TCRγδ1-Engineered αβT Cells Exhibit Effective Antitumor Activity

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TCRγδ1-engineered αβT cells with αβT receptors (TCRs) specific for tumors play an important role in adoptive T cell transfer (ATC) therapy for cancer. Here, we present a novel strategy to redirect peripheral blood-derived αβT cells against tumors via TCRγδ1 gene transduction. The broad-spectrum antitumor activity of TCRαβ cells in innate immunity is dependent on CDR3αβ. TCRγδ1-engineered αβT cells were prepared by lentiviral transduction and characterized by analyzing in vitro and in vivo cytotoxicity to tumors, ability of proliferation and cytokine production, and potential role in autoimmunity. Results show that TCRγδ1 genes were transduced to approximately 36% of polyclonal αβT cells. TCRγδ1-engineered αβT cells exhibited effective in vitro TCRγδ-dependent cytotoxicity against various tumor cells via the perforin-granzyme pathway. They also showed a strong proliferative capacity and robust cytokine production. TCRγδ1-engineered αβT cells neither expressed mixed TCR dimers nor bound/killed normal cells in vitro. More important, adoptive transfer of TCRγδ1-engineered αβT cells into nude mice bearing a human HepG2 cell line significantly suppressed tumor growth. Our results demonstrate a novel role for TCRγδ1 in gene therapy and ATC for cancer.

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INTRODUCTION

Adoptive T cell transfer (ACT) is a promising immunotherapy strategy for cancer. Chimeric antigen receptor (CAR)-modified T cells have shown promise in the treatment of B cell malignancy. (1) However, ACT immunotherapy for solid tumors faces the challenge of specificity when targeting tumors. To date, T cells have frequently been tested as effectors for ACT, including tumor infiltrating lymphocytes (TILs) for metastatic melanoma (2,3) and T cell receptor (TCR) gene-engineered T cells for other tumor types. (4,5) Although Rosenberg’s laboratory found that a mutation in erbB2 interacting protein triggered CD4 + TH1 cell activation and demonstrated a cure efficacy in ACT, the method is not feasible, given the high cost and complicated process. (6) Therefore, our laboratory and other groups have pursued TCRγδ gene therapy as an alternative approach.

Compared with TCRαβ, which is highly specific for its antigen, TCRγδ displays characteristics of innate immunity, directly recognizing many stress-induced antigens in an MHC-independent manner in the early stages of inflammation and tumorogenesis. (7) Human γδT cells are grouped into 2 major subsets, Vδ1 and Vδ2 T cells. Vδ1 T cells are common in mucosa, especially the submucosal areas of the gastrointestinal, respiratory and genitourinary tracts. They recognize MHC class I–related molecules A and B (MICA and MICB) and UL-16–binding proteins (ULBPs) expressed at variable levels on epithelial tumor cells and some leukemias and lymphomas. Vδ2 T cells belong to a minor subset of the total T cell pool in the peripheral blood, responding mainly to aminobisphosphonates/synthetic phosphoantigen. (8) Due to broad-spectrum tumor recognition of TCRγδ, TCRγδ gene transduction into effector T cells, such as αβT cells, may be an attractive therapeutic approach. It appears to resolve the fundamental problem of tumor targeting not found in TCRαβ. Previous studies by other groups and our laboratory have confirmed that TCRγδ2-transduced αβT cells, or TCRγδ2-modified peripheral blood mononuclear cells (PBMCs), mediate cytotoxicity against a broad range of tumor cell lines in vitro and suppress tumor growth in Daudi or SKOV3 tumor cell–bearing mice models. (9,10)

Preparing a large number of tumor-reactive T cells in a short time is a major challenge for ACT in cancer patients.
Transduction of tumor antigen–reactive TCR into T cells is one strategy to acquire sufficient T cells. Antigen-specific TCRαβ-modified CD8 + αβT cells display significant antitumor activity. (11) However, a potential disadvantage of TCRαβ-modified αβT cells is the formation of new mixed TCRs. Specifically, the introduced TCRα or TCRβ chains can pair with the endogenous TCRα or TCRβ chains to generate mixed TCR dimers. These mixed TCR dimers do not experience thymic negative selection during T cell development and elicit an autoimmune response. Bendle et al. first noted that OT-I TCRαβ-transduced CD8 + T cells triggered in vivo mispairing–mediated autoimmunity in C57BL/6 mice. (12) To prevent this, multiple approaches were used, including murinized TCR and cysteine-modified TCR, with γδT cells as recipient cells transduced with exogenous TCRβ. (13,14) TCRαβ-engineered γδT cells exhibited no evidence of TCR mispairing; TCRδ or TCRγ in γδT cells did not exchange chains with TCRαβ. (15,16) Other approaches, except for complete knockdown of TCRα and TCRβ chains, reduced but did not completely prevent mispairing. Nevertheless, other major drawbacks of TCRαβs as genetic donors include MHC restriction and single-antigen-peptide recognition, restricting the application to fewer patients. (17)

