Blockade of transforming growth factor-β signaling in tumor-reactive CD8+ T cells activates the antitumor immune response cycle

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Abstract

Transforming growth factor-β (TGF-β) is a potent immunosuppressant. Overproduction of TGF-β by tumor cells leads to evasion of host immune surveillance and tumor progression. Results of our early studies showed that adoptive transfer of tumor-reactive, TGF-β-insensitive CD8+ T cells into immunocompetent mice was able to eradicate lung metastasis of mouse prostate cancer. The present study was conducted with three objectives. (a) We tested if this technology could be applied to the treatment of solid xenograft tumors in allogeneic immunodeficient hosts. (b) We determined relevant variables in the tumor microenvironment with the treatment. (c) We tested if immune cells other than CD8+ T cells were required for the antitumor effect. Mouse prostate cancer cells, TRAMP-C2 of the C57BL/6 strain, grown in immunodeficient allogeneic hosts of BALB/c, were used as a xenograft model. Tumor-reactive CD8+ T cells from C57BL/6 mice were isolated, expanded ex vivo, and rendered insensitive to TGF-β by introducing a dominant-negative TGF-β type II receptor vector. Seven days following s.c. injection of TRAMP-C2 cells (5 × 105) into the flank of male BALB/c-Rag1–/– mice, tumor-reactive, TGF-β-insensitive CD8+ T cells (1.5 × 106) were transferred with and without the cotransfer of an equal number of CD8-depleted splenocytes from C57BL/6 donors. Naïve CD8+ T cells or green fluorescent protein-empty vector–transfected tumor-reactive CD8+ T cells were transferred as controls. Forty days following the transfer, the average tumor weight in animals that received cotransfer of tumor-reactive, TGF-β-insensitive CD8+ T cells and CD8-depleted splenocytes was at least 50% less than that in animals of all other groups (P < 0.05). Tumors in animals of the former group showed a massive infiltration of CD8+ T cells. This was associated with secretion of relevant cytokines, decreased tumor proliferation, reduced angiogenesis, and increased tumor apoptosis. Based on these results, we postulated a concept of antitumor immune response cycle in tumor immunology. [Mol Cancer Ther 2006;5(7):1733-43]

Introduction

Tumor immunology is characterized by an insufficient immune surveillance, as most tumors are able to evade the immune surveillance program of the host. Despite the ability of generating the reactivity of immune cells against tumor antigens, the immune surveillance program of the host can be overpowered by tumors with an eventual tumor progression (1). This is because tumor cells have acquired many mechanisms to evade the immune surveillance program of the host (2, 3). One of such possibilities has been the tumor-derived transforming growth factor-β (TGF-β), which is highly immunosuppressive (3–6). Most tumors secrete large amounts of TGF-β (7–9). TGF-β-producing tumor cells fail to elicit primary CTL responses despite retaining class I MHC expression molecules and tumor-specific antigens (4). Priming of T cells by dendritic cells or tumor cells can also be negatively influenced by TGF-β (10). Therefore, an ideal approach to activate the antitumor response will be to render the immune cells of the host insensitive to TGF-β.

In 2001, Gorelik and Flavell disrupted TGF-β signaling in CD4+ and CD8+ T cells through the transgenic expression of a truncated dominant-negative TGF-β type II receptor (TβRIIDN). Adoptive transfer of these T cells allowed the generation of an immune response capable of inhibiting metastasis in mice challenged with murine thymoma EL-4 and melanoma B16 cells (11). In 2002, we reported the inhibition of metastasis of mouse prostate cancer TRAMP-C2 and mouse melanoma B16 by transplanting TGF-β-insensitive bone marrow cells into mice (12, 13). In all studies described above, although antitumor response was encouraging, the nonspecific nature of the immune response, as most tumors are able to evade the immune surveillance program of the host. Despite the ability of generating the reactivity of immune cells against tumor antigens, the immune surveillance program of the host can be overpowered by tumors with an eventual tumor progression (1). This is because tumor cells have acquired many mechanisms to evade the immune surveillance program of the host (2, 3). One of such possibilities has been the tumor-derived transforming growth factor-β (TGF-β), which is highly immunosuppressive (3–6). Most tumors secrete large amounts of TGF-β (7–9). TGF-β-producing tumor cells fail to elicit primary CTL responses despite retaining class I MHC expression molecules and tumor-specific antigens (4). Priming of T cells by dendritic cells or tumor cells can also be negatively influenced by TGF-β (10). Therefore, an ideal approach to activate the antitumor response will be to render the immune cells of the host insensitive to TGF-β.

