SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
IMMUNOTHERAPY
IMMUNOSURVEILLANCE

Received
15 November 2013

Accepted
29 November 2013

Published
6 January 2014

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Anti-melanoma activity of T cells redirected with a TCR-like chimeric antigen receptor

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Genetically modified T cells to recognize tumor-associated antigens by transgenic TCRs or chimeric antigen receptors (CAR) have been successfully applied in clinical trials. However, the disadvantages of either TCR mismatching or the requirement of a surface tumor antigen limit their wider applications in adoptive T cell therapy. A TCR-like chimeric receptor, specific for the melanoma-related gp100/HLA-A2 complex was created by joining a TCR-like antibody GPA7 with the endodomains of CD28 and CD3-ζ chain. This TCR-like CAR, GPA7-28z, was subsequently introduced into human T cells. Retargeted T cells expressing GPA7-28z could exhibit efficient cytotoxic activities against human melanoma cells *in vitro* in the context with HLA-A2. Furthermore, infusion of GPA7-28z-transduced T cells suppressed melanoma progression in a xenograft mouse model. Redirecting human T cells with TCR-like CARs would be a promising alternative approach to TCR-mediated therapy for melanoma patients, which is also feasible for targeting a variety of other tumor antigens.

T cells, as promising effector cells for adoptive cell therapy, could detect transformed cells through the specific recognition between T-cell receptors (TCRs) and peptide/human leukocyte antigen (peptide/HLA) complexes[1]. These peptides are derived from tumor-associated antigens (TAAs) which are mutant proteins or over-expressed proteins exist in malignant cells[2]. An increasing number of TAAs have been identified by T-cell epitope cloning, together with advanced genomic, transcriptomic and proteomic technologies[3]. Among these TAAs, melanocyte differentiation antigen glycoprotein 100 (gp100) is of particular interest because it is over-expressed in melanoma (>90%)[4] and highly immunogenic[5]. The TCR α and β chains from the gp100-reactive T-cell clones have been isolated and subsequently used to transduce patients' lymphocytes, which induced a 19% objective tumor regression rate in 16 treated patients with melanoma[6]. Despite of its clinical efficacy, further development of adoptive therapy based on transgenic TCR has been limited due to the difficulty in TCR acquisition and the potential danger of TCR mispairing[7].

To obviate the obstacles of transgenic TCR, several groups including us generated antibodies with a TCR-like specificity instead of natural αβTCR[8–11]. These TCR-like antibodies bind TAA-derived peptide in a HLA-restricted manner, mimicking the recognition of TCR to a particular MHC complex on tumor cells. Utilizing phage-display selection, TCR-like antibodies could be feasibly selected entirely *in vitro*, dramatically increasing the efficiency of selection compared with isolation of TCR. Furthermore, like traditional monoclonal antibodies, TCR-like antibodies could also be fused with intracellular signal transduction moieties to generate TCR-like chimeric antigen receptors (CARs)[12,13]. Expressed on the surface of T cells, the TCR-like CAR could mediate specific interaction between T cells and target tumor cells. Once the TCR-like antibody binds to a tumor-associated peptide/HLA complex on cell surface of tumor, the intracellular domain of TCR-like CAR transduces activating and proliferating signals to trigger T cells to lyse target tumor cells. Therefore, this TCR-like CAR could execute TCR's function while circumventing the drawbacks of transgenic TCR.

Previously, we have successfully produced a TCR-like antibody GPA7 specific for $gp100_{209-217}$/HLA-A2 complex[11]. GPA7 was a single-domain antibody (sdAb), containing only one antigen-binding domain derived from llama VHH antibody. To utilize the melanoma-targeting property of GPA7, we fused GPA7 gene with an

intracellular signaling unit, thus generating a TCR-like CAR. Subsequently, this TCR-like CAR was introduced into human primary T cells. We demonstrated that the transgenic T cells could stably express TCR-like CARs and recognize $gp100_{209-217}$ epitopes on melanoma cells in a HLA-A2 restricted manner. Furthermore, TCR-like CARs containing GPA7 could mediate the enhanced cytotoxicity of transgenic T cells against HLA-A2-matched melanoma *in vitro* and in *vivo*. These findings would serve as a base for future clinical trials in patients with melanoma and creation of other TCR-like CARs for a variety of tumors.

