulsed with the tumor lysate and rendered TGF-β insensitive by dominant-negative TGF-β type II receptor. In addition, human RCC cell lines were generated using patients' tumor cells injected into SCID/beige mice.

Results: Using flow cytometry analysis, we confirmed the expression of the tumor-reactive, TGF-β–insensitive CD8+ T cells were the effector CD8+ cells (CD27−CDRA+). Adoptive transfer of autologous TGF-β–insensitive CD8+ T cells into tumor-bearing Hu-PBMC-SCID mice induced robust tumor-specific CTL responses *in vitro*, were associated with tumor apoptosis, suppressed lung metastasis, and prolonged survival times *in vivo*.

Conclusion: The one-to-one adoptive transfer strategy is an ideal *in vivo* murine model for studying the relationship between TGF-β and immunosurveillance in RCC *in vivo*. Furthermore, this technique may offer the promise of a novel therapeutic option for the treatment of human patients with RCC. *Clin Cancer Res; 16(1); 164–73. ©2010 AACR.*

Renal cell carcinoma (RCC) is the most common solid tumor of the kidney in adults. It has previously been reported that the overproduction of TGF-β by RCC cells may lead to tumor evasion from the host immune surveillance and subsequent tumor progression (1–4). Similarly, inhibition of transforming growth factor-βTGF-β signaling using a dominant-negative TGF-β type II receptor construct (TβRIIDN) generates an immune response capable of eradicating tumors in mice challenged with live tumor cells (5).

We have previously shown that murine CD8+ T cells that are rendered insensitive to TGF-β could activate the antitumor immune response cycle in prostate cancer and subsequently eradicate lung metastases (6, 7). Moreover, inhibition of TGF-β signaling in DC also enhanced the efficacy of DC-based vaccines (8, 9). Although such TGF-β inhibition strategies are promising therapeutic approaches,

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Translational Relevance

In this preclinic exploratory study, we describe a novel method based on blockade of transforming growth factor-β signaling in autologous human CD8$^+$ T cells to treat the human renal cell cancer (RCC) in an immunodeficient mouse. Our findings indicate that this adoptive transfer strategy is an ideal *in vivo* murine model for studying the relationship between transforming growth factor-β and immunosurveillance in RCC *in vivo*. Furthermore, this technique may offer the promise of a novel therapeutic option for the treatment of human patients with RCC.

little is known about its effects on human malignancies *in vivo*. Currently, the safety, efficacy, and complexity of immunomodulation humans are major concerns that inhibit the clinical application of TGF-β blocking strategies. Therefore, a preclinical model that uses and analyzes human tumor–reactive TGF-β–insensitive CD8$^+$ T cells and their effect on human RCC is needed.

The severe combined immunodeficient (SCID) mouse has been shown to be an ideal host for studying patient or healthy donor immune system responses. Since Moiser et al. (10) first reported reconstitution of SCID mice with human peripheral blood mononuclear cells (Hu-PBMC), this approach has been widely applied in the fields of cancer biology, autoimmunity, allergy, infections, and transplantation biology. Bonnet et al. (11) reported that CD8$^+$ CTL clones specific for minor histocompatibility antigen could inhibit the engraftment of human acute myeloid leukemia cells in immunodeficient nonobese/SCID (NOD/SCID) mice. In another study, researchers showed that i.v. administration of *in vitro* generated donor-derived CTLs into NOD/SCID mice could eradicate human acute lymphoblastic leukemia (12). Recently, researchers have shown that infusion of PBMCs obtained from patients with chronic lymphocytic leukemia into NOD/SCID mice could mimic many clinical characteristics of the disease (13). Given the success of these adoptive immunotherapy models for studying hematologic malignancies, it is possible that similar techniques may be developed to study solid tumors such as RCC.

In the present study, we isolated tumor-reactive CD8$^+$ T cells from RCC patients, activated them with autologous immature dendritic cells (DC) and tumor lysate, rendered them insensitive to TGF-β by infection with a TβRIIDN retrovirus, and subsequently tested their antitumor responses both *in vitro* and *in vivo*. By using the SCID mouse model humanized with PBMC from RCC patients (hu-PBMC-SCID), we were able to transfer the autologous TGF-β–insensitive CD8$^+$ T cells into RCC tumor–bearing animals without inducing severe graft versus host disease (GVHD). Our results show that these autologous TGF-β–insensitive CD8$^+$ T cells exhibit antitumor activity, decrease tumor burden, and suppress pulmonary metastases.

Materials and Methods

Patients and cell lines. Specimens were obtained from 125 patients who were diagnosed with RCC and underwent radical nephrectomy between September 2005 and December 2008 in Xijing Hospital. The study protocol was approved by the Ethics Committee of the Xijing Hospital, Fourth Military Medical University. Informed consent was obtained from all participants. A total of 125 fresh RCC patient tissues were obtained at the time of surgery and were subsequently implanted s.c. into nude mice as described previously (14). Ten different RCC specimens remained engrafted successfully 1 mo after transplantation. Histologic sections from the original patient tumors as well as from xenograft specimens were obtained and subjected to H&E analysis. Cells from the 10 different RCC xenografts were subsequently isolated, grown in culture, and passed over 100 times. These RCC cell lines were maintained in complete medium containing RPMI 1640 (HyClone) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 2 mmol/L L-glutamine, 50 μmmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma).

Immunohistochemical and immunofluorescence analysis for TGF-β1 expression in RCC tumor tissues and cell lines. Immunohistochemical analysis was done to assess TGF-β expression levels in both the surgical patient specimens as well as the 10 different RCC cell lines. To this end, paraffin-embedded sections (4 μm) obtained from either the surgical specimens or xenografts were deparaffinized and rehydrated. After quenching endogenous peroxidase and blocking with serum, tissue sections were incubated with anti–TGF-β1 monoclonal antibody (1:100 dilution; ab27969, Abcam Biotechnology). Sections were subsequently washed with PBS and then incubated with biotinylated goat-anti-mouse secondary antibody (1:500; Abcam Biotechnology). Peroxidase substrate solution containing 3,3′-diaminobenzidine was used for direct staining. Counter-staining was done with 10% hematoxylin. Nonimmune murine antibody was used as a negative control. For immunofluorescence analysis, cells were incubated with TGF-β1 monoclonal antibody for 2 h, and then immunostained with FITC-conjugated anti-mouse IgG (1:1,000, Abcam Biotechnology) for 1 h. Cellular nuclei were identified using 100 ng/mL DAPI and all cells were subsequently examined by fluorescence microscopy (Nikon Corp.).

