

TCR γ 4 δ 1-Engineered $\alpha\beta$ T Cells Exhibit Effective Antitumor Activity

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T cell engineering with T cell receptors (TCRs) specific for tumors plays an important role in adoptive T cell transfer (ATC) therapy for cancer. Here, we present a novel strategy to redirect peripheral blood-derived $\alpha\beta$ T cells against tumors via TCR γ 4 δ 1 gene transduction. The broad-spectrum antitumor activity of TCR δ 1 cells in innate immunity is dependent on CDR3 δ 1. TCR γ 4 δ 1-engineered $\alpha\beta$ T 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 TCR γ 4 δ 1 genes were transduced to approximately 36% of polyclonal $\alpha\beta$ T cells. TCR γ 4 δ 1-engineered $\alpha\beta$ T cells exhibited effective *in vitro* TCR $\gamma\delta$ -dependent cytotoxicity against various tumor cells via the perforin-granzyme pathway. They also showed a strong proliferative capacity and robust cytokine production. TCR γ 4 δ 1-engineered $\alpha\beta$ T cells neither expressed mixed TCR dimers nor bound/killed normal cells *in vitro*. More important, adoptive transfer of TCR γ 4 δ 1-engineered $\alpha\beta$ T cells into nude mice bearing a human HepG2 cell line significantly suppressed tumor growth. Our results demonstrate a novel role for TCR γ 4 δ 1 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 effector cells 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 TCR $\gamma\delta$ gene therapy as an alternative approach.

Compared with TCR $\alpha\beta$, which is highly specific for its antigen, TCR $\gamma\delta$ displays characteristics of innate immunity, directly recognizing many stress-induced antigens in an MHC-independent manner in the early stages of inflammation and tumorigenesis. (7) Human $\gamma\delta$ T cells are grouped into 2 major subsets, V δ 1 and V δ 2 T cells. V δ 1 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. V δ 2 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 TCR $\gamma\delta$, TCR $\gamma\delta$ gene transduction into effector T cells, such as $\alpha\beta$ T cells, may be an attractive therapeutic approach. It appears to resolve the fundamental problem of tumor targeting not found in TCR $\alpha\beta$. Previous studies by other groups and our laboratory have confirmed that TCR γ 9 δ 2-transduced $\alpha\beta$ T cells, or TCR γ 9 δ 2-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.

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Transduction of tumor antigen-reactive TCR into T cells is one strategy to acquire sufficient T cells. Antigen-specific TCR $\alpha\beta$ -modified CD8 + $\alpha\beta$ T cells display significant antitumor activity. (11) However, a potential disadvantage of TCR $\alpha\beta$ -modified $\alpha\beta$ T cells is the formation of new mixed TCRs. Specifically, the introduced TCR α or TCR β chains can pair with the endogenous TCR α or TCR β 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 TCR $\alpha\beta$ -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 $\gamma\delta$ T cells as recipient cells transduced with exogenous TCR $\alpha\beta$. (13,14) TCR $\alpha\beta$ -engineered $\gamma\delta$ T cells exhibited no evidence of TCR mispairing; TCR δ or TCR γ in $\gamma\delta$ T cells did not exchange chains with TCR $\alpha\beta$. (15,16) Other approaches, except for complete knockdown of TCR α and TCR β chains, reduced but did not completely prevent mispairing. Nevertheless, other major drawbacks of TCR $\alpha\beta$ s 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 TCR $\delta 1$ 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 TCR $\delta 1$ with GTM and TCR $\gamma 4$ chains were amplified and paired to form TCR $\gamma\delta 1$ receptor. We confirmed TCR $\gamma\delta 1$ -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 TCR $\gamma\delta 1$ has broad-spectrum tumor recognition. Due to the high prevalence and diversity of TCR $\gamma\delta 1$ ligands on malignant cells, we reasoned that TCR $\gamma\delta 1$ targeting various ligands as genetic donors to modify effector cells is a more promising approach compared with specific single-chain antibody fragments (scFv) or TCR $\alpha\beta$ 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 $\alpha\beta$ T cells against various tumors via a broadly tumor-reactive TCR $\gamma 4\delta 1$ gene transfer method.

Here we show for the first time that TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T cells exhibit significant antitumor activity *in vitro* and *in vivo*. Moreover, TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T cells neither expressed mixed TCR dimers nor bound/killed normal cells *in vitro*. These results indicate that TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T 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 mmol/L L-glutamine and 1 mmol/L sodium pyruvate. K562 and Raji cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FCS, 100 U/mL penicillin-streptomycin, 1 mmol/L L-glutamine and 1 mmol/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 TCR $\gamma 4$ and TCR $\delta 1$ chains with a tumor antigen-specific CDR3 region identified from TILs of gastric carcinoma tissues was been previously described. (18) Briefly, the V region of TCR $\delta 1$ was amplified using cDNA from the TILs of gastric carcinoma tissues as a template with V $\delta 1$ - and C $\delta 1$ -specific primers, and subcloned into a pGEM-T easy vector for sequence analysis. We identified one high-frequency CDR3 $\delta 1$ -specific binding to gastric carcinoma tissues as a critical region (termed GTM). Finally, full-length TCR $\delta 1$ with GTM and TCR $\gamma 4$ chains were amplified from cDNA of gastric tumor-derived $\gamma\delta$ TILs by PCR using full-length TCR $\delta 1$ and TCR $\gamma 4$ 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 TCR $\delta 1$ and TCR $\gamma 4$ genes were cloned individually and co-cloned into pCDH vectors containing the marker genes copGFP to obtain pCDH-TCR $\delta 1$, pCDH-TCR $\gamma 4$ and pCDH-TCR $\gamma 4\delta 1$. 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 $\alpha\beta$ T Cells and Lentiviral Transfer