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reaction led to the widespread inflammatory disease in the hosts (12). Most recently, we employed adoptive transfer of tumor-specific TGF-β-insensitive CD8+ T cells to tumor-bearing immunocompetent mice and were able to eradicate established lung metastasis of TRAMP-C2 tumors (14, 15). This approach showed no apparent development of the widespread inflammatory syndrome in the recipients and therefore offers a possibility for clinical application.

Although the above initial observations are encouraging, further characterization of this novel approach is necessary. (a) We would like to know if the system could be applied to the treatment of solid tumors. (b) We also would like to determine if this approach can be used to test antitumor efficacy in allogeneic hosts, so that we will be able to test clinical specimens in immunodeficient surrogate animals. (c) We would like to identify the major players in the current system of adoptive transfer of tumor-reactive, TGF-β-insensitive CD8+ T cells for cancer therapy. In the present study, we employed the above approach to investigate the ability of tumor-reactive, TGF-β-insensitive CD8+ T cells on primary solid tumors using the allogeneic immunodeficient mouse as a surrogate host. Here, we report solid tumor response, alteration of tumor microenvironment, and systemic and local cytokine response and postulate the concept of an antitumor immune response cycle.

Materials and Methods

Experimental Animal and Cell Lines

Male BALB/c-Rag1−/− strain Rag1 mice 6 to 8 weeks old were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in pathogen-free facilities at the Center for Comparative Medicine at Northwestern University Feinberg School of Medicine in accordance with established guidelines of the Animal Care and Use Committee of Northwestern University. TRAMP-C2 is an early-passage androgen-independent prostate cancer cell line derived from TRAMP mouse (C57BL/6 strain) that developed prostate cancer due to its prostate-specific expression of just SV40 T antigen that drives the prostate cancer development in that model (16). The mouse melanoma cell line B16-F10 was obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were cultured in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA).

Ex vivo Expansion of Tumor-Reactive, TGF-β-Insensitive CD8+ T Cells

Primed tumor-reactive CD8+ T cells were isolated from C57BL/6 mice that were vaccinated five times each with irradiated TRAMP-C2 cells (5 × 10^6 per mice per injection). The ex vivo culture was done as described previously (14). Tumor-reactive CD8+ T cells were rendered insensitive to TGF-β by infection with TiRIIDN-green fluorescent protein (GFP)-containing retrovirus as described previously (12, 13). Infection efficiency was assessed by GFP expression and flow cytometry and was always >90%. Naive spleen cells were isolated from the C57BL/6 mice and the depletion of CD8+ T cells was done by using MagCellect Magnet apparatus (R&D Systems, Minneapolis, MN) with a biotinylated antimouse CD8a antibody and MagCellect streptavidin ferrofluid (R&D Systems) according to the manufacturer’s protocol. In vitro cytotoxic assay was done by 51Cr release assay as described previously (13).

Challenge of the Mouse Prostate Cancer and Adoptive Transfer of CD8+ T Cells

BALB/c-Rag1−/− mice received an injection in the right flank with 5 × 10^5 TRAMP-C2 cells. Seven days later, adoptive transfer with CD8+ T cells was done. Each group (5–12 mice per group) received i.p. transfer of one of the following six groups of CD8+ T cells (1.5 × 10^7) with or without the same amount of naive CD8-depleted splenocytes. In group 1 (12 mice) and group 2 (10 mice), tumor-reactive, TGF-β-insensitive CD8+ T cells were transferred with or without CD8-depleted splenocytes, respectively. Group 3 (10 mice) and group 4 (10 mice) received tumor-reactive, TGF-β-sensitive CD8+ T cells infected with or without the cotransfer of CD8-depleted splenocytes, respectively. Group 5 (10 mice) and group 6 (5 mice) received naive CD8+ T cells with or without cotransfer of CD8-depleted splenocytes, respectively. Tumor size was measured weekly. Forty days after the adoptive transfer, all mice were sacrificed and the tumors were isolated for evaluation of the volume, weight, and histologic characteristics. Tumor volumes were estimated using the formula: volume = 0.5 × [(length + width) × length × width] × 0.5236.

Determination of Interleukin-2 and IFN-γ in Serum by ELISA

Blood was extracted from a central artery of mice from each treatment group. The supernatant was separated by allowing the whole blood to stand at room temperature for 2 hours. ELISA assays were carried out using the Quantikine mouse interleukin-2 (IL-2) and IFN-γ immunoassay kits (R&D Systems) according to the manufacturer’s protocol. Expression of cytokines in tumor parenchyma was evaluated by an immunofluorescent approach as discussed below.