Establishment of the Hu-PBMC-SCID mice model. To efficiently transfer CD8$^+$ T cells into RCC tumor–bearing animals without inducing severe GVHD, SCID mice were first humanized with PBMCs obtained from human patients with RCC. To this end, male or female SCID/beige mice age 6 to 8 wk were obtained from the Laboratory Animal Research Center of the Fourth Military Medical University and housed in sterile filter-top cage placed in a laminar backflow cabinet under specific pathogen-free conditions. The SCID-beige mice were divided into 10 different groups. Each group was injected with PBMCs that were derived from 1 of the 10 different patients that were

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used for generation of the RCC cell lines. Autologous PBMCs were purified from each patient's blood using a Ficoll-HyPaque (Pharmacia) gradient after platelet depletion and washing as previously described (8). One day before PBMCs injection, mice were sublethally irradiated with 3.5 Gy [(60Co) source Gammatron F 80S, Simens]. Each mouse received 0.3 mL of the PBMCs (2×10^7 cells) suspended in PBS through i.p. injection.

ELISA determination of TGF-β levels in RCC cell lines and human immunoglobulin IgG levels in SCID-beige mice. The pooled conditioned medium was collected and concentrated by using YM-3 Centriprep Centrifugal Filter Devices (Millipore). TGF-β1 ELISA was carried out using the Quantikine Human TGF-β1 Immunoassay kit from R&D Systems (Minneapolis). The total number of cells in each flask was counted using a Coulter Counter and levels of TGF-β1 were reported as pg/1×10^5 cells/48 h. Human prostate cancer cell line PC-3 (American Type Culture Collection) was used as a control.

Four weeks after PBMCs injection into SCID-beige mice, 100 μL of tail vein blood were obtained and a sandwich ELISA was done to quantify serum human IgG as previously described (15). Briefly, microculture plates were coated with affinity-purified goat anti-human IgG (Abcam Biotechnology). Affinity-purified alkaline phosphatase-conjugated goat anti-human IgG (Abcam Biotechnology) was used to detect human IgG. The absorbance at 405 nm was quantified on an ELISA reader.

Generation of patient autologous tumor–reactive TGF-β–insensitive CD8⁺ T cell. With the use of CD8⁺ Microbeads (Miltenyi Biotec), patient's CD8⁺ T cells were positively selected from whole blood with a purity of >98%. CD8⁺ T cells was expanded with using autologous patient's DCs pulsed with the tumor lysate in the presence of recombinant human interleukin-2 (500 U/mL; PeproTech) as previously described (8).

There were two types of CD8⁺ T cells used for experimentation: (*a*) tumor-reactive TGF-β–insensitive CD8⁺ T cells that were rendered insensitive to TGF-β by infection using a retrovirus containing TβRIIDN-green fluorescent protein (GFP)7 and (*b*) naive CD8⁺ T cells isolated from PBMC (controls). The efficiency of infection of TβRIIDN was 85% (green fluorescent protein–positive cells: total cells) under fluorescence microscopy (Nikon Corp.; Supplementary Fig. S1A). Under the treatment of TGF-β (10 ng/mL for 16 h), phosphorylation of Smad-2 was observed in naive CD8⁺ T cells but not in tumor-reactive CD8⁺ T cells infected with the TβRIIDN. This result indicates that these TβRIIDN-infected CD8⁺ T cells were insensitive to TGF-β (Supplementary Fig. S1B).

Flow cytometric analysis: TGF-β–insensitive CD8⁺ T cell characterization. Immunophenotypical characterization of TGF-β–insensitive CD8⁺ T cell and naive CD8⁺ T cell were done using FITC-conjugated anti-CD27 monoclonal antibody (Cell Signaling) and anti-CD45RA monoclonal antibody (Cell Signaling) before administration to the mice. Cells were stained with monoclonal antibody in PBS, 0.2% bovine serum albumin, and 50 μmol/L EDTA for 20 min at 4°C and either directly analyzed or sorted into defined populations on a FACSVantae SE, using CellQuest software (BD Bioscience; ref. 16).

^{51}Chromium release assays. The two types of CD8⁺ T cells (TGF-β–insensitive CD8⁺ and naive CD8⁺ T cells) were subjected to a standard ^{51}Chromium release (^{51}Cr) assay as previously described (6). The 10 different RCC cell lines and, as a negative control, the human PC-3 prostate carcinoma cell line were used as targets. Target cells were labeled with 100 μ Ci ^{51}Cr/10^5 cells. Different groups of CD8⁺ T cells were added to U-bottomed plates containing 5,000 cells per well with various E/T ratios ranging from 1:1 to 100:1. Equal volumes of RPMI 1640 and 1 mol/L HCl were added to other wells as the negative and positive controls, respectively. After a 4-h incubation, 100 μL of supernatants was harvested from each well and the ^{51}Cr released was measured using a γ counter. The percent cell lysis was calculated according to the formula as previously described (6): percent-specific ^{51}Cr release = (experimental release − spontaneous release) × 100/(maximum release − spontaneous release)].

Adoptive transfer of TGF-β–insensitive CD8⁺ T cell in tumor-bearing Hu-PBMC-SCID mice. There are 30 Hu-PBMC-SCID mice that received an injection in the right flank using 5×10^6 of patient's autologous RCC tumor cell line (day 0). Tumors were found in all these mice at approximately 2 to 3 mm in diameter and were palpable 14 d later. On day 14, adoptive transfer with patient's autologous CD8⁺ T cells was done in the tumor-bearing Hu-PBMC-SCID mice. Three groups (10 mice per group) received i.p. injection with different types of adoptive transfer composed of either (*a*) TGF-β–insensitive CD8⁺ T cells (1×10^7), (*b*) naive CD8⁺ T cells (1×10^7), or (*c*) PBS (0.5 mL). The adoptive transfer methodology was repeated on day 21. Tumor growth and animal survival was monitored daily after vaccination.