Our laboratory previously identified complementary determining region 3 (CDR3) at a high frequency in TCRδ1 chain from TILs in human gastric cancer. This high-frequency CDR3, termed GTM, is a critical region for tumor antigen recognition; its binding to gastric tumors reached 88.89%. The full-length TCRδ1 with GTM and TCRγ4 chains were amplified and paired to form TCRγ4δ1 receptor. We confirmed TCRγ4δ1-Fc fusion proteins bind to a large panel of tumor cell lines and tissues through their TCRs, including gastric carcinoma BGC823, kidney cancer G401, lung adenocarcinoma GLC-82, colonic carcinoma HT29 and ovarian cancer SKOV3. These fusion proteins strongly bind MICA and ULBP5 in enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) assay. (18) MICA is a stress-inducible cell surface antigen expressed on epithelial tumors in lung, colon, prostate, kidney, breast and ovary carcinomas. (19) Our previous data demonstrated that TCRγ4δ1 has broad-spectrum tumor recognition. Due to the high prevalence and diversity of TCRγ4δ1 ligands on malignant cells, we reasoned that TCRγ4δ1 targeting various ligands as genetic donors to modify effector cells is a more promising approach compared with specific single-chain antibody fragments (scFv) or TCRαβ targeting only single antigens. In addition, it prevented tumor immune escape resulting from loss or deficiency of single antigens during tumor progression. Thus, we propose a promising strategy to redirect peripheral blood-derived αβT cells against various tumors via a broadly tumor-reactive TCRγ4δ1 gene transfer method.

Here we show for the first time that TCRγ4δ1-engineered αβT cells exhibit significant antitumor activity in vitro and in vivo. Moreover, TCRγ4δ1-engineered αβT cells neither expressed mixed TCR dimers nor bound/killed normal cells in vitro. These results indicate that TCRγ4δ1-engineered αβT cells are a promising effector in ACT for cancer therapy.

MATERIALS AND METHODS

Cells

Human tumor cell lines (HepG2, BGC-803, K562, Raji, fetal liver cells ccc-HEL-1, adrenal cortical reticular epithelial cells 1308.1.86, renal epithelial cell line 293T) were obtained from the American Type Culture Collection (ATCC). HepG2, BGC-803, ccc-HEL-1, 1308.1.86 and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, USA) supplemented with 10% fetal bovine serum (FCS), 100 U/mL penicillin-streptomycin, 1 mM L-glutamine and 1 mM/L sodium pyruvate. K562 and Raji cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FCS, 100 U/mL penicillin-streptomycin, 1 mM/L L-glutamine and 1 mM/L sodium pyruvate. Human PBMCs, from healthy donors who provided informed consent, were isolated by density gradient centrifugation on Ficoll-Hypaque (TianjinHaoyang, China) and cultured in RPMI-1640 medium supplemented with 10% FCS and 200 IU/mL recombinant human interleukin-2 (IL-2).

Construction of Lentiviral Vectors and Production of Lentiviral Supernatant

The full-length sequence of TCRγ4 and TCRδ1 chains with a tumor antigen–specific CDR3 region identified from TILs of gastric carcinoma tissues was previously described. (18) Briefly, the V region of TCRδ1 was amplified using cDNA from the TILs of gastric carcinoma tissues as a template with Vδ1 and Cδ1-specific primers, and subcloned into a pGEM-T easy vector for sequence analysis. We identified one high-frequency CDR3δ1-specific binding to gastric carcinoma tissues as a critical region (termed GM). Finally, full-length TCRγ4 and TCRδ1 chains were amplified from cDNA of gastric tumor–derived γδTILs by PCR using full-length TCRγ4 and TCRδ1 primers directed at 5’-end region and 3’-end region, then cloned into pREP9 and pREP7 vectors for sequence analysis. After sequence was identified, especially for GTM, the TCRδ1 and TCRγ4 genes were cloned individually and co-cloned into pCDH vectors containing the marker genes copGFP to obtain pCDH-TCRδ1, pCDH-TCRγ4 and pCDH-TCRγ4δ1. These recombinant lentiviral vectors and pCDH vector were co-transfected into 293T cells with psPAX2 and PMD2.G vectors, respectively, using Lipofectamine 2000 (Invitrogen, USA). The lentiviral supernatant was collected, concentrated and stored at –80°C.

Generation of αβT Cells and Lentiviral Transfer

The γδT cells of PBMCs were removed by positive selection using FITC-labeled γδ-T-cell–specific immunomagnetic beads (Miltenyi, Germany). PBMCs without γδT cells were stimulated for 72 h with anti-CD3 and anti-CD28 in the presence of 200 IU/mL IL-2. The αβT cells were transfected with TCRγ4δ1-lentivirus,
TCRδ1-lentivirus, TCRγδ-lentivirus and mock-lentivirus, using 10 μg/mL polybrene (Sigma, USA) and then cultured in RPMI-1640 with 10% FCS plus 400 IU/mL IL-2 for 7 d. TCRγδ1, TCRδ1 and TCRγ4 surface molecule staining was performed separately with anti-human TCRαβ-PE and anti-human TCRγ-APC antibodies (Biolegend, USA), anti-human Vδ1-FITC antibody (Thermo, USA), and rabbit anti-human TCRγ primary antibody (Santa Cruz, USA) and goat anti-rabbit IgG-FITC secondary antibody (ZSGB-bio, China). Immunofluorescence was measured with an Accuri 6 Flow cytometer and analyzed by BD Accuri C6 software.