Pathologic Evaluation and Immunohistochemical Staining

After mice were euthanized, the tumor from each animal was excised, fixed in formalin, and embedded in paraffin. Sections (4 μm) were obtained. Routine H&E staining was done on every fifth serial section. All H&E sections were evaluated by at least two independent investigators. CD31, Ki-67, and Bcl-2 were used for immunohistochemical staining in conjunction with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s protocol. After deparaffinization, quenching of endogenous peroxidase activity, and normal serum pre-blocking, the sections were incubated in either diluted mouse monoclonal antibody against CD31 (1:100; Upstate, Lake Placid, NY), Ki-67 (1:200; DAKO, Carpinteria, CA), or Bcl-2 (1:100; Upstate) for 2 hours at room temperature. This was followed by incubation with biotinylated goat

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anti-mouse secondary antibody (1:200; Vector Laboratories) for 2 hours. Peroxidase substrate solution 3,3'-diaminobenzidine (DAKO) was used for direct staining. Harris hematoxylin solution was used for counterstaining.

**Immunofluorescent Staining and Apoptosis Assay**

Unstained paraffin-embedded serial sections of spleen and tumor were used for immunofluorescent staining to detect presence of transferred CD8+ T cells in spleen tissue (nuclear-CD8-GFP protein triple staining) and tumor nodules [nuclear-CD8-terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) triple staining]. Nuclear-IFN-γ/IL-2 double staining was also done on these sections to analyze secretion of cytokines in tumor parenchyma. The primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Immunofluorescent costaining was done by using the assay as described previously (12–14). The TUNEL apoptosis assay kit (Upstate) was used according to the manufacturer’s protocol. Briefly, slides were treated with proteinase K for 30 minutes at 37°C and incubated with a terminal deoxynucleotidyl transferase end labeling cocktail (terminal deoxynucleotidyl transferase buffer, biotin-dUTP, and terminal deoxynucleotidyl transferase at a ratio of 90:5:5) for 120 minutes at 37°C. This was followed by overlaying an avidin-FITC (green) solution and incubated in the dark for 60 minutes at 37°C. Slides were then incubated with 50 μL blocking buffer at room temperature for 20 minutes followed by a rat monoclonal antibody for CD8 labeled with Texas red (2 μg/mL; Santa Cruz Biotechnology) in the dark for 30 minutes at room temperature. All slides were stained with Vectashield mounting medium (blue; Vector Laboratories) for nuclear counterstaining. Slides were examined with a Nikon TE2000-U fluorescent microscope (Nikon Corp., Tokyo, Japan). Images were digitized by Photoshop 7.0. The intensity of the fluorescent signal was standardized by the standard fluorescent index (positive lymphocytes or signal/100 tumor cells/1,000 μm²: −, <5; ±, 6–10; +, 11–30; ++, 31–50; ++++, 51–70; ++++, >70).

**Statistical Methods**

ANOVA and multiple range tests were done to determine differences of means among different treatment groups. P < 0.05 was considered statistically significant. SPSS 10.0.7 software package (SPSS, Inc., Chicago, IL) was used for analysis.

**Results**

**Reduced Tumor Burden in Allogeneic Immunodeficient Hosts**

The specific tumor-killing ability of the tumor-reactive, TGF-β-insensitive CD8+ T cells was shown by the in vitro CTL assay (Fig. 1A). These cells showed a 5- and 25-fold greater tumor-killing activity than the TGF-β-sensitive counterparts and naive CD8+ T cells, respectively. Both TGF-β-sensitive and TGF-β-insensitive tumor-reactive CD8+ T cells showed a reduced tumor-killing activity when incubated with an irrelevant cell line, mouse B16-F10 melanoma cells (Fig. 1B). In the group treated with cotransfer of tumor-reactive, TGF-β-insensitive CD8+ T cells and CD8-depleted splenocytes, 2 of 12 mice were free of tumor, and the tumor burden in the remaining 10 mice was 50% less than that of animals in other groups (P < 0.05; Fig. 1C and E). The average tumor volumes and tumor weights were not significantly different within the other five groups. In the group treated with the tumor-reactive, TGF-β-insensitive (TGFRIIDN) CD8+ T cells with or without the cotransfer of CD8-depleted splenocytes (group 2), the tumor burden was not significantly different from that of wild-type tumor-reactive CD8+ T cells or naive groups with or without the cotransfer of CD8-depleted splenocytes (groups 3–6). Furthermore, tumor growth rate curves were generated and shown in Fig. 1F based on the tumor volume measurement weekly. The tumor growth rates correspond to the final tumor volumes (Fig. 1C and D) in each group. The tumor growth rate was significantly inhibited by treatment of TGF-β-insensitive (TGFRIIDN) CD8+ T cells with the cotransfer of CD8-depleted splenocytes (group 1) when compared with other five groups. The data would suggest that the transfer of CD8-depleted splenocytes improves the efficacy of adoptive transfer with the modified CD8+ T cells.