To evaluate the effects of TGF-β on cellular metastasis, the pulmonary metastasis model was done as previously described (6). Briefly, a single injection of 5×10^6 RCC cells were injected through the tail vein. On day 7, the tumor-bearing mice (5 mice per group) were inoculated with either TGF-β–insensitive or naive CD8⁺ T cells (1×10^7 cells) or PBS through i.p. injection. Forty days after adoptive transfer, all mice were sacrificed and the tumors were isolated for evaluation of the volume (volume = length × width2 × π/6), weight, and histologic analysis. Lung tissues were also harvested.

ELISA assay for INF-γ. The sera of the mice were obtained using tail vein blood. Because IFN-γ is commonly used as a measure of T-cell activation, serum levels of INF-γ were determined using an ELISA kit (R&D Systems) according to an established protocol. Serum samples were stored at −70°C until the assay.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining for tumor apoptosis. Paraffin-embedded tumor sections (obtained from mice that underwent adoptive transfer) were used for apoptosis assays. The nuclear and terminal deoxynucleotidyl

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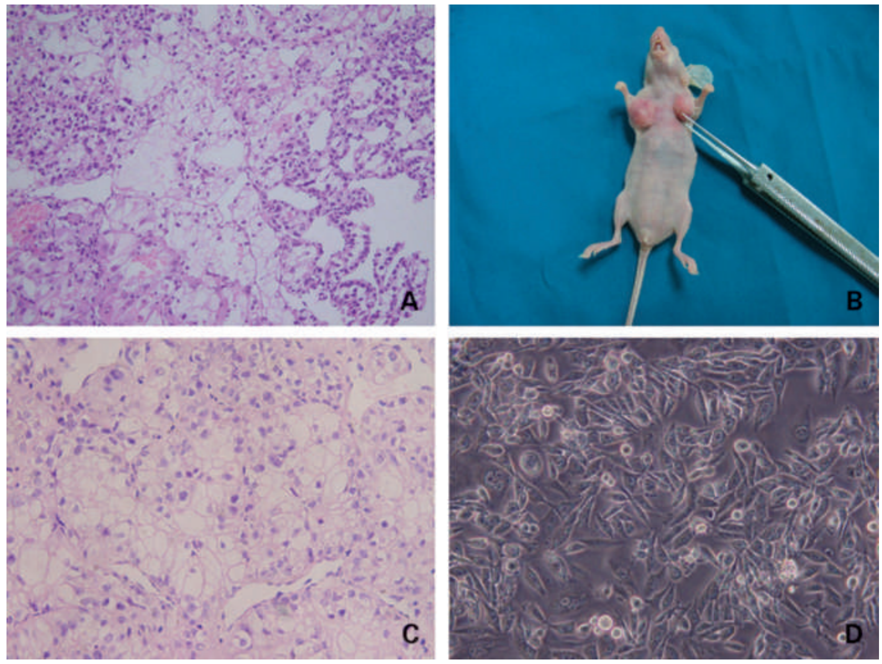

Fig. 1. Histologic and morphologic analysis of RCC *in vivo* and *in vitro*. *A,* original RCC tumor specimen, obtained from patients and confirmed by H&E analysis. *B,* analysis of patient RCC tumor tissues engrafted in SCID/beige mice. *C,* H&E analysis of the xenograft tissues in mice show similar morphologic and histologic patterns compared with the original tumor tissues. *D,* phase images of 1 of the 10 different immortalized cultured RCC cell lines. (magnification: *A* and *C,* ×40; *B,* ×20).

transferase-mediated dUTP nick end labeling (TUNEL; R & D System) apoptosis assays were done as described previously. The intensity of the fluorescent signal was standardized by the standard fluorescent index (positive lymphocytes or signal/100 tumor cells/1,000 μm^2) as follows: –, <5; ±, 6 to 10; +, 11 to 30; ++, 31 to 50; +++, 51 to 70; ++++, >70 as described previously (7).

Statistical analysis. Numerical data were expressed as mean ± SD. ANOVA and χ^2 tests were done to determine the differences in the means among the various treatment groups. $P < 0.05$ was considered statistically significant. The SPSS 10.0.2 software package (SPSS, Inc.) was used for analysis. The Kaplan-Meier survival curve was analyzed by the log-rank test with the Graphpad Prism 5.0 software (Graphpad Software, Inc.).

Results

Establishment and characterization of RCC cell lines. Tumors obtained from human patients with RCC were injected into nude mice and used to establish 10 different RCC cell lines. All RCC cell lines were stably cultured and were able to withstand repeated cryopreservation and thawing. Histologic analysis of xenografts obtained from mice was similar to the histologic evaluation of the original patient tissue specimen (Supplementary Table S1; Fig. 1). In addition, cultured cells appeared to retain many of the morphologic properties observed *in vivo*.

TGF-β1 expression in RCC xenograft in mice and cell lines. We next sought to examine the expression patterns of TGF-β1 in RCC xenografts as well as the 10 immortalized human RCC cell lines. Immunohistochemical analysis of RCC xenografts obtained from SCID-beige mice shows strong expression of TGF-β1 throughout the cytoplasm and cellular membranes of neoplastic cells (Fig. 2A and B). Similar staining patterns were observed by immunofluorescence staining of RCC cell lines (Fig. 2C and D). This result is consistent with ELISA analysis (Supplementary Fig. S2).

Establishment and analysis of Hu-PBMC-SCID mice. To develop an adoptive transfer murine model to study the effects of TGF-β1 inhibition on RCC tumor progression, SCID mice were first humanized with PBMCs obtained from 10 different human patients with RCC. Of note, PBMCs were obtained from the same patients whose tumors were used to establish the RCC cell lines. Four weeks after PBMCs were injected into SCID mice, human immunoglobulins could be detected in 300 of 440 (68.2%) Hu-PBMC-SCID mice sera. The IgG levels of each group averaged between 0.8 and 2.2 mg/mL, which are in agreement with results of previous studies (15). There were no significant differences in the success rate of PBMC engraftment and the levels of IgG were similar between each group of mice ($P > 0.05$; Fig. 3A). Furthermore, we found no evidence of severe xenogenic GVHD.