**Binding of TCRγδ1-Fc to Tested Cells**

TCRγδ1-Fc was constructed by fusing the complete extracellular domain of TCRγδ1 to the constant region of human IgG1 (18) and expressed by Sino Biological Inc. For flow cytometry: To examine the binding of TCRγδ1-Fc with various cells, including HepG2, BGC-803, K562, Raji, PBMCs, ccc-Hel-1, 1308.1.86 and 293T cells, the tested cells were individually incubated with TCRγδ1-Fc for 60 min at 4°C. FITC-conjugated goat anti-human IgG antibody (ZSGB-bio, China) was added and incubated for 30 min at 4°C; human IgG-Fc was used as a control. Immunofluorescence was measured with an Accuri 6 Flow cytometer (BD, USA) and analyzed using BD Accuri C6 software. Confocal microscopy: Tumor cells including HepG2, BGC-803, K562 and Raji were plated on slides with or without polylysine overnight and fixed with 4% cold paraformaldehyde. Fixed cells were incubated with TCRγδ1-Fc protein for 30 min at 4°C. FITC-conjugated goat anti-human IgG antibody was then added and incubated for 30 min at 4°C, human IgG-Fc was used as the control. Slides were examined with a confocal laser microscope (Leica, USA).

**Cytokine Production and Proliferation Capacity**

Cytokine production: Flow cytometry was used to determine the expression of IL-2 and IFN-γ in TCRγδ1-engineered αβT cells against target cells including HepG2, PBMCs, 1308.1.86, ccc-Hel-1 and 293T cells. Effector cells comprised TCRγδ1-engineered αβT cells, mock-engineered αβT cells, TCRγδ1-engineered αβT cells with resting treatment and mock-engineered αβT cells with resting treatment. Resting effector cells were cultured in RPMI-1640 medium with 5% FCS for 24 h. PBMCs as target cells were labeled by carboxyfluorescein diacetate N-succinimidyl ester (CFSE). Effector cells were incubated with target cells at an effector-to-target ratio of 3:1 in the presence of Brefeldin A (BFA) (Biosciences, USA) for 8 h. Cells were fixed and perméabilized with 1 x cytofix/cytoperm buffer (BD, USA) for 30 min, and then stained with anti-human IL-2-PE or anti-human IFN-γ-APC (Biolegend, USA) for 30 min in the dark. Cells were washed with 1X permeabilization/wash buffer and analyzed on an Accuri 6 flow cytometer. Flow cytometry data were based on gating at the effector cell population. Data analysis was performed with BD Accuri C6 software. Proliferation: Forty-eight-well plates were treated with 1 μg/mL anti-TCRγ antibody for 2 h at 37°C. After washing, 5 × 10^5 mock- and TCRγδ1-engineered αβT cells were plated separately in RPMI-1640 medium with 200 IU/mL IL-2 for 8 h. Cells were then harvested and stained with anti-human Ki67 PEcy5 (BD, USA) according to the intracellular cytokine staining protocol and analyzed by flow cytometry.

**Cytotoxicity**

HepG2, BGC-803, K562, Raji, PBMCs, 1308.1.86, ccc-Hel-1 and 293T as target cells were added to 96-well plates at a density of 1 x 10^4 per well. Transfected effector cells including mock- and TCRγδ1-engineered αβT cells were incubated with target cells at an effector-to-target ratio of 10:1 for 6 h; each condition was plated in triplicate. The three controls were background group, spontaneous release group and maximal release group. We detected the cytotoxicity by a lactate dehydrogenase (LDH) assay, using the Cytotox 96 (Promega, USA) nonradioactive cytotoxicity assay reagent kit instructions. Cytotoxicity was calculated using the following formula:

\[
\text{% of cytotoxicity} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})} \times 100
\]

Antitumor activity of TCRγδ1-engineered αβT cells in vivo. Mice: Animal experiments were performed according to the Animal Use Committee guidelines of the Experiment Animal Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Athymic BALB/c nu/nu mice (female, 4–6 wks) were obtained from the Laboratory Animal Center of the Chinese National Institute for the Control of Pharmaceutical and Biological Products, and housed under specific pathogen-free condition. Animal tumor model: To investigate the antitumor activity of TCRγδ1-engineered αβT cells in vivo, approximately 2 x 10^6 HepG2 cells in 100 μL of PBS was subcutaneously injected into the rear right flank of BALB/c nu/nu mice. After the tumor grew to approximately 100 mm^3, mice bearing HepG2 tumors were randomly divided into 2 groups and intratumorally injected with TCRγδ1-engineered αβT cells (1 x 10^6, n = 8) or mock-engineered αβT cells (1 x 10^6, n = 8) every 3 d a total of 4 times. Tumors were measured with an electronic caliper every 3 d, and the tumor volumes were calculated using the following formula:

\[
\text{Tumor volume (mm}^3\text{)} = (\text{long diameter, mm}) \times (\text{short diameter, mm}) \div 2
\]

The tumor growth inhibition ratio (D%) was estimated according to the following formula: D% = (Vc - Vt) / Vc x 100%

Vc and Vt represent the means of tumor volumes of the control and treatment mice, respectively. On the last day of the experiment, the animals were euthanized by the cervical vertebra exarticulation method, and the tumors were excised and photographed.
Statistical Analysis

Data from the in vitro experiments are presented as the mean ± standard deviation (SD). Analysis of variance and independent samples t-test were used to analyze data. For in vivo experiments, the tumor volume was assessed by analysis of variance and paired t-test, and the data are presented as the mean ± SEM. *p* value < 0.05 is regarded as significant.