**Adoptively Transferred Tumor-Reactive, TGF-β-Insensitive CD8+ T Cells Persisted in the Spleen of the Host**

Adoptively transferred CD8+ T cells were detected in the spleen of recipient animals (Fig. 2A and B), suggesting that these CD8+ T cells were able to persist in recipient hosts at least at the time of sacrifice, which was 40 days since the initial adoptive transfer. In contrast, in animals that received the wild-type tumor-reactive CD8+ T cells or naive CD8+ T cells with or without the cotransfer of CD8-depleted splenocytes (groups 2–6), only occasional CD8+ T cells were detected in the spleen (Fig. 2A and B). These results suggested that, in immunodeficient mice (Rag1−/−), cotransfer of wild-type CD8+ T cells and CD8-depleted splenocytes was unable to manifest an engraftment of transferred cells in the recipients unless tumor-reactive, TGF-β-insensitive CD8+ T cells were cotransferred with the CD8-depleted splenocytes.

**Drastic Histologic Changes in Tumor Tissues (H&E, Ki-67, Bcl-2, and CD31 Staining)**

Three main histologic features in tumors of animals in group 1 differed from those of the other five groups (Fig. 3; Table 1). (a) The tumors in mice that received cotransfer of tumor-reactive, TGF-β-insensitive CD8+ T cells and CD8-depleted splenocytes (group 1) had heavy infiltration of lymphocytes into the tumor parenchyma. (b) There was a significant increase of spindle-shaped cells, suggesting degeneration of cancer cells. (c) There was a significantly less number of mitosis (0.5 versus 3–5 per ×400 field). These findings are consistent with the immunohistochemical staining for Ki-67. As shown in Fig. 3, most tumor cells (>90%) in animals of groups 2 to 5 stained strongly with Ki-67 and Bcl-2. In sharp contrast, the degenerative tumor cells in mice of group 1 showed far less intensity and density of the...
Figure 1. *In vivo* antitumor activity of tumor-reactive, TGF-β-insensitive CD8+ T cells. Three types of CD8+ T cells were used for *in vitro* chromium release assay: naive CD8+ T cells from untreated C57BL/6 mice (Naive), tumor-reactive control CD8+ T cells (GFP), and tumor-reactive, TGF-β-insensitive CD8+ T cells (TβRIIDN). A, TRAMP-C2 mouse prostate cancer cells were used as the targets. B, B16-F10 mouse melanoma cells were used as targets. Columns, average observation obtained from eight wells; bars, SD. P < 0.05, TβRIIDN CD8+ T cells versus the GFP and naive CD8+ T cells. Six groups of CD8+ T cells (1.5 × 10^7) with or without the same amount of naive CD8+ T cell–depleted spleen cells were used. In groups 1 and 2, tumor-reactive, TGF-β-insensitive CD8+ T cells infected with the TβRIIDN-GFP viral particles were transferred with or without CD8+-depleted spleen cells, respectively. In groups 3 and 4, tumor-reactive, TGF-β-sensitive CD8+ T cells were infected with the GFP viral particles only with or without the cotransfer of CD8+-depleted spleen cells, respectively. In groups 5 and 6, naive CD8+ T cells were adoptively transferred with or without cotransfer of CD8+-depleted spleen cells, respectively. In groups 5 and 6, naive CD8+ T cells were adoptively transferred with or without cotransfer of CD8+-depleted spleen cells, respectively. TRAMP-C2 cells (5 × 10^5) were challenged to recipient mice. At 7 d following tumor challenge, the different subtypes of CD8+ T cells were transferred through i.p. injection with or without cotransfer of spleen cells. Animals were sacrificed at 40 d following the adoptive transfer. C, representative gross features of prostate cancer samples from tumor-bearing mice at 40 d following adoptive transfer. D, weight of the tumor of each group. E, volume of the tumor of each group. TβRIIDN-treated mice completely abolished tumors in 2 mice, with the remaining 10 bearing the smallest and lightest tumor burden. P < 0.05, TβRIIDN + spleen group versus the GFP group, naive group, and TβRIIDN only group in both weight and volume. Furthermore, tumor growth rate was inhibited significantly in TβRIIDN-treated mice with cotransfer of CD8-depleted splenocytes. F, curve was generated based on the tumor volume measurement weekly. The tumor growth rate is corresponding to the final tumor volumes (C and D) in each group.
same markers. For CD31, as illustrated in Fig. 3, tumors from animals in groups 2 to 5 contained significantly more CD31+ cells than those from animals in group 1. Quantitative analysis revealed that the microvessel densities (CD31+) in tumors of animals in groups 1 to 6 were 26 ± 8/mm², 177 ± 37/mm², 154 ± 45/mm², 196 ± 22/mm², and 164 ± 41/mm², and 121 ± 28/mm², respectively (Table 1). These observations are consistent with the results of the TUNEL assay (Fig. 3). Results of immunohistochemical staining for Bcl-2 showed scant staining in tumors of animals in group 1 in comparison with that in tumors of groups 2 to 5, which stained strongly for Bcl-2. A quantitative summary of expression of H&E, Ki-67, CD31, and Bcl-2 is listed in Table 1.