Phenotypic analysis of TGF-β–insensitive and naive $CD8^+$ T cells. To analyze the effects of TGF-β on immune surveillance of RCC, $CD8^+$ T cells were isolated using the 10 patients' PBMCs. Some of these $CD8^+$ cells were infected with a retroviral construct containing a dominant negative TGβRII-green fluorescent protein construct. Next, the phenotypes of both the TGF-β1–insentsitive and naïve $CD8^+$

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T-cell lines were characterized. To this end, flow cytometry analysis showed that the expression of the costimulatory molecules CD27 and CD45RA were different in the two $CD8^+$ T-cell groups. In the TGF-β–insensitive $CD8^+$ T cells, the dominant phenotype was the $CD27^+CD45RA^-$, which is phenotype of effector $CD8^+$ T cells. However, most of naive $CD8^+$ T cells were identified as $CD27^+CD45RA^+$, which is consistent with unprimed $CD8^+$ T cells (Supplementary Table 2; Fig. 3B and C).

***TGF-β–insensitive CD8$^+$ T cells show significant antitumor responses* in vitro *and* in vivo.** The specific tumor-killing ability of the autologous TGF-β–insensitive $CD8^+$ T cells (described above) was first examined using an *in vitro* CTL assay. To this end, TGF-β–insensitive $CD8^+$ T cells exhibited a 5-fold higher tumor-killing activity compared with naïve $CD8^+$ T cells [75.5% versus 15.8% at an effector/target (E/T) cell ratio of 100:1; Fig. 4A]. No apparent lytic activity was observed when the assay was done using the PC-3–negative control cell line. (Fig. 4B).

In vivo assays further confirmed the antitumor abilities of TGF-β–insensitive $CD8^+$ T cells. Adoptive transfer of either TGF-β–insensitive $CD8^+$ T cells or naïve $CD8^+$ T cells into Hu-PMBC-SCID mice inoculated with RCC tumors from the same human patients (see Materials and Methods) were used in this regard. Forty days after adoptive transfer, mice injected with TGF-β–insensitive $CD8^+$ T cells exhibited average tumor volumes and tumor weights that were significantly decreased compared with mice injected with either naive $CD8^+$ T cells or PBS ($P < 0.05$; Fig. 4C and D). Taken together, TGF-β–insensitive $CD8^+$ T cells decreased tumor growth and were therefore associated with antitumor activity *in vivo*.

Finally, it was of interest to determine whether inhibition of TGF-β in $CD8^+$ T cells could inhibit cellular metastasis of RCC. Therefore, each one of the 10 different RCC cell lines was independently injected into Hu-PMBC-SCID mice tail veins. Seven days after tail vein injection, mice were inoculated with either TGF-β–insensitive or naive

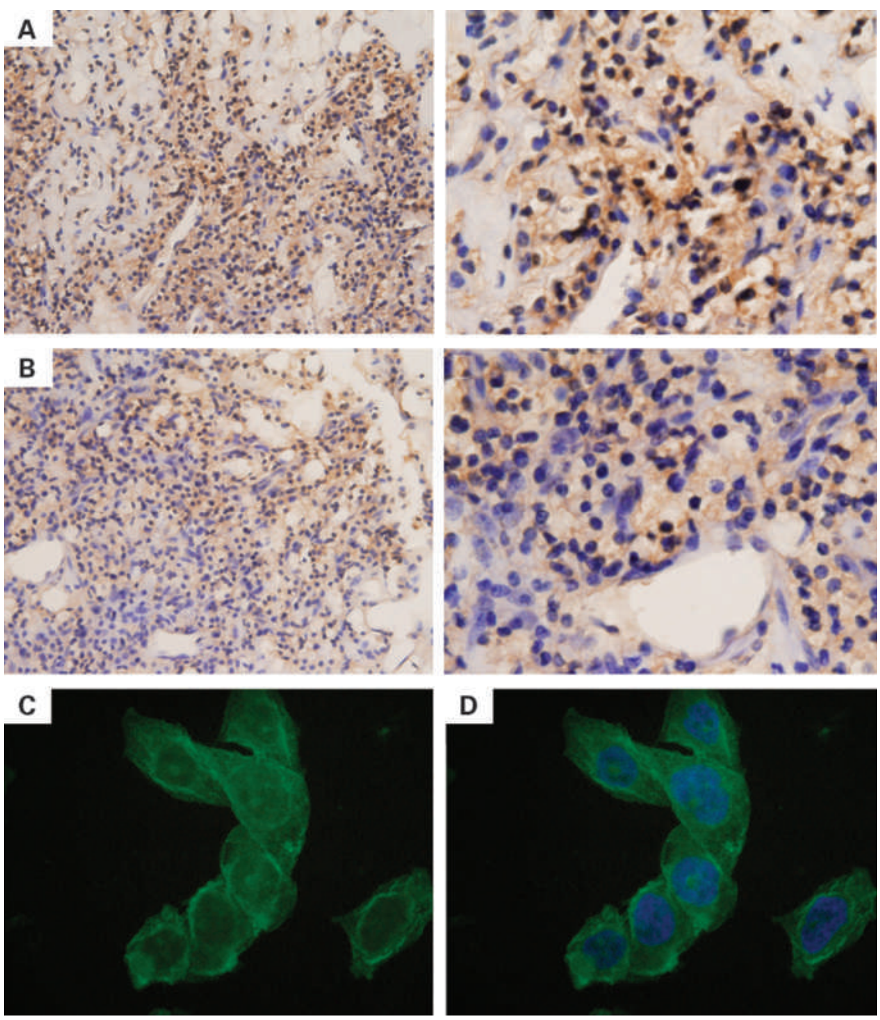

Fig. 2. TGF-β1 expression in RCC tumor tissues and cell lines. *A* to *D*, immunohistochemical expression of TGF-β1 in RCC tumor tissues. *A*, TGF-β1 expression was observed throughout the cytoplasm of neoplastic cells in histologic sections taken from surgical specimens. *B*, TGFβ-1 expression in the xenograft was similar to that of original RCC tissues in RCC xenograft in nude mice. *C* to *D*, immunofluorescence analysis of TGF-β1 expression in RCC cell lines show staining throughout the cytoplasm. This staining is similar to our previous results (7). *C*, TGF-β1 expression was observed throughout the cytoplasm of tumor cells in culture (FITC; *green*). *D*, merged image of TGF-β1 expression in the RCC cell line. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (*blue*; magnification: *A* and *B*, *left*, ×20; *right*, ×40; *C* and *D*, ×100).