Results

Construction and expression of TCR-like chimeric receptor. To create engineered T cells specifically targeting melanoma-related antigen gp100/HLA-A2, we constructed a TCR-like CAR based on GPA7 antibody. GPA7 was a sdAb selected from a llama-derived VHH library by phage display. This antibody could specifically bind $gp100_{209-217}$/HLA-A2 complex in a TCR-like manner with a moderate affinity (K_D = 183 nM)[11]. Then, a TCR-like CAR was constructed based on the specificity of GPA7 sdAb. The GPA7-28z CAR was generated to comprise a GPA7 sdAb, human IgG1 hinge region, and transmembrane-intracellular domains of CD28 linked to the signaling motif of CD3-ζ chain (Figure 1a). Subsequently, PBMCs from healthy donors were transduced with lentiviruses encoding GPA7-28z. Three days after transduction, the expression of gp100/HLA-A2-specifc CAR was measured by flow cytometry using gp100/HLA-A2 tetramer and anti-hIgG1 antibody (Figure 1b). About 35% of cells transduced with GPA7-28z lentiviral vector were CAR positive.

***In vitro* antigen-specific expansion of GPA7-28z-postive T cells.** A fast expansion protocol (Figure 2a) was developed because the CAR-positive fraction of transduced cell culture is not big enough at 72 hours post-infection time point and non-specific expansion of engineered T cells is relatively slow. After transduction, cell cultures were re-stimulated immediately with irradiated gp100-loaded T2 cells. gp100-pulsed T2 cells presented high level of gp100/HLA-A2 complex could specifically activate GPA7-28z-positve T cells, thus enhancing antigen-specific expansion. In this setting, GPA7-28z-transduced T cell increased up to 25 ~ 30 folds after one-round of stimulation (the cell number was counted on day 14), while mock-transduced T cells retained the same expansion kinetics as that under non-specific expansion protocol. The fraction of GPA7-28z-positve T cells was also raised to around 70% (Figure 2b). These results indicate that the proliferation of T cells expressing GPA7-28z CAR could be specifically triggered by T2 cells loaded with gp100 peptide. In addition, the ratio of GPA7-28z$^+$ CD8$^+$ T cells to GPA7-28z$^+$ CD4$^+$ was about 3.5.

GPA7-28z mediates peptide specific response toward gp100-loaded T2 cells and melanoma cells in a HLA-A2 restricted manner. To analyze the response specifically triggered by peptide-loaded T2 cells, the expanded T cells were initially tested for specific IFN-γ release against T2 cells either pulsed with gp100 peptide or irrelevant peptide by ELISPOT assay. After incubation with gp100-pulsed T2 cells, GPA7-28z T cells secreted large amount of IFN-γ cytokine (Figure 3a). As expected T2 cells loaded with flu peptide as control failed to stimulate GPA7-28z transduced T cells ($P < 0.01$, compared with gp100-pulsed T2).

To evaluate the ability of GPA7-28z T cells to respond to melanoma cells, IFN-γ secretions in ELISPOT assays were compared for stimulation of GPA7-28z T cells after co-cultured with various melanoma cells. Granzyme B release is a quantitative indicator of cytotoxic activity for T cells. In response to HLA-A2-positive melanoma cell lines Malme-3m and MEL-624, GPA7-28z-transduced T cells significantly up-regulated the granzyme B secretion, compared with