Infiltration of CD8⁺ T Cells into the Tumor Parenchyma and Induced Tumor Cell Apoptosis

The most prominent histologic feature of the tumor tissue in this study was the evidence of infiltration of many tumor-reactive, TGF-β-insensitive (GFP⁺) CD8⁺ T cells
with concomitant apoptosis in tumor tissues in animals of group 1 (Fig. 4A and B). The degree of infiltration by different types of lymphocytes was evaluated by the standard fluorescent index, which corresponded to the fluorescent intensity criterion (positive lymphocytes/100

\[ C_0 \] ; <5; \[ F \]; 5–10; +, 11–30; ++, 31–50; ++++, 51–70; ++++, >70; Fig. 4A). Almost all of these infiltrated CD8+ T cells were GFP+, consistent with the knowledge that all these CD8+ T cells were adoptively transferred. Results of the TUNEL assay revealed that tumor cells/1,000 \( \mu m^3 \): –, <5; ±, 6–10; +, 11–30; ++, 31–50; ++++, 51–70; ++++, >70; Fig. 4A). Almost all of these infiltrated CD8+ T cells were GFP+, consistent with the knowledge that all these CD8+ T cells were adoptively transferred. Results of the TUNEL assay revealed that

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Table 1. Characteristics of histologic finding

<table>
<thead>
<tr>
<th>CD8-depleted splenocytes*</th>
<th>Tumor-reactive, TGF-β-insensitive CD8+ T cells (TβRIIDN)</th>
<th>Tumor-reactive, TGF-β-insensitive CD8+ T cells (GFP)</th>
<th>Naive CD8+ T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitosis</td>
<td>0.5 ± 0.12</td>
<td>4.1 ± 1.41</td>
<td>4.6 ± 0.87</td>
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<tr>
<td>Degeneration of cancer cells</td>
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<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Infiltration of lymphocytes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Spindly change of cancer cells</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Ki-67</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CD31</td>
<td>26 ± 8</td>
<td>154 ± 45</td>
<td>164 ± 41</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>+</td>
<td>+++</td>
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</table>

NOTE: Quantitative analysis revealed that the microvessel densities in tumors of TβRIIDN + spleen, TβRIIDN only, GFP + spleen, GFP only, naive + spleen, naive only were 26 ± 8/mm\(^2\), 177 ± 37/mm\(^2\), 154 ± 45/mm\(^2\), 196 ± 22/mm\(^2\), 164 ± 41/mm\(^2\), and 121 ± 28/mm\(^2\) respectively. In addition, immunohistochemical staining for Bcl-2 showed the least intense staining in TβRIIDN + spleen group. The characteristics of H&E and quantitative expression of Ki-67, Bcl-2, and CD31 of different groups are listed.

*Large-sized window is with a magnification of ×100; small-sized window is with a magnification of ×400.

†Mitosis was expressed by the number of mitosis for each ×400 field.

‡Evaluation of the staining of Ki-67 and Bcl-2: ±, <5%; +, 5-30%; ++, 30-50%; ++++, >50%.

§Quantitative analysis of CD31 was evaluated by microvessel density per mm\(^2\).
apoptosis in tumor cells was detected only in animals of group 1. Tumors in the animals of the other five groups showed little or no infiltration of CD8+ T cells and showed no evidence of apoptosis in tumor cells.