RESULTS

The Lentiviral Vector Efficiently Transduced TCRγδ1 into Peripheral Blood-Derived αβ Cells

We previously identified a high-frequency CDR3 dominant sequence (CDR3δ1: CAFLPHADKLIFGKG), termed GTM, in TCRδ1 chain from TILs in human gastric cancer through RT-PCR and analysis of a large number of CDR3δ1 sequences. We confirmed that the CDR3δ1 peptide played a crucial role in tumor antigen recognition and bound to a wide variety of tumor cell lines and tissues similar to intact TCRγδ1. (18) The full-length TCRδ1 with GTM and TCRγ4 chains were amplified from cDNA of one case of gastric tumor–derived γδTILs by RT-PCR. A high-frequency CDR3δ1 sequence, termed GTM, was identified by sequence analysis. The full-length TCRδ1 with GTM and TCRγ4 chains were amplified from cDNA of one case of gastric tumor–derived γδTILs by PCR and paired to form TCRγδ1. (B) Schematic diagram of the lentiviral vector containing the TCRδ1 and TCRγ4 genes. The TCRδ1 and TCRγ4 genes were amplified by PCR and co-cloned into pCDH-TCRγ4 lentiviral vector. (LTR, long terminal repeats; CMV, cytomegalovirus promoter.) (C) The cell-surface expression of TCRγδ1 in αβ T cells. Peripheral blood-derived αβ cells were lentivirally transduced with either mock lentiviral vectors or lentiviral vectors with TCRγδ1, then these cells were separately stained with APC-conjugated anti-TCRδ1 and PE-conjugated anti-TCRγ4 mAbs and analyzed by flow cytometry. The percentage of positive cells for each quadrant is indicated. (TCR, T-cell receptor; CDR, complementary determining region; TIL, tumor-infiltrating lymphocyte.)

TCRγδ1-engineered αβT cells were cytotoxic to several tumor cells in vitro.

To assess binding of TCRγδ1 to tumor cells in vitro, we determined the binding capacity of TCRγδ1-Fc fusion proteins to various tumor cells by flow cytometry and confocal microscopy. Results show significant binding activity with HepG2, BGC-803 and K562 tumor cells, with binding rates of 94.23%, 91.46% and 72.05%, respectively (Figure 2A and 2B). No binding was observed in Raji tumor cells.

LDH assay was performed to assess the cytolytic capacities of TCRγδ1-engineered αβT cells to tumor cells. We found significant cytotoxicity to HepG2 (55.78 ± 10.39%) and BGC-803 (67.36 ± 5.30%) tumor cells, and less activity against K562 tumor cells (41.33 ± 1.28%) at an effector-to-target ratio of 10:1. In comparison, mock-engineered αβT cells had significantly less cytotoxicity against HepG2 22.26 ± 12.56% (*p* = 0.024), BGC-803 32.56 ± 15.01% (*p* = 0.019) and K562 19.29 ± 5.25% (*p* = 0.002) (Figure 2C). Raji cells were not lysed and served as a negative control. We found that the cytotoxicity of TCRγδ1-engineered αβT cells was proportionate to the binding ability of TCRγδ1-Fc to tumor cells. This indicates that TCRγδ1-engineered αβT cells exert cytolytic activity against HepG2, BGC-803 and K562 tumor cells in a TCRγδ1-dependent manner.

Cytotoxicity of TCRγδ1-engineered αβT cells to tumor cells is related to perforin-granzyme. Previous reports showed that T cells kill tumors through 2 main mechanisms, Fas-FasL and
perforin-granzyme pathways. Fas-FasL is involved in the transmission of cell death signaling via binding of Fas molecules on the tumor cell surface with ligands on T cells. Perforin-granzyme involves synergy between perforin and granzyme molecules released by T cells. Granzyme B is more abundant, with higher enzyme activity, compared with other types of granzymes. (20,21) Thus, we measured the expression of FasL and granzyme B by flow cytometry on mock- and \( \text{TCR}^\gamma_4\delta_1 \)-engineered \( \alpha\beta \)T cells following incubation with HepG2 cells for 6 h. We found no significant difference in FasL expression between the 2 groups. Results show that the percentage of \( \text{TCR}^\gamma_4\delta_1 \)-engineered \( \alpha\beta \)T cells expressing granzyme B was 40.66%, significantly higher than the control group (25.66%; Figure 2D). These data suggest that \( \text{TCR}^\gamma_4\delta_1 \)-engineered \( \alpha\beta \)T cells kill tumor cells via the perforin-granzyme pathway.