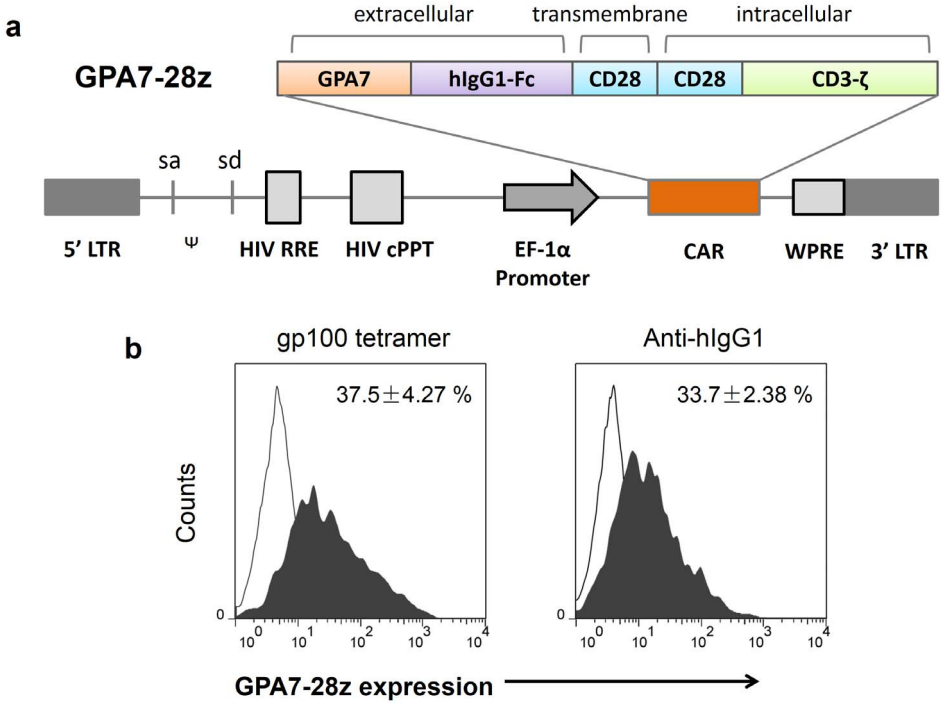

Figure 1 | Lentiviral vector design and expression of GPA7-28z transgene in human primary T cells. (a) Schemetic diagram of lentiviral vector used for GPA7-28z expression. GPA7 represents a single domain antibody; hIgG1-Fc, CH2-CH3 hinge domains of human IgG1; CD28, CD28 transmembrane and intracellular domain; CD3-ζ, CD3 ζ chain intracellular domain. (b) Cell surface expression of chimeric antigen receptor GPA7-28z by human primary T cells 72 hours after transduction. CAR-transduced cells were stained with gp100-HLA-A2 tetramer-PE and a mouse anti-human IgG1-FITC to recognize the CAR (filled black histograms). Mock-transduced cell culture was stained as control (open histograms). Representatives of flow cytometry plots of three independent repeats are shown. Frequencies of positive cells are shown as mean ± s.d. (n = 3) in the upper right corner.