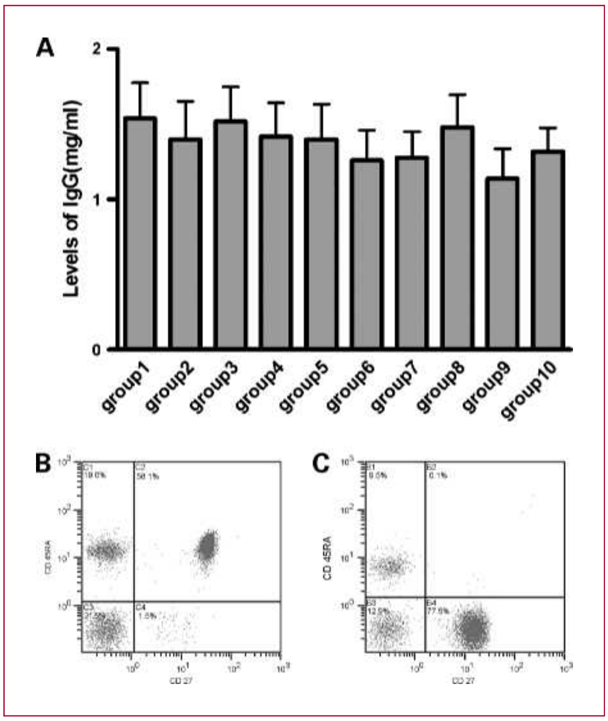

Fig. 3. The human immunoglobulin IgG levels in the hu-PBMC-SCID mice and CD27/CD45RA phenotype of autologous patient's CD8+ T cells. *A*, an ELISA assay was done to quantify serum human IgG in the 10 different hu-PBMC-SCID mice. No significant differences were observed between any two groups of mice ($P > 0.05$). *B*, naive CD8$^+$ T cells and TGF-β–insensitive CD8$^+$-T cells were costained with FITC-conjugated anti-CD27 and anti-CD45RA antibodies. Four distinct populations could be defined for CD8$^+$-T cells obtained from the 10 patients used in this study: (*a*) CD27$^+$CD45RA$^+$ cells, (*b*) CD27$^+$CD45RA$^-$ cells, (*c*) CD27$^-$CD45RA$^+$ cells, and (*d*) CD27$^-$CD45RA$^-$ cells; the dominant phenotype in TGF-β–insensitive CD8$^+$-T cells was CD27$^+$CD45RA$^-$, the effector phenotype. *C*, in contrast, the dominant phenotype of naive CD8$^+$-T cells was CD27$^+$CD45RA$^+$, the unprimed CD8$^+$-T-cell phenotype.

CD8$^+$ T cells or with PBS as a control. Interestingly, all the animals died in the PBS-treated group before day 30, 80% of mice died in the naïve CD8$^+$ T–treated group before day 32 of the experiment due to poor health conditions, whereas all the mice survived in the TGF-β–insensitive CD8$^+$ T cell–treated group at the end of the experiment (Fig. 5A). According to the long-rank test, there were significant differences among three groups ($P < 0.05$; Fig. 5B).

TGF-β–insensitive CD8$^+$ T cells induced high serum levels of INF-γ. Activated CD8$^+$ T cells release INF-γ, and therefore, measurement of INF-γ levels can be used to determine the activity of T lymphocytes *in vivo*. Therefore, serum levels of INF-γ were determined in Hu-PMBC-SCID mice that were inoculated with RCC tumors and underwent adoptive transfer with either TGF-β–insensitive or naive CD8$^+$ T cells. The serum levels of INF-γ exhibited significant ($P < 0.01$) increases in mice with TGF-β–insensitive CD8$^+$ T cells, but not in mice treated with either naive CD8$^+$ T cells or PBS. (Fig. 5C). The increased levels of serum INF-γ observed in the TGF-β–insensitive CD8$^+$ T cell–treated group indicated that immune cells were more strongly activated in these hosts.

TGF-β–insensitive CD8$^+$ T-cell–induced tumor cell apoptosis. The above experiments suggest that TGF-β–insensitive CD8$^+$ T cells are associated with increased immunomodulatory functions *in vivo*, which result in tumor shrinkage and decreased metastases. To further confirm this increased activity, TUNEL assays were used to show that autologous TGF-β–insensitive CD8$^+$ T cells could induce tumor cell apoptosis in the TβRIIDN group (+++, 52 apoptosis signal/100 tumor cells/1,000 μm^2). However, almost no apoptotic cells was observed in the naive CD8$^+$ T cell (–, 2 apoptosis signal/100 tumor cells/1,000 μm^2) or PBS groups (–, 0 apoptosis signal/100 tumor cells/1,000 μm^2; Fig. 6).

Discussion

In this study, we used a one-to-one adoptive immunotherapy strategy to treat human RCC with patients' own CD8$^+$ T cells in a SCID mouse model. This preclinical study show that adoptive transfer of autologous TGF-β–insensitive CD8$^+$ T cells can induce tumor apoptosis that subsequently decreases tumor burden and suppress pulmonary metastases. These tumor-reactive, TGF-β–insensitive CD8$^+$ T cells show a strong antitumor ability both *in vitro* and *in vivo*.

Nude mice engrafted with RCC primary xenograft present advantages to study tumor biological behaviors. However, the success rate in established xenograft animal model is not always satisfactory due to the difference on histoincompatibility between human and animal (17). Here, we reported that the establishment of 10 RCC cell lines derived from human patient tumors. Only 10 of 125 patient tumors were able to be used in this regard (8.0% success rate), which is lower than that of other reports (18, 19). Because of this reason, we used the nude mice to establish the RCC cell lines, and then used the SCID/beige mice to assess tumor burden and immune cell reconstitution. We believe that this provides improved immunohistocompatibility compared with other methods that directly implant human tumor tissues directly into SCID mice.