The $\gamma\delta$ T cells of PBMCs were removed by positive selection using FITC-labeled $\gamma\delta$ T-cell-specific immunomagnetic beads (Miltenyi, Germany). PBMCs without $\gamma\delta$ T cells were stimulated for 72 h with anti-CD3 and anti-CD28 in the presence of 200 IU/mL IL-2. The $\alpha\beta$ T cells were transfected with TCR $\gamma 4\delta 1$ -lentivirus,

TCR δ 1-lentivirus, *TCR γ 4*-lentivirus and mock-lentivirus, using 10 μ g/mL polybrene (Sigma, USA) and then cultured in RPMI-1640 with 10% FCS plus 400 IU/mL IL-2 for 7 d. TCR γ 4 δ 1, TCR δ 1 and TCR γ 4 surface molecule staining was performed separately with anti-human TCR $\alpha\beta$ -PE and anti-human TCR $\gamma\delta$ -APC antibodies (Biolegend, USA), anti-human V δ 1-FITC antibody (Thermo, USA), and rabbit anti-human TCR γ 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 TCR γ 4 δ 1-Fc to Tested Cells

TCR γ 4 δ 1-Fc was constructed by fusing the complete extracellular domain of TCR γ 4 δ 1 to the constant region of human IgG1 (18) and expressed by Sino Biological Inc. For flow cytometry: To examine the binding of TCR γ 4 δ 1-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 TCR γ 4 δ 1-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 by 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 TCR γ 4 δ 1-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 *TCR γ 4 δ 1*-engineered

$\alpha\beta$ T cells against target cells including HepG2, PBMCs, 1308.1.86, ccc-HEL-1 and 293T cells. Effector cells comprised *TCR γ 4 δ 1*-engineered $\alpha\beta$ T cells, mock-engineered $\alpha\beta$ T cells, *TCR γ 4 δ 1*-engineered $\alpha\beta$ T cells with resting treatment and mock-engineered $\alpha\beta$ T 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 permeabilized with 1 \times 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 μ g/mL anti-TCR $\gamma\delta$ antibody for 2 h at 37°C. After washing, 5 \times 10⁵ mock- and *TCR γ 4 δ 1*-engineered $\alpha\beta$ T 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 \times 10⁴ per well. Transfected effector cells including mock- and *TCR γ 4 δ 1*-engineered $\alpha\beta$ T 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 *TCR γ 4 δ 1*-engineered $\alpha\beta$ T 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 *TCR γ 4 δ 1*-engineered $\alpha\beta$ T cells *in vivo*, approximately 2 \times 10⁶ HepG2 cells in 100 μ L of PBS was subcutaneously injected into the rear right flank of BALB/c nu/nu mice. After the tumor grew to approximately 100 mm³, mice bearing HepG2 tumors were randomly divided into 2 groups and intratumorally injected with *TCR γ 4 δ 1*-engineered $\alpha\beta$ T cells (1 \times 10⁶, n = 8) or mock-engineered $\alpha\beta$ T cells (1 \times 10⁶, 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:

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

The tumor growth inhibition ratio (D%) was estimated according to the following formula: D% = (Vc - Vt) / Vc \times 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 \pm 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 \pm SEM. *p* value < 0.05 is regarded as significant.

RESULTS

The Lentiviral Vector Efficiently Transduced TCR $\gamma\delta 1$ into Peripheral Blood-Derived $\alpha\beta$ T Cells

We previously identified a high-frequency CDR3 dominant sequence (CDR3 $\delta 1$: CAFLPHADKLIFGKG), termed GTM, in TCR $\delta 1$ chain from TILs in human gastric cancer through RT-PCR and analysis of a large number of CDR3 $\delta 1$ sequences. We confirmed that the CDR3 $\delta 1$ peptide played a crucial role in tumor antigen recognition and bound to a wide variety of tumor cell lines and tissues similar to intact TCR $\gamma\delta 1$. (18) The full-length TCR $\delta 1$ with GTM and TCR $\gamma 4$ chains were amplified from cDNA of a gastric tumor-derived $\gamma\delta$ TILs by PCR and paired to form TCR $\gamma\delta 1$ (18) (Figure 1A). Using the amplified TCR $\delta 1$ and TCR $\gamma 4$ DNAs from pREP9-TCR $\delta 1$ with GTM and pREP7-TCR $\gamma 4$, we constructed pCDH-TCR $\gamma\delta 1$, a lentiviral vector harboring the TCR $\delta 1$ and TCR $\gamma 4$ chain DNAs. The TCR $\gamma 4$ gene was controlled by the cytomegalovirus (CMV) promoter (Figure 1B).