**Up-Regulation of Systemic and Local Levels of IFN-γ and IL-2**

An increase in serum level of IL-2 and IFN-γ (Fig. 5A and B) was observed in animals cotransferred with tumor-reactive, TGF-β-insensitive CD8+ T cells and CD8-depleted splenocytes (group 1), suggesting the presence of activated immune cells. Increased levels of IFN-γ and IL-2 were also noted by immunofluorescent analysis in the tumor parenchyma, which correlated with serum levels (Fig. 5C). IFN-γ and IL-2 was localized around CD8+ cells, which imply that these cytokines were produced by these immune cells. In comparison, IFN-γ

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**Figure 4.** Infiltration of lymphocytes into tumor parenchyma and apoptosis of tumor cells. **A**, representative tissue sections were simultaneously stained for cell nucleus (blue), CD8+ T cells (red), and apoptosis (green). The tumor site was identified by the nuclear staining (blue). CD8+ T cells were localized in the tumor parenchyma that also stained for tumor apoptosis (green) in the TjRIIDN + spleen group. The majority of the apoptotic cells were tumor cells (green) and not CD8+ T cells (yellow). Magnification, × 400. In contrast, tumors in animals that received CD8+ cells that were either naive, GFP alone, or treated with TjRIIDN only did not exhibit significant infiltration of CD8+ T cells or tumor cell apoptosis within the tumor parenchyma. In contrast to GFP-infected and naive CD8+ T cells, only TjRIIDN + spleen group CD8+ T cells effectively infiltrated into both tumor parenchyma. **B**, quantitative analysis CD8+ T cell infiltration. **C**, TUNEL assay of tumor tissue.

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**Table:**

<table>
<thead>
<tr>
<th>Group</th>
<th>Nuclear (DAPI)</th>
<th>CD8 (TR)</th>
<th>TUNEL (FITC)</th>
<th>Merged images (CD8+TUNEL)</th>
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*For tumor tissue: positive signal/1000 tumor cells/1000 μm2: -- <5, +: 6-10, ++: 11-50, +++: 51-70, ++++: >70

*+p<0.05; **p<0.01; ***p<0.001
and IL-2 expression in tumors of animals in other five groups (groups 2–6) was negligible. A summary of cytokine expression was described as a standard fluorescent index listed in Fig. 5D.

**Discussion**

Results of the present study have provided three important pieces of information. (a) Our results have shown that it is feasible to use immunodeficient allogeneic mice as surrogate hosts for the treatment of xenograft tumors by adoptive transfer of tumor-reactive, TGF-β-insensitive autologous CD8⁺ T cells. (b) We have indicated that the present treatment protocol created a tumor microenvironment that favorably eliminated the s.c. solid tumors. (c) We showed that the adoptively transferred tumor-reactive, TGF-β-insensitive CD8⁺ T cells alone were insufficient for an antitumor response unless they are support by other immune cells.

By using an allogeneic system, it is necessary for us to determine if graft-versus-host disease develops. Based on...
our results, it does not seem that within the timeline of this study there was any evidence of graft-versus-host disease. In the present study, the cotransferred CD8-depleted splenocytes seemed necessary to assist the tumor-reactive, TGF-β-insensitive CD8⁺ T cells to acquire the antitumor effector function. The cotransfer of CD8-depleted splenocytes was important, as CD8⁺ T cells are cytotoxic effector cells, which possess the ability to mediate apoptosis of target cells in the host. Had we cotransferred splenocytes that contained CD8⁺ T cells, these CD8⁺ T cells, on activation, would mediate apoptosis of the nontumor cells of the hosts, leading to autoimmune disease. Such examples are abundant in the literature. Our own experience has indicated that transfer of non-tumor-specific CD8⁺ T cells to recipients will lead to widespread inflammatory disease (12, 13). In the present study, the situation is more critical than the syngeneic systems, as the recipient is allogeneic to the transferred CD8⁺ T cells. Therefore, it is important that when we cotransfer splenocytes they must be CD8 depleted. Similarly, transfer of naive splenocytes will not be feasible, as they include CD8⁺ T cells.

In the normal prostate, TGF-β1 signaling inhibits cell growth and induces apoptosis in epithelial cells (17, 18) and thus serves as a tumor suppressor. In prostate cancer, TGF-β promotes progression of advanced tumors through several paracrine and autocrine mechanisms (19). TGF-β secreted by tumor cells can facilitate tumor progression through stimulating proteins, such as vascular endothelial growth factor. TGF-β also induces expression of platelet-derived growth factor, connective tissue growth factor, and matrix metalloproteinases, all of which create conditions favorable for tumor invasion and angiogenesis (20). Further, TGF-β, being a potent immune suppressor, inhibits the immune system and facilitates tumor progression (4, 5, 21). The crucial role of TGF-β in maintaining immune system homeostasis is highlighted by the multifocal inflammatory disease that results from the genetic disruption of the TGF-β1 allele in transgenic mice (22, 23). Conditional elimination of TGF-β1 signaling in T cells (24) or in bone marrow cells (25) results in a widespread inflammatory response.