Since the first report of a successful transfer of normal human immune cells to SCID mice, the reconstitution of a human immune system in mice for studying human immune reaction *in vivo* has been extensively studied (10). The SCID/beige mouse model has a combined defect of the T/B cell system and the innate immune system, and is therefore advantageous for studying human immune cell reconstitution (20). Berney et al. (21) reported that after i.p. injection, the engraftment of human PBMC in SCID/beige mouse is largely improved, and although human B cells are virtually absent from the circulation, they are present in lymphoid organs and significant levels of human IgG are found in the blood. Our experiments confirmed this finding without inducing severe GVHD.

The effectiveness of adoptive therapy as a treatment strategy may depend on generating and administering

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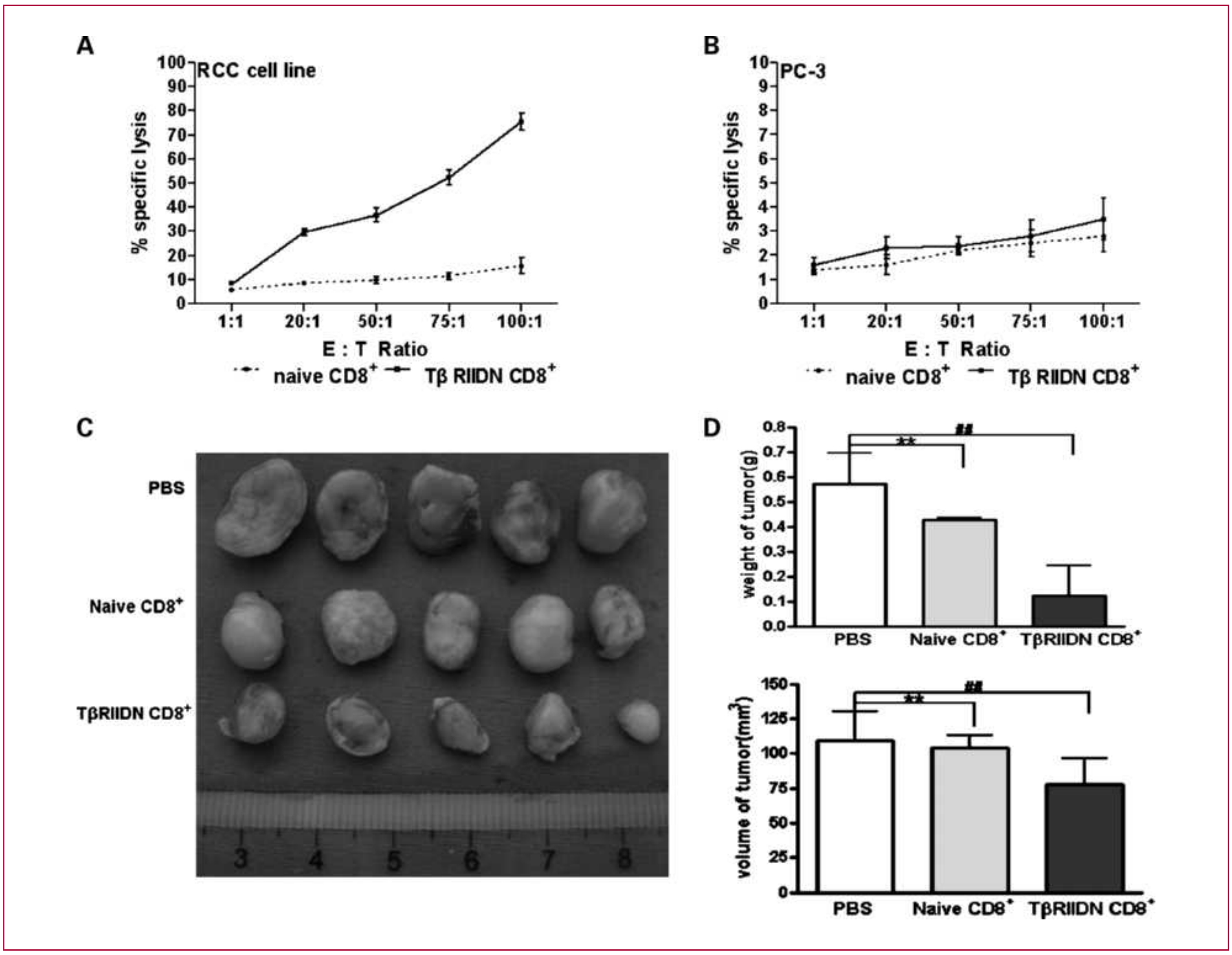

Fig. 4. TGF-β–insensitive CD8+ T cells exhibit antitumor activity both *in vitro* and *in vivo. A* and *B, in vitro* CTL assays. Naive CD8+ T cells and TβRIIDN CD8+ T cells were cocultured with ^{51}Cr-labeled targets at the specified E/T-ratios. *A,* RCC cell lines were used as targets. *B,* PC-3 human prostate cancer cells were used as targets as a negative control. The results suggest that the specific lysis induced by TβRIIDN CD8+ T cells was 5-fold higher compared with naive CD8+ T cells at a 100:1 E/T ratio. No lytic activities were observed against PC-3 cells by either type of CD8+ T cells. Adoptive transfer of naive CD8+ T cells and TβRIIDN CD8+ T cells in hu-PBMC-SCID mice. *C,* representative gross tumor features from tumor-bearing mice at 40 d following adoptive transfer in mice that underwent adoptive transfer with naive CD8+ T cells, TβRIIDN CD8+ T cells, or PBS (control). *D,* weight of the tumor in each group (*top*). The average tumor weight was significantly lower for the TβRIIDN group (0.124 ± 0.117 g) versus the naive CD8+ T-cell group (0.428 ± 0.077 g) or the PBS group (0.570 ± 0.122 g; $P = 0.001$ versus naive CD8+ group; $P < 0.0001$ versus PBS group). Bottom, the average volume of the tumor in each group. Compared with the naive CD8+ group (104.00 ± 9.03 mm^3) and the PBS group (109.00 ± 21.48 mm^3), the average tumor volume in the TβRIIDN group (96.80 ± 21.62 mm^3) was the smallest ($P = 0.001$ versus naive CD8+ group; $P < 0.0001$ versus PBS group).

antigen specific T cells in ways that mimic physiologic conditions to preserve normal function. For example, adoptive T-cell therapy using antigen-specific CD8+ T-cell clones for the treatment of patients with metastatic melanoma showed that the transferred T cells could persist *in vivo*, and show specific migration and antitumor effect (22). In this study, antigen-specific CD8+ T cells were generated *in vitro* by cyclical stimulation with autologous DCs plus tumor lysate and low-dose interleukin-2. Using this cocktail approach, we obtained large number of activated CD8+ T cells for further experiments. By infecting with a retrovirus containing TβRIIDN, this type of CD8+ T cells was rendered TGF-β insensitive. Phenotypic analysis showed that this type of CD8+ T cells were CD27+CD45RA−, which indicated the effector-type CTLs (16, 23–25). The CTL assay also confirmed its potent ability to kill target tumor cells *in vitro*.