To examine whether peripheral blood-derived $\alpha\beta$ T cells can be lentivirally engineered with TCR $\gamma\delta 1$, a lentiviral vector with TCR $\gamma\delta 1$ was transduced into pre-activated peripheral blood-derived $\alpha\beta$ T cells. TCR $\gamma\delta 1$ expression was determined by staining with specific TCR $\gamma\delta$ and TCR $\alpha\beta$ antibodies. Approximately 36% of polyclonally activated $\alpha\beta$ T cells expressed TCR $\gamma\delta 1$ (Figure 1C), indicating that TCR $\gamma\delta 1$ s were efficiently transduced.

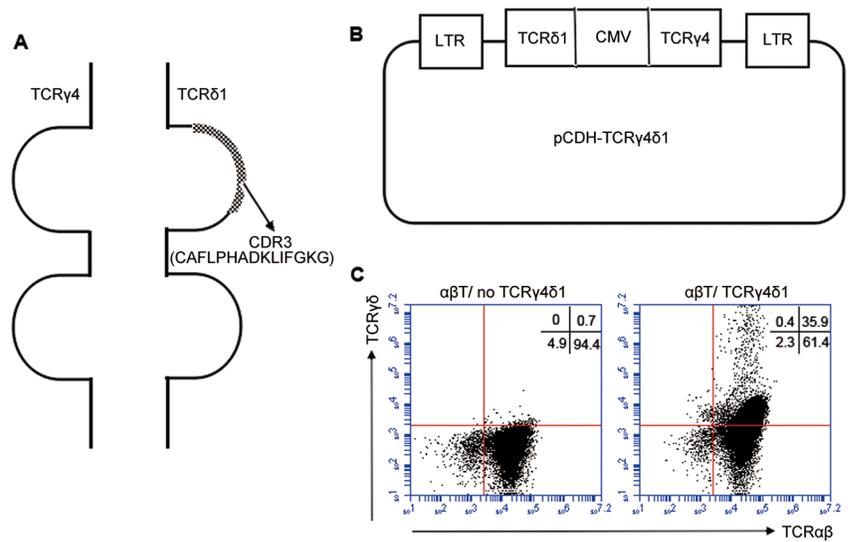


Figure 1. The lentiviral vector efficiently transduced TCR $\gamma\delta 1$ into peripheral blood-derived $\alpha\beta$ T cells. (A) Schematic diagram of TCR $\gamma\delta 1$ receptor. The V region of TCR $\delta 1$ was amplified using RNA from the $\gamma\delta$ TILs of gastric carcinoma tissues by RT-PCR. A high-frequency CDR3 $\delta 1$ sequence, termed GTM, was identified by sequence analysis. The full-length TCR $\delta 1$ with GTM and TCR $\gamma 4$ chains were amplified from cDNA of one case of gastric tumor-derived $\gamma\delta$ TILs by PCR and paired to form TCR $\gamma\delta 1$. (B) Schematic diagram of the lentiviral vector containing the TCR $\delta 1$ and TCR $\gamma 4$ genes. The TCR $\delta 1$ and TCR $\gamma 4$ genes were amplified by PCR and co-cloned into pCDH lentiviral vector. (LTR, long terminal repeats; CMV, cytomegalovirus promoter.) (C) The cell-surface expression of TCR $\gamma\delta 1$ in $\alpha\beta$ T cells. Peripheral blood-derived $\alpha\beta$ T cells were lentivirally transduced with either mock lentiviral vectors or lentiviral vectors with TCR $\gamma\delta 1$, then these cells were separately stained with APC-conjugated anti-TCR $\gamma\delta$ and PE-conjugated anti-TCR $\alpha\beta$ 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.)

TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells were cytotoxic to several tumor cells *in vitro*. To assess binding of TCR $\gamma\delta 1$ to tumor cells *in vitro*, we determined the binding capacity of TCR $\gamma\delta 1$ -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 TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells to tumor cells. We found significant cytotoxicity to HepG2 (55.78 \pm 10.39%) and BGC-803 (67.36 \pm 5.30%) tumor cells, and less activity against K562 tumor cells (41.33 \pm 1.28%)

at an effector-to-target ratio of 10:1. In comparison, mock-engineered $\alpha\beta$ T cells had significantly less cytotoxicity against HepG2 22.26 \pm 12.56% (*p* = 0.024), BGC-803 32.56 \pm 15.01% (*p* = 0.019) and K562 19.29 \pm 5.25% (*p* = 0.002) (Figure 2C). Raji cells were not lysed and served as a negative control. We found that the cytotoxicity of TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells was proportionate to the binding ability of TCR $\gamma\delta 1$ -Fc to tumor cells. This indicates that TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells exert cytolytic activity against HepG2, BGC-803 and K562 tumor cells in a TCR $\gamma\delta 1$ -dependent manner.