**\( \text{TCR}^\gamma_4\delta_1 \)-engineered \( \alpha\beta \)T cells produced cytokines IL-2 and IFN-\( \gamma \) when stimulated by HepG2 tumor cells.** The cytokines IL-2 and IFN-\( \gamma \) play important roles in antitumor activity. (22,23) We analyzed the IL-2 and IFN-\( \gamma \) expression of resting and nonresting \( \text{TCR}^\gamma_4\delta_1 \)-engineered \( \alpha\beta \)T cells and mock-engineered \( \alpha\beta \)T cells by flow cytometry after incubation with HepG2 tumor cells and treatment with BFA for 6 h. Results show that the percentage of resting \( \text{TCR}^\gamma_4\delta_1 \)-engineered \( \alpha\beta \)T cells expressing IL-2 and IFN-\( \gamma \) was 30.38% and 31.56%, respectively, higher than control groups (9.49% and 10.43%, respectively; Figure 3A). The percentage of nonresting \( \text{TCR}^\gamma_4\delta_1 \)-engineered \( \alpha\beta \)T cells producing IL-2 and IFN-\( \gamma \) was 33.66% and 31.16%, respectively, higher than the control group (14.63% and 10.78%, respectively; Figure 3B). Two different statuses of \( \text{TCR}^\gamma_4\delta_1 \)-engineered \( \alpha\beta \)T cells produced similar amounts of cytokines. In addition, \( \text{TCR}^\gamma_4\delta_1 \)-engineered \( \alpha\beta \)T cells expressed Ki67, a proliferation marker, significantly higher than control after stimulation with anti-\( \gamma\delta \)TCR antibody (Figure 3C). These findings indicate that \( \text{TCR}^\gamma_4\delta_1 \)-engineered \( \alpha\beta \)T cells
produce cytokines and proliferate similar to γδT cells. 

**TCRγδ1-engineered αβT cells neither expressed mixed TCR dimers nor bound/killed normal cells in vitro.**

Previous studies have shown that the TCR receptor is transported to the surface of T cells after the formation of complete TCR dimers-CD3 complex. (24) Thus, we transduced TCRγ4, or TCRδ1 chains alone, into αβT cells and analyzed surface expression. TCRγδ1-engineered αβT cells served as a positive control. We found no TCRγ4 or TCRδ1 expression on the surface of TCRγ4- or TCRδ1-engineered αβT cells. In contrast, TCRγδ1 was expressed on the surface of TCRγδ1-engineered αβT cells (Figure 4A). These data suggest that the introduced TCRγ4 or TCRδ1 chains did not pair with the endogenous TCRα or TCRβ chains to generate mixed TCR dimers. This feature would be beneficial in reducing the risk of autoimmunity. We also assessed the binding activity of TCRγδ1-Fc and the cytotoxicity of TCRγδ1-engineered αβT cells to some normal cells by flow cytometry and LDH assay. We found that TCRγδ1-Fc did not bind to PBMCs, 1308.1.86, ccc-HEL-1 or 293T cells (Figure 4B). The cytotoxicity of TCRγδ1-engineered αβT cells toward PBMCs, 1308.1.86, ccc-HEL-1 and 293T cells was 2.79% ± 2.04%, 8.11% ± 3.52%, 11.57% ± 1.89% and 15.53% ± 0.68% at an effector-to-target ratio of 10:1, respectively, compared with 4.04% ± 0.54% (p = 0.365), 9.10% ± 6.14% (p = 0.821), 8.13% ± 3.17% (p = 0.181) and 15.44% ± 0.30% (p = 0.850) of mock-engineered αβT cells, respectively (Figure 4C). The cytotoxicity of TCRγδ1-engineered αβT cells was in accordance with the binding ability of TCRγδ1-Fc to normal cells. We further evaluated the ability of mock- or TCRγδ1-engineered αβT cells to produce IFN-γ in response to normal cells by flow cytometry. No significant difference was observed between the 2 groups (Figure 4D). Taken together, these results indicate that TCRγδ1-engineered αβT cells lack an autoimmune response to several normal cells in vitro. 

**TCRγδ1-engineered αβT cells displayed antitumor activity in vivo.**

To assess the antitumor effect of TCRγδ1-engineered αβT cells in vivo, mice were subcutaneously injected with 2 × 10⁶ HepG2 cells in the rear right flank. After the tumor grew to approximately 100 mm³, mock- and TCRγδ1-engineered αβT cells were intratumorally injected into nude mice every 3 d a total of 4 times. Tumors were measured by a caliper every 3 d, starting on d 0. The tumor growth curve and the excised tumors show that TCRγδ1-engineered αβT cells had significantly higher tumor inhibiting effect compared with the control group. 