Studies have shown that CD4+ T cells are required for the cytolytic activity of CD8+ T cells (5, 26, 27). Our previous studies have also shown that the adoptive transfer of tumor-reactive, TGF-β–insensitive CD8+ T cells alone were insufficient for an antitumor response unless they are supported by other immune cells (7). In this study, we established the Hu-PBMC-SCID mouse model instead of transferring TGF-β–insensitive CD8+ T cells directly to SCID mouse. Results of pathologic evaluation show that tumor-reactive TGF-β–insensitive CD8+ T cells persist in tumor-bearing hosts and subsequently reduce tumor burden. The circulating level of INF-γ, which a critical cytokine for antitumor activity in the host, is also elevated in TGF-β–insensitive CD8+ T cell–treated hosts (28). Furthermore, the TUNEL assay shows that patient's autologous TGF-β–insensitive CD8+ T cells could induce tumor tissue apoptosis. However, in naive CD8+ T cell–treated hosts, there is no significant tumor apoptosis found in tumor parenchyma.

Many studies have shown that blockade of TGF-β signal in cancer offers a promising immunotherapy strategy (29, 30). Results of our previous studies have also shown that adoptive transfer of murine-derived TGF-β–insensitive CD8+ T cells or DCs represent an efficient immunotherapeutic strategy in the treatment of murine prostate cancer (6–9). In the present study, using this one-to-one adoptive transfer strategy, we further confirm that tumor-reactive TGF-β–insensitive CD8+ T cells is sufficient for tumor rejection.

We did not observe the complete resolution of RCC tumors in the TGF-β–insensitive CD8+ T cell–treated mice. However, these TGF-β–insensitive CD8+ T cells were almost 100% effective in preventing pulmonary metastases.