Cytotoxicity of TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells to tumor cells is related to perforin-granzyme. Previous reports showed that T cells kill tumors through 2 main mechanisms, Fas-FasL and

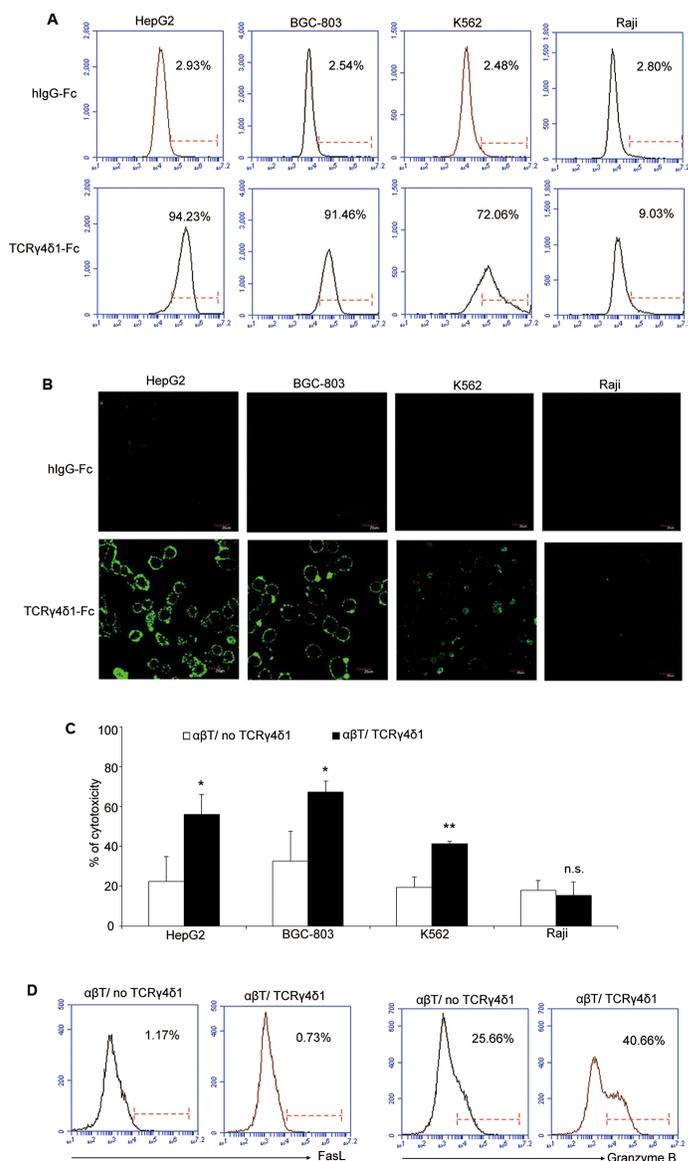


Figure 2. Cytotoxicity of *TCRγ4δ1*-engineered $\alpha\beta$ T cells to tumor cells *in vitro*. (A) Flow cytometry profiles of the binding of *TCRγ4δ1*-Fc fusion protein to the tumor cell lines HepG2, BGC-803, K562 and Raji. After incubation with *TCRγ4δ1*-Fc, tested cells were stained with FITC-conjugated goat anti-human IgG and assessed by flow cytometry. (B) Confocal images of the binding of the *TCRγ4δ1*-Fc fusion protein to the tumor cell lines HepG2, BGC-803, K562 and Raji. After incubation with *TCRγ4δ1*-Fc, tested cells were stained with FITC-conjugated goat anti-human IgG and detected by laser scanning confocal microscopy. (C) Cytolytic activity of *TCRγ4δ1*-engineered $\alpha\beta$ T cells against tumor cells. *TCRγ4δ1*-engineered $\alpha\beta$ T cells were incubated with HepG2, BGC-803, K562 and Raji target cells at an effector-to-target ratio of 10:1 for 6 h. Cytotoxicity was analyzed by the LDH cytotoxicity detection kit. The data are representative of 3 independent experiments and expressed as the mean \pm SD. *, $P < 0.05$; **, $P < 0.01$ (ns, no significance). (D) Expression of FasL and granzyme B in *TCRγ4δ1*-engineered $\alpha\beta$ T cells was determined by flow cytometry. Mock- and *TCRγ4δ1*-engineered $\alpha\beta$ T cells were cultured with HepG2 at an effector-to-target ratio of 10:1 for 6 h, and the cellular surface molecule FasL and intracellular granzyme B were analyzed by flow cytometry. A representative FCM analysis of cytotoxic molecules is shown. (LDH, lactate dehydrogenase.)

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 *TCRγ4δ1*-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 *TCRγ4δ1*-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 *TCRγ4δ1*-engineered $\alpha\beta$ T cells kill tumor cells via the perforin-granzyme pathway.

***TCRγ4δ1*-engineered $\alpha\beta$ T cells produced cytokines IL-2 and IFN- γ when stimulated by HepG2 tumor cells.** The cytokines IL-2 and IFN- γ play important roles in antitumor activity. (22,23) We analyzed the IL-2 and IFN- γ expression of resting and nonresting *TCRγ4δ1*-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 *TCRγ4δ1*-engineered $\alpha\beta$ T cells expressing IL-2 and IFN- γ was 30.38% and 31.56%, respectively, higher than control groups (9.49% and 10.43%, respectively; Figure 3A). The percentage of nonresting *TCRγ4δ1*-engineered $\alpha\beta$ T cells producing IL-2 and IFN- γ was 33.66% and 31.16%, respectively, higher than the control group (14.63% and 10.78%, respectively; Figure 3B). Two different statuses of *TCRγ4δ1*-engineered $\alpha\beta$ T cells produced similar amounts of cytokines. In addition, *TCRγ4δ1*-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 *TCRγ4δ1*-engineered $\alpha\beta$ T cells