**Figure 3.** Cytokine production and proliferative capacity of TCRγδ1-engineered αβT cells. (A) Expression of IL-2 and IFN-γ in resting TCRγδ1-engineered αβT cells. Resting mock- and TCRγδ1-engineered αβT cells were incubated with HepG2 at an effector-to-target ratio of 3:1 for 6 h with BFA, and the expression of intracellular IL-2 and IFN-γ was determined by flow cytometry. (B) Expression of IL-2 and IFN-γ in the nonresting TCRγδ1-engineered αβT cells. The nonresting mock- and TCRγδ1-engineered αβT cells were incubated with HepG2 at an effector-to-target ratio of 3:1 for 6 h with BFA, and expression of intracellular IL-2 and IFN-γ was determined by flow cytometry. (C) Expression of intracellular Ki67 in TCRγδ1-engineered αβT cells. Mock- and TCRγδ1-engineered αβT cells were stimulated with plate-bound anti-TCRδ for 6 h, and the expression of Ki67 was examined by flow cytometry. Shown is a representative FCM analysis of cytokines and Ki67. (IL, interleukin; IFN, interferon; BFA, brefeldin A.)
significantly retarded the tumor growth of human HepG2 tumor cells in a nude mouse model. The tumor growth inhibition ratio was above 60% after d 9 and reached 73.26% at d 27. 

Figure 4. Determination of TCRγ4 or δ1 expression and binding/activity to normal cells of TCRγ4δ1-engineered αβT cells. (A) Cell surface expression of TCRδ1 and TCRγ4 chains on the TCRδ1- or TCRγ4-engineered αβT cells. TCRδ1- and TCRγ4-engineered αβT cells were separately stained with anti-human Vδ1-FITC antibody, and with rabbit anti-human TCRδ primary antibody and goat anti-rabbit IgG-FITC secondary antibody, and then assessed by flow cytometry. TCRγ4δ1 expression on the surface of TCRγ4δ1-engineered αβT cells served as a positive control. (B) Binding of TCRγ4δ1-Fc to normal cells including PBMCs, 1308.1.86, ccc-HEL-1 and 293T cells. After incubation with TCRγ4δ1-Fc, these normal cells were stained with FITC-conjugated goat anti-human IgG and then evaluated by flow cytometry. (C) Cytotoxicity of TCRγ4δ1-engineered αβT cells to PBMCs, 1308.1.86, ccc-HEL-1 and 293T. TCRγ4δ1-engineered αβT cells were incubated with these normal cells at an effector-to-target ratio of 10:1 for 6 h, and the cytolytic activity was detected by LDH assay. The data are representative of 3 independent experiments and expressed as the mean ± SD (ns, no significance). (D) Expression of IFN-γ in the TCRγ4δ1-engineered αβT cells. Mock- and TCRγ4δ1-engineered αβT cells were individually incubated with PBMCs, 1308.1.86, ccc-HEL-1 and 293T at an effector-to-target ratio of 3:1 for 6 h with BFA, and expression of intracellular IFN-γ was determined by flow cytometry. Shown is a representative FCM analysis of the IFN-γ and TCR chains. (PBMC, peripheral blood mononuclear cell.)

The tumor growth inhibition ratio was above 60% after d 9 and reached 73.26% at d 27. TCRγ4δ1-engineered αβT cells significantly retarded the tumor growth of human HepG2 tumor cells in a nude mouse model.
of a retroviral transfection method. First, lentiviruses mediate the integration of their genome into the DNA of the host dividing and nondividing cells, whereas retroviruses only infect dividing cells. (26–28) We found that lentiviral vector efficiently transduced TCRγδ1 genes into αβT cells. Approximately 36% of polyclonally activated αβT cells were lentivirally transduced with TCRγδ1. Second, lentiviruses have a lower risk of insertional mutagenesis compared with retroviruses. Lentiviruses preferentially concentrate in high gene density regions and integrate into transcriptional units, while retroviruses prefer to insert in the neighborhood of transcription start sites of active genes. (29–31) Third, lentiviral vectors are less prone to activate adjacent genes compared with retroviral vectors. (32,33)

We used peripheral blood–derived αβT cells as targets for transduction of TCRγδ1 based on several considerations. First, the efficiency of TCRγδ-surface expression is controlled by formation of the TCR-CD3 complex, and recognition signaling of TCRγδ and tumor antigens is delivered to intracellular structures in a CD3-dependent manner. CD3 molecules are naturally expressed in the αβT cells. Thus, it is not necessary to redirect TCRγδ-engineered αβT cells with the CD3 gene. (24) Second, transfer of TCRγδ to αβT cells, or transfer of TCRαβ to γδT cells, generates a large quantity of tumor-reactive T cells without the expression of mixed TCR dimers, avoiding an autoimmune response. (9,17) Therefore, TCRγδ1-engineered αβT cells avoid the expression of potentially harmful mixed TCR dimers. Formation of TCR dimers–CD3 complex is required for TCR transport to the surface of T cells. Here, we found that TCRδ1 and TCRγ chains were not expressed on the surface of TCRγδ1- and TCRγ4-transduced αβT cells, indicating that the introduced TCRδ1 and TCRγ4 chains did not pair with endogenous TCRα or TCRβ chains, which could reduce the risk of autoimmunity. Third, the main aim of the construction of TCR-modified T cells was to obtain tools to deliver genes into T cells, including the viral transfection system, electroporation and the transposase system. (25) Viral methods are common for gene delivery. We chose the lentiviral transduction system to avoid the drawbacks