In the present study, tumor-reactive, TGF-β-insensitive CD8⁺ T cells mediated complete regression of established solid tumors in 2 of 12 (16.7%) mice and inhibited >50% of the tumor burden in the remaining animals. This effect may be due to a reversal of the tumor-promoting microenvironment, which warrants further discussion. (a) Although tumor-derived TGF-β suppresses a variety of immune cells, we found that suppression of CTLs by TGF-β was the most critical (20). There was an 8-fold increase in tumor-reactive, TGF-β-insensitive CD8⁺ T cells that migrated into spleen tissue and the tumor parenchyma compared with those in control groups. This phenomenon was confirmed, in the present study, by the histologic analysis of spleen and tumor specimens. (b) The transferred tumor-reactive, TGF-β-insensitive CD8⁺ T cells induced systemic expression of IL-2 and IFN-γ. It is likely that the up-regulation of these cytokines significantly enhanced the tumor-killing ability. (c) The present treatment protocol resulted in an inhibition of tumor cell proliferation as indicated by a decrease in Ki-67 staining intensity. This observation is consistent with the study of lung metastasis in which proliferating cell nuclear antigen expression was inhibited by the infiltration of tumor-reactive, TGF-β-insensitive CD8⁺ T cells. (d) The present result indicated that TGF-β-insensitive CD8⁺ T cells played a negative role in tumor angiogenesis (14). Angiogenesis is an important prognostic factor in cancer survival (26, 27). An increase in small vessels, as assessed by CD31 staining, correlated with lymph node involvement and was an independent predictor of survival in cancer patients. Various studies have highlighted the importance of CD8⁺ T cell, IL-2, and IFN-γ expression in inhibiting metastasis through blocking tumor angiogenesis (28–33). Our results showed that tumors in mice cotransferred with tumor-reactive, TGF-β-insensitive CD8⁺ T cells and CD8-depleted splenocytes were poorly vascularized. (e) Adoptive transfer of tumor-reactive wild-type CD8⁺ T cells could not effectively inhibit tumor growth regardless whether they were cotransferred with CD8-depleted splenocytes. Because these CD8⁺ T cells are the same as the conventional tumor-reactive CD8⁺ T cells (34, 35), this observation suggests that, in the face of high levels of tumor-derived TGF-β, adoptive transfer of conventional tumor-reactive wild-type CD8⁺ T cells would have limited antitumor efficacy. (f) Tumors in mice cotransferred with tumor-reactive, TGF-β-insensitive CD8⁺ T cells and CD8-depleted splenocytes showed markedly increased apoptosis, which coincided with an inhibition of Bcl-2 expression. Inhibitors of Bcl-2, such as ABT-737, can induce apoptosis in cancer cells and are potential agents in anticancer therapeutics (36–39). The expression of Bcl-2 was inhibited when a large number of tumor-reactive, TGF-β-insensitive CD8⁺ T cells infiltrated into the tumor parenchyma. In addition, cytokines, such as IL-2 and IFN-γ, may have played a coordinated role in the observed increase in tumor apoptosis. The regression of tumor cells, the presence of spindle-shaped cancer cells, the reduced mitotic figures, and the decreased expression of Bcl-2 may all contribute toward changes in the tumor microenvironment dictated by the presence of tumor-reactive, TGF-β-insensitive CD8⁺ T cells.

Tumor-reactive, TGF-β-insensitive CD8⁺ T cells showed a strong tumor-killing ability in vitro. Although CD8⁺ T cells are the cytotoxic effectors, CD4⁺ T cells are likely required to facilitate the effector function of CD8⁺ cells. Furthermore, CD4⁺ T cells can mediate CD8-independent antitumor function and memory (11, 40–42). Our in vivo results are consistent with these reports. Although we have not delineated the subtype of the immune cells in the spleen, the cotransferred CD8-depleted splenocytes included CD4⁺ cells, macrophages, and dendritic cells. This supports at least two possibilities. (a) Cotransfer of CD8-depleted splenocytes was required for the prolonged survival of CD8⁺ T cells. These splenocytes may contain antigen presentation function to CD8⁺ T cells. Based on our observation, TGF-β-insensitive CD8⁺ T cells (TJRIDN only
group) alone could not be maintained unless CD8-depleted splenocytes were cotransferred. (b) Only with the cotransfer of CD8-depleted splenocytes could the CD8\(^+\) T cells reach the tumor parenchyma. This is likely due to the helper function of CD4\(^+\) T cells, which primed CD8\(^+\) T cells to acquire the antitumor effector function. Therefore, blockade of TGF-\(\beta\) signaling in tumor-reactive CD8\(^+\) T cells provides an effective antitumor function, which should be translated to the treatment of clinical cancer cases.