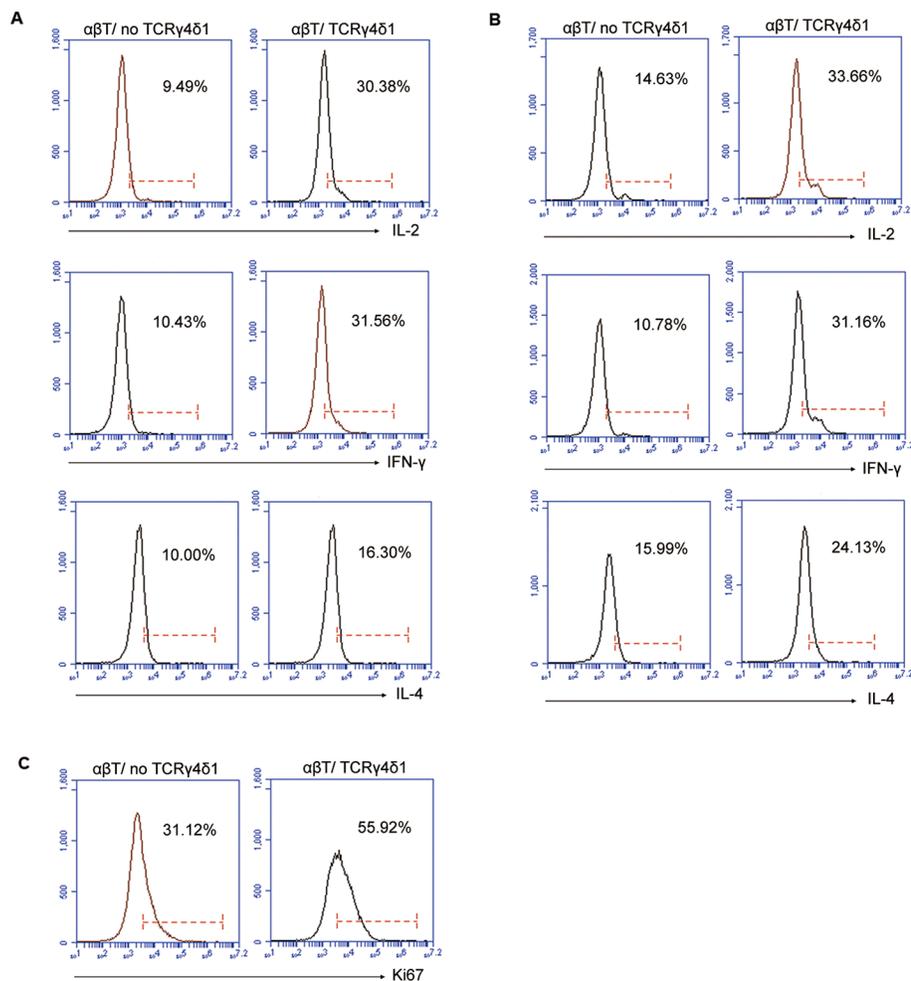


Figure 3. Cytokine production and proliferative capacity of TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells. (A) Expression of IL-2 and IFN- γ in resting TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells. Resting mock- and TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells were incubated 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 TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells. The nonresting mock- and TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T 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 TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells. Mock- and TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells were stimulated with plate-bound anti-TCR $\gamma\delta$ 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.)

produce cytokines and proliferate similar to $\gamma\delta$ T cells.

TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T 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 TCR $\gamma 4$, or TCR $\delta 1$ chains alone, into $\alpha\beta$ T cells and analyzed surface expression. TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T cells served as a positive control. We found no TCR $\gamma 4$ or TCR $\delta 1$ expression on the surface of TCR $\gamma 4$ - or TCR $\delta 1$ -engineered $\alpha\beta$ T cells. In contrast,

TCR $\gamma\delta 1$ was expressed on the surface of TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T cells (Figure 4A). These data suggest that the introduced TCR $\gamma 4$ or TCR $\delta 1$ chains did not pair with the endogenous TCR α or TCR β chains to generate mixed TCR dimers. This feature would be beneficial in reducing the risk of autoimmunity. We also assessed the binding activity of TCR $\gamma\delta 1$ -Fc and the cytotoxicity of TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T cells to some normal cells by flow cytometry and LDH assay. We found that TCR $\gamma\delta 1$ -Fc did not bind to PBMCs, 1308.1.86, ccc-HEL-1 or 293T cells (Figure 4B). The cytotoxicity of TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T cells toward PBMCs, 1308.1.86, ccc-HEL-1 and 293T cells was $2.79\% \pm 2.04\%$, $8.11\% \pm 3.52\%$, $11.57\% \pm 1.89\%$ and $15.53\% \pm 0.68\%$ at an effector-to-target ratio of 10:1, respectively, compared with $4.04\% \pm 0.54\%$ ($p = 0.365$), $9.10\% \pm 6.14\%$ ($p = 0.821$), $8.13\% \pm 3.17\%$ ($p = 0.181$) and $15.44\% \pm 0.30\%$ ($p = 0.850$) of mock-engineered $\alpha\beta$ T cells, respectively (Figure 4C). The cytotoxicity of TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T cells was in accordance with the binding ability of TCR $\gamma\delta 1$ -Fc to normal cells. We further evaluated the ability of mock- or TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T 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 TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T cells lack an autoimmune response to several normal cells *in vitro*.

TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T cells displayed antitumor activity *in vivo*.