**DISCUSSION**

In this study, αβT cells were redirected by lentiviral transduction of the TCRγδ1 gene to generate effective tumor-reactive T cells. To date, genetically engineered T cells have been developed via several methods, common for gene delivery. We chose the lentiviral transduction system to avoid the drawbacks of a retroviral transfection method. First, lentiviruses mediate the integration of their genome into the DNA of the host dividing and nondividing cells, whereas retroviruses only infect dividing cells. (26–28) We found that lentiviral vector efficiently transduced TCRγδ1 genes into αβT cells. Approximately 36% of polyclonally activated αβT cells were lentivirally transduced with TCRγδ1. Second, lentiviruses have a lower risk of insertional mutagenesis compared with retroviruses. Lentiviruses preferentially concentrate in high gene density regions and integrate into transcriptional units, while retroviruses prefer to insert in the neighborhood of transcription start sites of active genes. (29–31) Third, lentiviral vectors are less prone to activate adjacent genes compared with retroviral vectors. (32,33)

We used peripheral blood–derived αβT cells as targets for transduction of TCRγδ1 based on several considerations. First, the efficiency of TCRγδ-surface expression is controlled by formation of the TCR-CD3 complex, and recognition signaling of TCRγδ and tumor antigens is delivered to intracellular structures in a CD3-dependent manner. CD3 molecules are naturally expressed in the αβT cells. Thus, it is not necessary to redirect TCRγδ-engineered αβT cells with the CD3 gene. (24) Second, transfer of TCRγδ to αβT cells, or transfer of TCRαβ to γδT cells, generates a large quantity of tumor-reactive T cells without the expression of mixed TCR dimers, avoiding an autoimmune response. (9,17) Therefore, TCRγδ1-engineered αβT cells avoid the expression of potentially harmful mixed TCR dimers. Formation of TCR dimers–CD3 complex is required for TCR transport to the surface of T cells. Here, we found that TCRδ1 and TCRγ chains were not expressed on the surface of TCRγδ1- and TCRγ4-transduced αβT cells, indicating that the introduced TCRδ1 and TCRγ4 chains did not pair with endogenous TCRα or TCRβ chains, which could reduce the risk of autoimmunity. Third, the main aim of the construction of TCR-modified T cells was to obtain tools to deliver genes into T cells, including the viral transfection system, electroporation and the transposase system. (25) Viral methods are common for gene delivery. We chose the lentiviral transduction system to avoid the drawbacks

**Figure 5.** Antitumor activity of TCRγδ1-engineered αβT cells in vivo. Mice were subcutaneously injected with 2 × 10⁶ HepG2 cells in the rear right flank. When tumor volume reached approximately 100 mm³, mock- and TCRγδ1-engineered αβT cells were injected intratumorally every 3 d a total of 4 times, and the tumor volume in the right flank of each mouse was measured every 3 d until 30 d. (A) Mice treated with TCRγδ1-engineered αβT cells exhibited significant suppression of tumor volume compared with mice treated with mock-engineered αβT cells (P = 0.001). Black arrows indicate the treatment time; each point represents the mean ± SEM, **, P < 0.01. (B) The tumor growth inhibition ratio was estimated. (C) Image of the excised tumors in the experiment evaluating the influences of TCRγδ1-engineered αβT cells on antitumor activity in HepG2 tumor-bearing nude mice.
a sufficient number of tumor-reactive T cells for cancer immunotherapy. The αβ T cells, as genetic recipients, comprise 42% to 69% of PBMCs and rapidly expand upon stimulation of anti-CD3 and anti-CD28 antibodies. We observed that TCRγδ1-engineered αβ T cells significantly proliferated upon stimulation of anti-γδ TCR antibody. These data support our design of using αβ T cells as the targets of TCRγδ1 transduction.

T cells mediate antitumor reactivity in a TCR-dependent manner, driven by the binding of TCR with tumor antigen. Therefore, it is of utmost importance to screen for T cell receptors with a high affinity for tumor antigen and a broad spectrum of tumor recognition. Previously, we identified a high-frequency CDR3 sequence in the TCRγδ chain from TILs in human gastric cancer and found that this CDR3 was crucial to antigen recognition. We also found that TCRγδ1-Fc fusion proteins had a strong binding ability to the tumor-associated antigens MICA and UL-16 binding protein 5 (ULBP5) via ELISA and SPR assays. (18) These results suggest that TCRγδ1 possess a high affinity to antigen recognition. In addition, multiple tumor-expressed antigens, such as MICA, MHC class I chain-related molecules B (MICB) and various ULBPs, are recognized by the TCR of human Vδ1 T cells and are constitutively expressed at variable levels on many epithelial tumor cells and some leukemias and lymphomas. (19,34,35) We therefore explored the binding ability of TCRγδ1 and a variety of tumors, particularly epithelial tumors. Our previous study reported that TCRγδ1 displayed strong binding to BGC-823, G401, GLC-82, HT29 and SKOV3 cells and tissues. (18) In this study, we found consistent results showing that TCRγδ1s also significantly bound other tumor cells, including HepG2, BGC-803, K562 (Figures 2A and B). Our results support the notion that TCRγδ1 recognizes a large panel of tumors, especially epithelium-derived tumors. In summary, TCRγδ1 has a broad spectrum of tumor recognition.