Based on the above results, we postulate the concept of an antitumor immune response cycle (Fig. 6). This antitumor immune response cycle represents a new paradigm in antitumor immunology and contains three major components: (a) tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells; (b) the autologous tumor; and (c) the immune system of the host (Fig. 6). This concept will be briefly discussed below. (a) Results of the present study have indicated that tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells are necessary for an effective antitumor immune response, as they are the only immune cells that are able to infiltrate into the tumor parenchyma and mediate tumor apoptosis. (b) Results of our past studies have indicated that the tumor itself is an important participant of this antitumor immune response cycle. The importance of autologous tumor has been shown by our earlier study (14, 15), which showed that in tumor-free hosts the tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells were unable to persist in the spleen. (c) As shown by the present results, the presence of CD8-depleted immune cells is also necessary to manifest an effective antitumor immune response. This statement is based on the observation in that, when tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells were transferred alone, they were insufficient in the growth of the established s.c. TRAMP-C2 tumor. The system requires the cotransfer of CD8-depleted splenocytes in order for the transferred tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells to mount an antitumor function (Fig. 6A). The immune response cycle can only be activated by tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells infiltrating into tumor parenchyma and inducing apoptosis of tumor cells (Fig. 6B).

Up to this point, we have established three salient aspects of this new system. (a) The aspect was to show that adoptive transfer of tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells was able to eradicate established autologous tumors (14). (b) We showed that infiltration of transferred tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells into the tumor parenchyma and to mediate tumor cell apoptosis was an important event in this system (15). (c) In this article, we report that the mere transfer of tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells was insufficient to mediate an antitumor response. Cotransfer of CD8-depleted splenocytes was necessary for the antitumor function of the transferred CD8\(^+\) T cells. In fact, the present observation was critical in that it allowed us to postulate the “antitumor immune response cycle.” (d) We also obtain critical information from the present study that it is feasible for us to use allogeneic host for syngeneic tumor treatment with transfer of syngeneic immune cells. Such information will be missed if the present study was not conducted.

In an immunocompetent host, because the wild-type CD8\(^+\) T cells are unable to play any role in the antitumor immune response, they are not considered a part of this antitumor immune response cycle (Fig. 6A). The sequence of events of this antitumor immune response cycle starts with the infiltration of the tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells into the tumor parenchyma to mediate tumor apoptosis and to release tumor-associated antigens into the circulation, thus allowing a continuous exposure of these antigens to the immune system of the host. It is likely that antigen-presenting cells, which are included in the CD8-depleted splenocytes, are important players in this process. Because antigen-presenting cells and CD4\(^+\) helper T cells are wild-type, they are unable to infiltrate into the tumor parenchyma. Their action must take place outside the tumor parenchyma. Therefore, the activation and priming of transferred tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells must also take place outside of the tumor parenchyma. The primed CD8\(^+\) T cells, because they have been rendered TGF-\(\beta\)-insensitive, are able to infiltrate into the tumor parenchyma (Fig. 6B). Such an antitumor immune response cycle will remain active until all tumor cells are eliminated.

In summary, results of the present study have provided three pieces of novel concept information. (a) Our results have shown that it is feasible to use immunodeficient mice as surrogate hosts for the treatment of solid cancer xenograft tumors with adoptive transfer of tumor-reactive, TGF-\(\beta\)-insensitive autologous CD8\(^+\) T cells. (b) There are changes in the tumor microenvironment secondary to adoptive transfer of tumor-reactive, TGF-\(\beta\)-insensitive CD8\(^+\) T cells as shown by changes in tumor histology,
cytokine secretion, tumor cell proliferation, angiogenesis, and apoptosis. (c) We proposed a concept of antitumor immune response cycle, which consists of tumor-reactive, TGF-β-insensitive CD8+ T cells, the autologous tumor, and the immune system of the host. In an immunocompetent tumor-bearing host, by virtue of adaptive transfer of tumor-reactive, TGF-β-insensitive CD8+ T cells, the anti-tumor immune response cycle will be activated. This novel concept, although overly simplified, may lead to the development of effective antitumor therapeutic strategies in the near future.

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Blockade of transforming growth factor-β signaling in tumor-reactive CD8+ T cells activates the antitumor immune response cycle

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