To assess the antitumor effect of TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T cells *in vivo*, mice were subcutaneously injected with 2×10^6 HepG2 cells in the rear right flank. After the tumor grew to approximately 100 mm³, mock- and TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T 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 TCR $\gamma 4\delta 1$ -engineered $\alpha\beta$ T cells had significantly higher tumor inhibiting effect compared with the control group

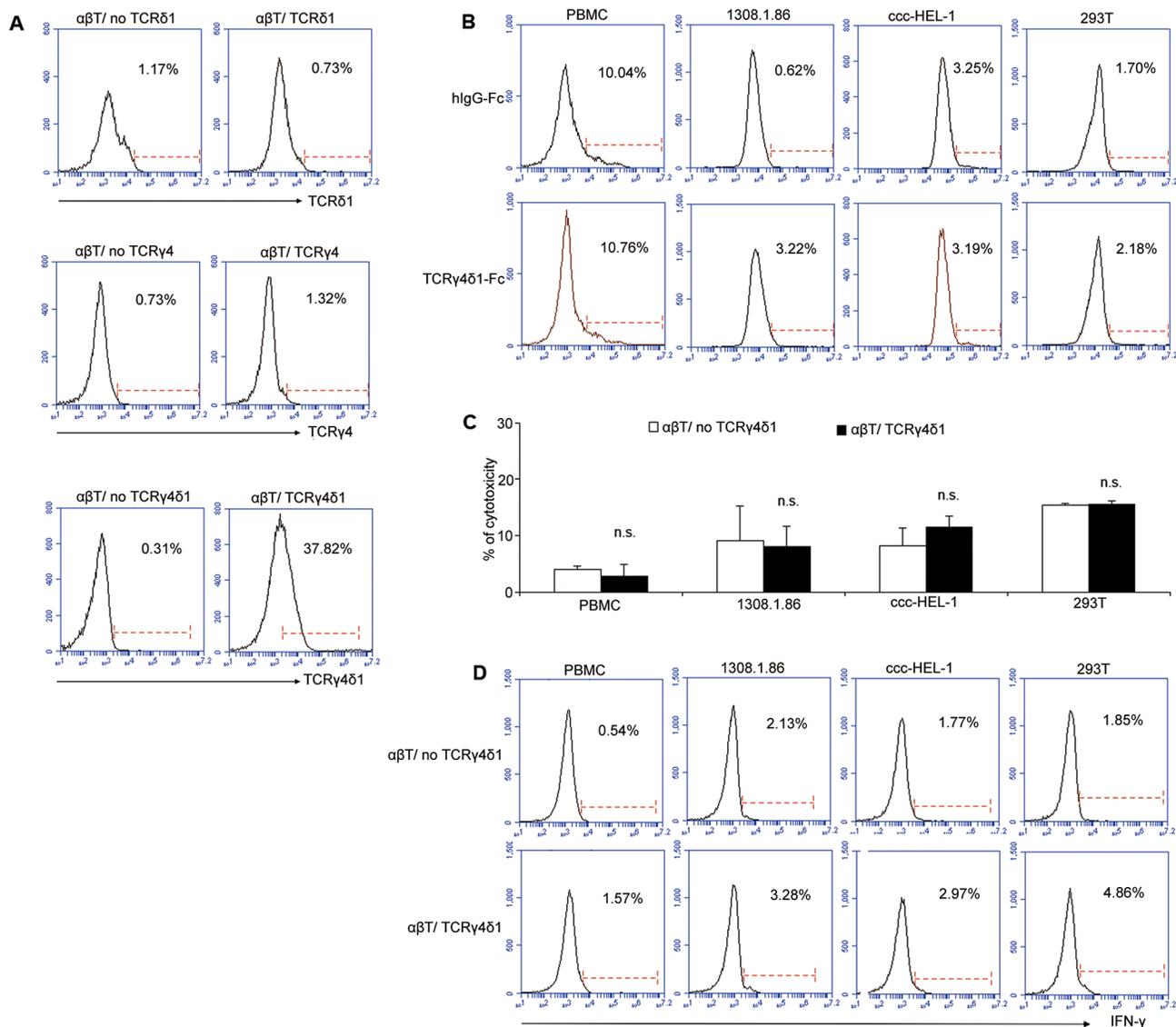


Figure 4. Determination of TCR γ 4 or δ 1 expression and binding/activity to normal cells of TCR γ 4 δ 1-engineered $\alpha\beta$ T cells. (A) Cell surface expression of TCR δ 1 and TCR γ 4 chains on the TCR δ 1- or TCR γ 4-engineered $\alpha\beta$ T cells. TCR δ 1- and TCR γ 4-engineered $\alpha\beta$ T cells were separately stained with anti-human V δ 1-FITC antibody, and with rabbit anti-human TCR γ primary antibody and goat anti-rabbit IgG-FITC secondary antibody, and then assessed by flow cytometry. TCR γ 4 δ 1 expression on the surface of TCR γ 4 δ 1-engineered $\alpha\beta$ T cells served as a positive control. (B) Binding of TCR γ 4 δ 1-Fc to normal cells including PBMCs, 1308.1.86, ccc-HEL-1 and 293T cells. After incubation with TCR γ 4 δ 1-Fc, these normal cells were stained with FITC-conjugated goat anti-human IgG and then evaluated by flow cytometry. (C) Cytotoxicity of TCR γ 4 δ 1-engineered $\alpha\beta$ T cells to PBMCs, 1308.1.86, ccc-HEL-1 and 293T. TCR γ 4 δ 1-engineered $\alpha\beta$ T 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 \pm SD (ns, no significance). (D) Expression of IFN- γ in the TCR γ 4 δ 1-engineered $\alpha\beta$ T cells. Mock- and TCR γ 4 δ 1-engineered $\alpha\beta$ T 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.)