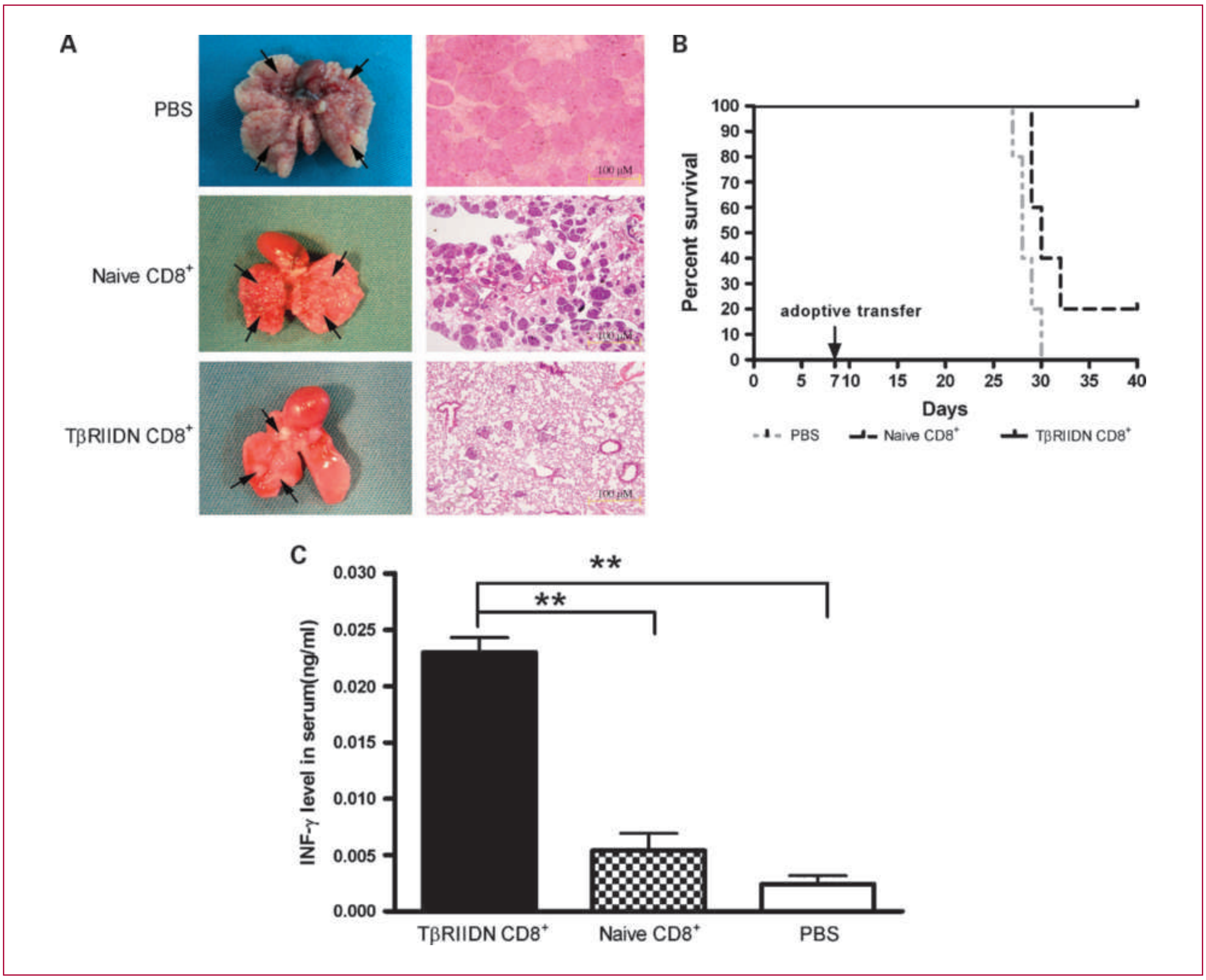

Fig. 5. *In vivo* analysis of RCC metastasis in TβRIIDN CD8+ T cell, naive CD8+ T cell, and PBS treatment groups. *A,* representative gross feature and H&E staining of lung metastasis from tumor-bearing mice following the three different treatments. *PBS group*: gross analysis of pulmonary tissues revealed innumerable metastases; H&E staining showed that many tumor nodules were merged and lung tissue suffered consolidation. These features made it almost impossible to differentiate between normal lung and tumor tissue. *Naive CD8+ group*: a large number of metastases were also observed in the lung tissues in this group; H&E staining showed tumor nodules that were merged, and only small parts of normal lung tissue could be easily differentiated. *TβRIIDN CD8+group*: very few metastatic tumors were visualized grossly; H&E staining confirmed the paucity of tumor nodules. Finally, the lung tissue retained normal physiologic features in this group. *B,* Kaplan-Meier survival curve of pulmonary metastatic tumor–bearing mice after different treatment regimens. Mice in the TβRIIDN CD8+ group were all alive at the end of the experiment (40 d). In contrast, all the mice in the PBS group died due to poor health conditions. Eighty percent of mice in the naive CD8+ group died. $P < 0.05$ according to the long-rank test for the TβRIIDN group compared with the naive CD8+ and PBS groups. *C,* compared with the naive CD8+ group (0.0054 ± 0.0034 ng/mL) and the PBS group (0.0024 ± 0.0017 ng/mL), there were significantly increased levels of INF-γ in the TβRIIDN group (0.023 ± 0.0029 ng/mL; $P < 0.001$ versus naive CD8+ group; $P < 0.001$ versus PBS group). **, $P < 0.001$. Columns, mean; bars, SD.

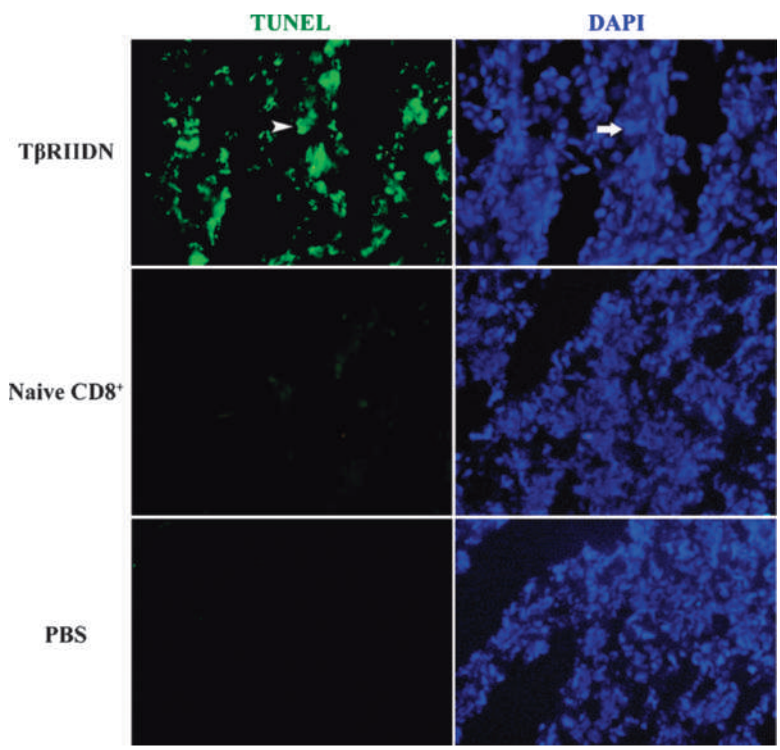

Fig. 6. Analysis of apoptosis in the three different treatment groups. A TUNEL assay was used to assess for apoptotic cells. The intensity of the fluorescent signal was standardized by the standard fluorescent index (positive lymphocytes or signal/100 tumor cells/1,000 μm^2) as follows: −, <5; ±, 6 to 10; +, 11 to 30; ++, 31 to 50; +++, 51 to 70; ++++, >70. Representative tumor tissue sections from different treated group were simultaneously stained for cell nucleus (*blue*) and apoptosis (*green*). Frequent tumor apoptotic sites (*green*) were only found in the TβRIIDN group (+++, 52 apoptosis signal/100 tumor cells/1,000 μm^2); no apparent apoptotic cells were observed in the other two groups.

The exact mechanisms of these antitumor effects are currently unknown and should be further explored.

It has been shown that the dominant negative TGF-β receptor type II is an effective method to inhibit TGF-β signaling (30). This technique is effective in our murine model and is associated with RCC tumor apoptosis and inhibition of metastasis. However, before its clinical application, the safety should be first considered. Although we did not observe any toxic reactions in humanized animal during the experiment period, to gain full appraisal of the safety and efficiency, more clinical trials and further experiments should be done.

The present translational project proposes the following new potential advances when compared with our previous studies and other studies in the same field. For example, this new systemic immunotherapy based on adoptive transfer of autotrophic CD8$^+$ T cells for both localized and metastatic models in immunodeficient mice mimic similar human RCC disease. To the best of our knowledge, these novel approaches have yet to be reported. These findings will hopefully establish the groundwork for additional clinical research in this field. Specifically, the success of this project will establish a preclinical foundation for the treatment of RCC recurrence and metastasis after surgical treatment. For example, we envision that in the future, it may be possible to preserve naive CD8$^+$ T cells after surgical operation and render them insensitive to TGF-β before adoptively transferring these specific CD8$^+$ T cells back to the same patient for immunotherapy.

In summary, results of the present study showed that the adoptive transfer of patient's autologous TGF-β–insensitive CD8$^+$ T cells to RCC-bearing humanized SCID mouse could decrease tumor burden, suppress pulmonary metastasis, prolong survival time, and show the superior antitumor responses *in vitro* and *in vivo*. This approach may lead to the highly effective treatment for patients with recurrence or metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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