Tumor-infiltrating Vδ1 T cells and peripheral blood-derived Vδ1 T cells exerted remarkable cytotoxicity against epithelium-derived tumors and some blood-derived tumors, such as renal carcinoma, colorectal cancer, pancreatic cancer and lymphoid leukemia. (34,36,37) However, the proliferative capacity and function of γδ T cells are significantly impaired in cancer patients. (38) Thus, TCRγδ1-engineered αβ T cells might be an attractive alternative strategy to kill tumors. Here, we found that TCRγδ1-engineered αβ T cells displayed striking cytolytic activity against HepG2, BGC-803 and K562 in a TCRγδ-dependent manner. Importantly, adoptive transfer of TCRγδ1-engineered αβ T cells into nude mice bearing a human HepG2 cell line significantly suppressed tumor growth. These findings display a similar effect when compared with previous reports showing that TCRγδ2-modified αβ T cells/PBMCs exerted cytotoxicity against a variety of tumors. (9,10) However, Vδ1 γδ T cells reside preferentially in the intestine and in the skin epithelium, and their Vδ1 TCRs recognize protein antigens, notably MICA and ULBP3, which are generally expressed on epithelial tumor cells. In contrast, Vγδ2 cells mainly appear in the blood and their TCRγδ2 recognizes phosphoantigens. (39) This suggests that TCRγδ1-engineered αβ T cells preferentially kill epithelial tumors compared with TCRγδ2-engineered αβ T cells/PBMCs.

Furthermore, we showed that the transduced TCRγδ1 chain gene did not pair with the endogenous TCRα or TCRβ chains to generate new mixed TCR heterodimers. This is advantageous in reducing the risk of autoimmunity. TCRγδ1 primarily recognizes stress-induced molecules, such as MICA and ULBPs. The expression of these stress-induced molecules is upregulated under heat shock, viral or mycobacterial infection, or oxidative stress. (40-42) Expression of MICA and MICB on epithelial cells can be stress-induced to regulate protective responses through the Vδ1 γδ T cells. (43) Thus, cancer patients with severe inflammation should avoid adoptive TCRγδ1-engineered αβ T cell transfer therapy unless inflammation is eliminated. These stress-induced molecules are rarely expressed on normal cells under physiological conditions. Most normal human tissues do not express MICA, but glandular and gastric epithelial cells do, which occurred in the intracellular location and was not transported to the cell surface. (44,45) Here, we show that PBMCs, ccc-HEL-1, 1308.1.86 and 293T normal cells did not induce any activation of TCRγδ1-engineered αβ T cells in a TCRγδ-dependent manner, measured by IFN-γ expression and cytotoxicity. These analyses support the hypothesis that TCRγδ1-engineered αβ T cells do not attack normal cells through TCRγδ1 recognition, implying that TCRγδ1 possesses a high specificity for tumors. Although we cannot preclude autoimmunity resulting from allologous TCRαβ recognition or complicated human physiological conditions in vivo, TCRγδ1-engineered αβ T cells are a relatively safe strategy for cancer therapy. Our further research will focus on knockdown of allologous TCRαβ receptors and in vivo autoimmunity.

TCRγδ1-engineered αβ T cells are effective killer cells that express much IL-2 and IFN-γ upon incubation with HepG2 cells. IL-2 displays pleiotropic effects on the immune system, promoting T cell proliferation and differentiation, downregulation of IL-2-induced expansion of regulatory T cells, and inhibition of tumor growth. (46,47) IFN-γ secretion enhances the immunogenicity of tumors by increasing MHC expression on tumor cells and TCR recognition of tumor cells, and inhibits the induction of CD4 + CD25 + regulatory T cells, providing a positive feedback loop for antitumor reactivity. Importantly, IFN-γ is beneficial for the suppression of tumor cells in vivo. (23,48,49)

The main challenge of this study was to improve the transduction rate and expression level of TCRγδ1 in αβ T cells. Previous reports suggested that the creation of high-avidity TCR-engineered
T cells was determined by the TCR cell-surface makeup of T cells, and that transport of the TCR to the plasma membrane depended on complete assembly of the TCR/CD3 complex. (24,50) In TCRγδ1-engineered αβT cells, competition between TCRγδ1 and TCRαβ for association with CD3 could restrict their expression levels on the cell surface. Thus, the upregulation of TCRγδ1 cell surface expression is advantageous in reducing the surface expression of endogenous TCRαβ. In summary, the increased transduction rate and expression level of TCRγδ1 in αβT cells will enhance the efficacy of TCRγδ1-engineered αβT cells and decrease the risk of autoimmunity resulting from allogeneic TCRαβ recognition.

CONCLUSION
Our results highlight a novel TCRγδ-engineering αβT cell-effector ACT therapy for solid tumors, with safety and strong antitumor efficacy, and with the clinical potential to treat cancer patients.

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DISCLOSURE
The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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