($P = 0.001$; Figures 5A and C). The tumor growth inhibition ratio was calculated, and its curve is illustrated in Figure 5B.

The tumor growth inhibition ratio was above 60% after d 9 and reached 73.26% at d 27. TCR γ 4 δ 1-engineered $\alpha\beta$ T cells

significantly retarded the tumor growth of human HepG2 tumor cells in a nude mouse model.

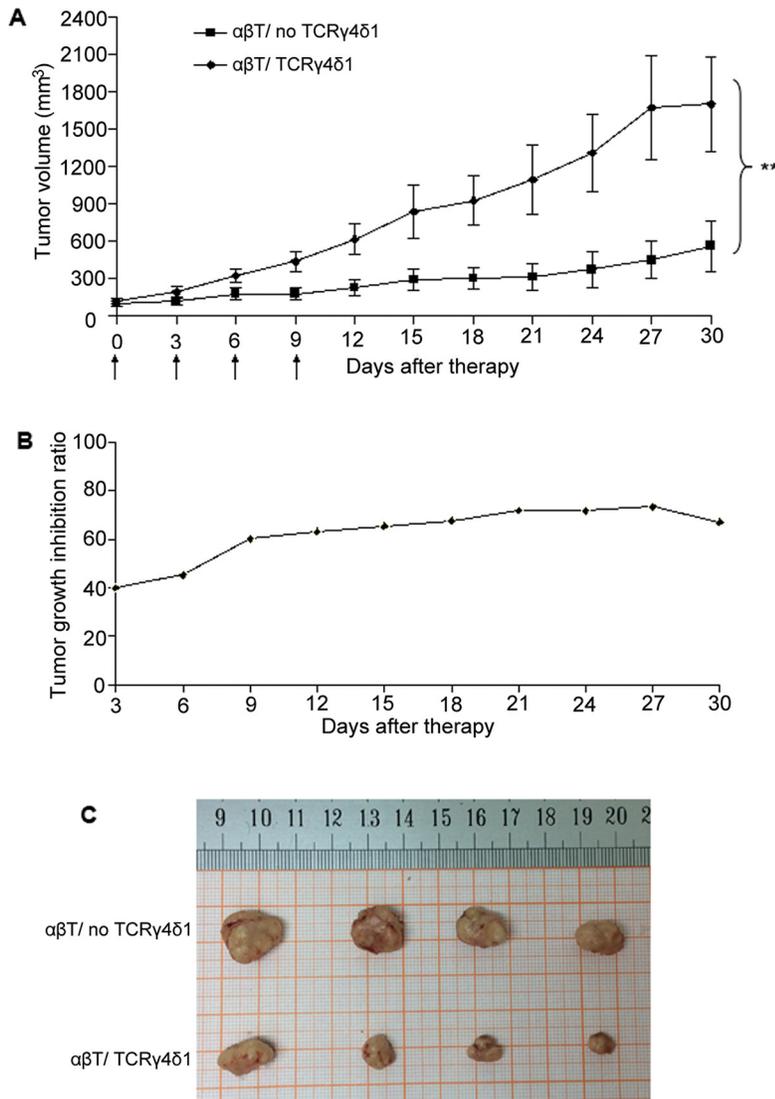


Figure 5. Antitumor activity of TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells *in vivo*. Mice were subcutaneously injected with 2×10^6 HepG2 cells in the rear right flank. When tumor volume reached approximately 100 mm³, mock- and TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells were injected 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 TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells exhibited significant suppression of tumor volume compared with mice treated with mock-engineered $\alpha\beta$ T cells ($P = 0.001$). Black arrows indicate the treatment time; each point represents the mean \pm 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 TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells on antitumor activity in HepG2 tumor-bearing nude mice.

DISCUSSION

In this study, $\alpha\beta$ T cells were redirected by lentiviral transduction of the TCR $\gamma\delta 1$ gene to generate effective tumor-reactive T cells. To date, genetically engineered T cells have been developed via several

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

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 TCR $\gamma\delta 1$ genes into $\alpha\beta$ T cells. Approximately 36% of polyclonally activated $\alpha\beta$ T cells were lentivirally transduced with TCR $\gamma\delta 1$. 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 $\alpha\beta$ T cells as targets for transduction of TCR $\gamma\delta 1$ based on several considerations. First, the efficiency of TCR $\gamma\delta 1$ -surface expression is controlled by formation of the TCR-CD3 complex, and recognition signaling of TCR $\gamma\delta 1$ and tumor antigens is delivered to intracellular structures in a CD3-dependent manner. CD3 molecules are naturally expressed in the $\alpha\beta$ T cells. Thus, it is not necessary to redirect TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells with the CD3 gene. (24) Second, transfer of TCR $\gamma\delta 1$ to $\alpha\beta$ T cells, or transfer of TCR $\alpha\beta$ to $\gamma\delta$ T cells, generates a large quantity of tumor-reactive T cells without the expression of mixed TCR dimers, avoiding an autoimmune response. (9,17) Therefore, TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T 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 TCR $\delta 1$ and TCR $\gamma 4$ chains were not expressed on the surface of TCR $\delta 1$ - and TCR $\gamma 4$ -transduced $\alpha\beta$ T cells, indicating that the introduced TCR $\delta 1$ and TCR $\gamma 4$ chains did not pair with endogenous TCR α or TCR β chains, which could reduce the risk of autoimmunity. Third, the main aim of the construction of TCR-modified T cells was to obtain

a sufficient number of tumor-reactive T cells for cancer immunotherapy. The $\alpha\beta$ 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 *TCR $\gamma\delta$ 1*-engineered $\alpha\beta$ T cells significantly proliferated upon stimulation of anti- $\gamma\delta$ TCR antibody. These data support our design of using $\alpha\beta$ T cells as the targets of *TCR $\gamma\delta$ 1* 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 TCR δ 1 chain from TILs in human gastric cancer and found that this CDR3 was crucial to antigen recognition. We also found that TCR $\gamma\delta$ 1-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 TCR $\gamma\delta$ 1 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 V δ 1 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 TCR $\gamma\delta$ 1 and a variety of tumors, particularly epithelial tumors. Our previous study reported that TCR $\gamma\delta$ 1 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 TCR $\gamma\delta$ 1s also significantly bound other tumor cells, including HepG2, BGC-803, K562 (Figures 2A and B). Our results support the notion that TCR $\gamma\delta$ 1 recognizes a large panel of tumors, especially epithelium-derived tumors. In summary, TCR $\gamma\delta$ 1 has a broad spectrum of tumor recognition.

Tumor-infiltrating V δ 1 T cells and peripheral blood-derived V δ 1 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 $\gamma\delta$ T cells are significantly impaired in cancer patients. (38) Thus, *TCR $\gamma\delta$ 1*-engineered $\alpha\beta$ T cells might be an attractive alternative strategy to kill tumors. Here, we found that *TCR $\gamma\delta$ 1*-engineered $\alpha\beta$ T cells displayed striking cytolytic activity against HepG2, BGC-803 and K562 in a TCR $\gamma\delta$ -dependent manner. Importantly, adoptive transfer of *TCR $\gamma\delta$ 1*-engineered $\alpha\beta$ 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 *TCR γ 9 δ 2*-modified $\alpha\beta$ T cells/PBMCs exerted cytotoxicity against a variety of tumors. (9,10) However, V δ 1 $\gamma\delta$ T cells reside preferentially in the intestine and in the skin epithelium, and their V δ 1 TCRs recognize protein antigens, notably MICA and ULBP3, which are generally expressed on epithelial tumor cells. In contrast, T γ 9 δ 2 cells mainly appear in the blood and their TCR γ 9 δ 2 recognizes phosphoantigens. (39) This suggests that *TCR $\gamma\delta$ 1*-engineered $\alpha\beta$ T cells preferentially kill epithelial tumors compared with *TCR γ 9 δ 2*-engineered $\alpha\beta$ T cells/PBMCs.

Furthermore, we showed that the transduced TCR γ 4 or TCR δ 1 chain gene did not pair with the endogenous TCR α or TCR β chains to generate new mixed TCR heterodimers. This is advantageous in reducing the risk of autoimmunity. TCR $\gamma\delta$ 1 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 V δ 1 $\gamma\delta$ T cells. (43) Thus, cancer patients

with severe inflammation should avoid adoptive *TCR $\gamma\delta$ 1*-engineered $\alpha\beta$ 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 *TCR $\gamma\delta$ 1*-engineered $\alpha\beta$ T cells in a TCR $\gamma\delta$ -dependent manner, measured by IFN- γ expression and cytotoxicity. These analyses support the hypothesis that *TCR $\gamma\delta$ 1*-engineered $\alpha\beta$ T cells do not attack normal cells through TCR $\gamma\delta$ 1 recognition, implying that TCR $\gamma\delta$ 1 possesses a high specificity for tumors. Although we cannot preclude autoimmunity resulting from allologous TCR $\alpha\beta$ recognition or complicated human physiological conditions *in vivo*, *TCR $\gamma\delta$ 1*-engineered $\alpha\beta$ T cells are a relatively safe strategy for cancer therapy. Our further research will focus on knock-down of allologous TCR $\alpha\beta$ receptors and *in vivo* autoimmunity.

TCR $\gamma\delta$ 1-engineered $\alpha\beta$ 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 TCR $\gamma\delta$ 1 in $\alpha\beta$ 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 TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells, competition between TCR $\gamma\delta 1$ and TCR $\alpha\beta$ for association with CD3 could restrict their expression levels on the cell surface. Thus, the upregulation of TCR $\gamma\delta 1$ cell surface expression is advantageous in reducing the surface expression of endogenous TCR $\alpha\beta$. In summary, the increased transduction rate and expression level of TCR $\gamma\delta 1$ in $\alpha\beta$ T cells will enhance the efficacy of TCR $\gamma\delta 1$ -engineered $\alpha\beta$ T cells and decrease the risk of autoimmunity resulting from allogeneic TCR $\alpha\beta$ recognition.

CONCLUSION

Our results highlight a novel TCR $\gamma\delta$ -engineering $\alpha\beta$ T 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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