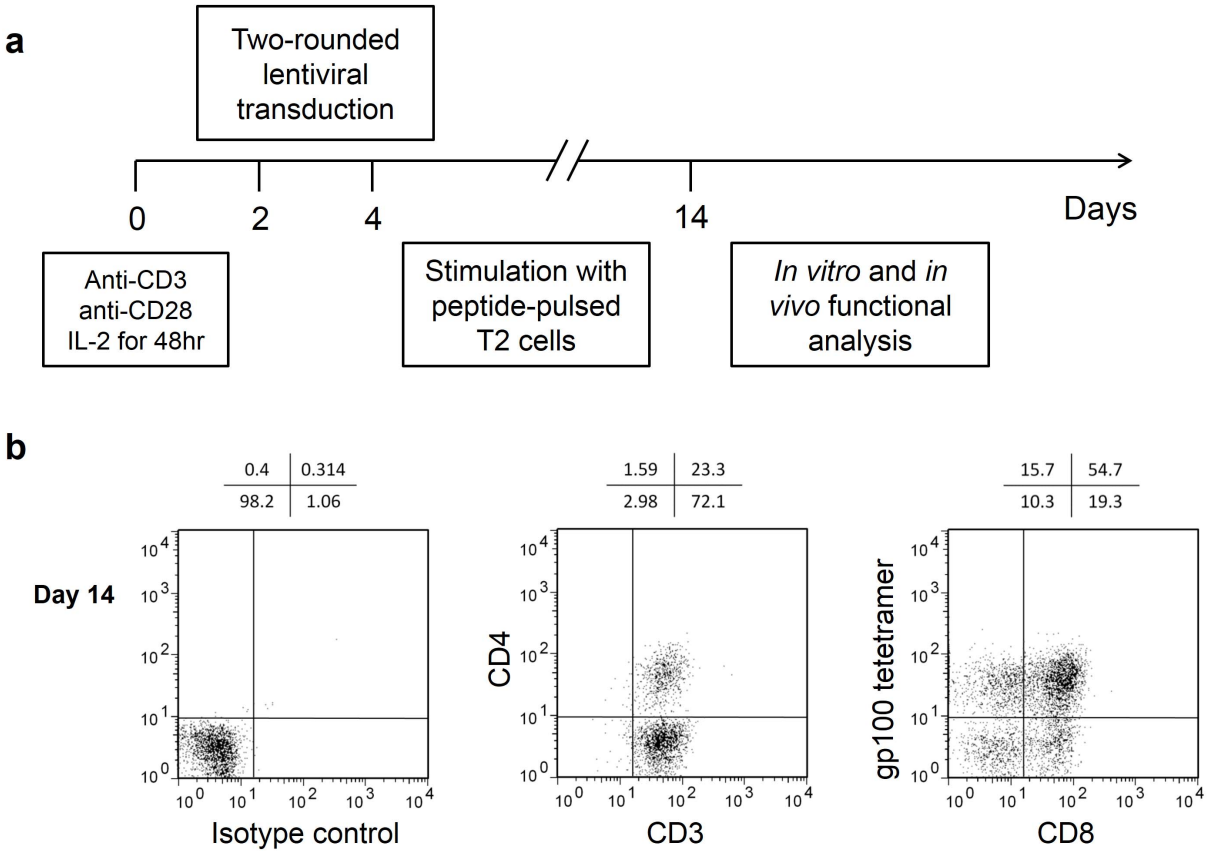

Figure 2 | Co-culture of GPA7-CD28/ζ transduced PBMC with gp100-pulsed T2 cells. (a) Schematic illustration of PBMC stimulation, transduction and expansion protocol. After lentiviral transduction, PBMC were cultured and re-stimulated with irradiated antigen-loaded T2 cells from day 4. (b) Cell surface phenotype of transduced T-cell cultures after a round of re-stimulation with gp100-loaded T2 cells. Anti-CD3-PE, anti-CD4-FITC, anti-CD8-FITC and PE-labeled gp100-HLA-A2 tetramer were used for characterization. Control was isotype-stained cells. Frequencies for each population were indicated above panels. A representative of three independent repeats from flow cytometry plots is shown.

mock-transduced T cells ($P < 0.01$) (Figure 3b). To assess the potential of gene-modified T cells for clinical application, two primary melanoma cells from patients, named Primary 1 and Primary 2 were tested for stimulation of the modified T cells. The expression of HLA-A2 complex on cell surface of Primary 1 and Primary 2 were confirmed[11]. Similar to an established A2-positive melanoma, Primary 1 and Primary 2 stimulated GPA7-28z-expressing T cells to produce a comparable amount of granzyme B. Background responses of mock-transduced T cells or of engineered T cells to HLA-A2-negative targets (MEL-888, SK-MEL-28 and B16F10) were relatively low, confirming the specificity of the interaction.

Both GPA7-28z and mock-transduced T cells could be stimulated by PHA, confirming their capacity to respond. Co-incubation of GPA7-28z transduced T cells with established HLA-A2-positive melanoma cell lines (Malme-3m and MEL-624) or primary melanoma cells (Primary 1 or Primary 2) induced high level of IFN-γ secretion (Figure 3c). As expected, GPA7-28z T cells did not respond to HLA-A2-negative melanoma cell lines MEL-888, SK-MEL-28 and B16F10. Similarly, mock-transduced T cells as control did not produce IFN-γ after HLA-A2 positive melanoma stimulation, demonstrating specific receptor-ligand interaction was required.

Primary human GPA7-28z T cells exhibited enhanced anti-tumor activity *in vitro*. The peptide-specific lysis of engineered T cell was initially examined in a 4-hour cytotoxicty assay. The cytotoxicity of GPA7-28z T cells against T2 cells loaded with various concentrations of peptides was evaluated at E/T ratio of 1. On Figure 4a it shows that GPA7-28z could mediate T cells to selectively lyse gp100-pulsed T2 cells even at a low peptide concentration of 10^{-4} nM. A clear dose-effect response was demonstrated for gp100 peptide. A similar experiment was performed using flu peptide as a negative control. There was no dose-effect of specific lysis at various peptide concentrations observed. Functional analysis of T cells referred to the peptide concentration producing a 50% killing of the maximum response (EC_{50}). Therefore, the peptide titration revealed that GPA7-28z T cells possessed a functional avidity of 0.3 nM.

To further demonstrate that a GPA7-28z-mediated T cell response toward gp100 was restricted in HLA-A2 presentation, we prepared T2 cells loaded with or without 20 μM of gp100 and then incubated them with GPA7-28z T cells to test their specific lysis rates. To confirm specificity a mouse anti-HLA-A2 antibody BB7.2 was used to block HLA-A2 -restricted recognition. T2 cells without peptide, but with antibody present, served as a control for any potential non-specific antibody-mediated cytotoxicity. As shown in Figure 4b, Admistration of anti-HLA-A2 antibody could significantly block the specific lysis of GPA7-28z T cells ($P < 0.01$, $n = 3$). This result demonstrated that the specific cytotoxicity mediated by TCR-like CAR GPA7-28z was HLA-A2 -restricted.

Furthermore, all HLA-A2-positive melanoma cells were efficiently lysed by GPA7-28z-transduced T cells, demonstrated in a 4-hour cytotoxicty assay (Figure 4c). Mock-transduced T cells showed negligible cytotoxicity against the same target cells ($P < 0.05$). HLA-A2-negative melanoma cells could hardly be lysed by either GPA7-28z or mock-transduced T cells ($P > 0.05$).

***In vivo* anti-tumor activity of retargeted T cells against established melanoma xenografts.** We next evaluated the ability of GPA7-28z T cells to induce regression of an established human melanoma *in vivo*.

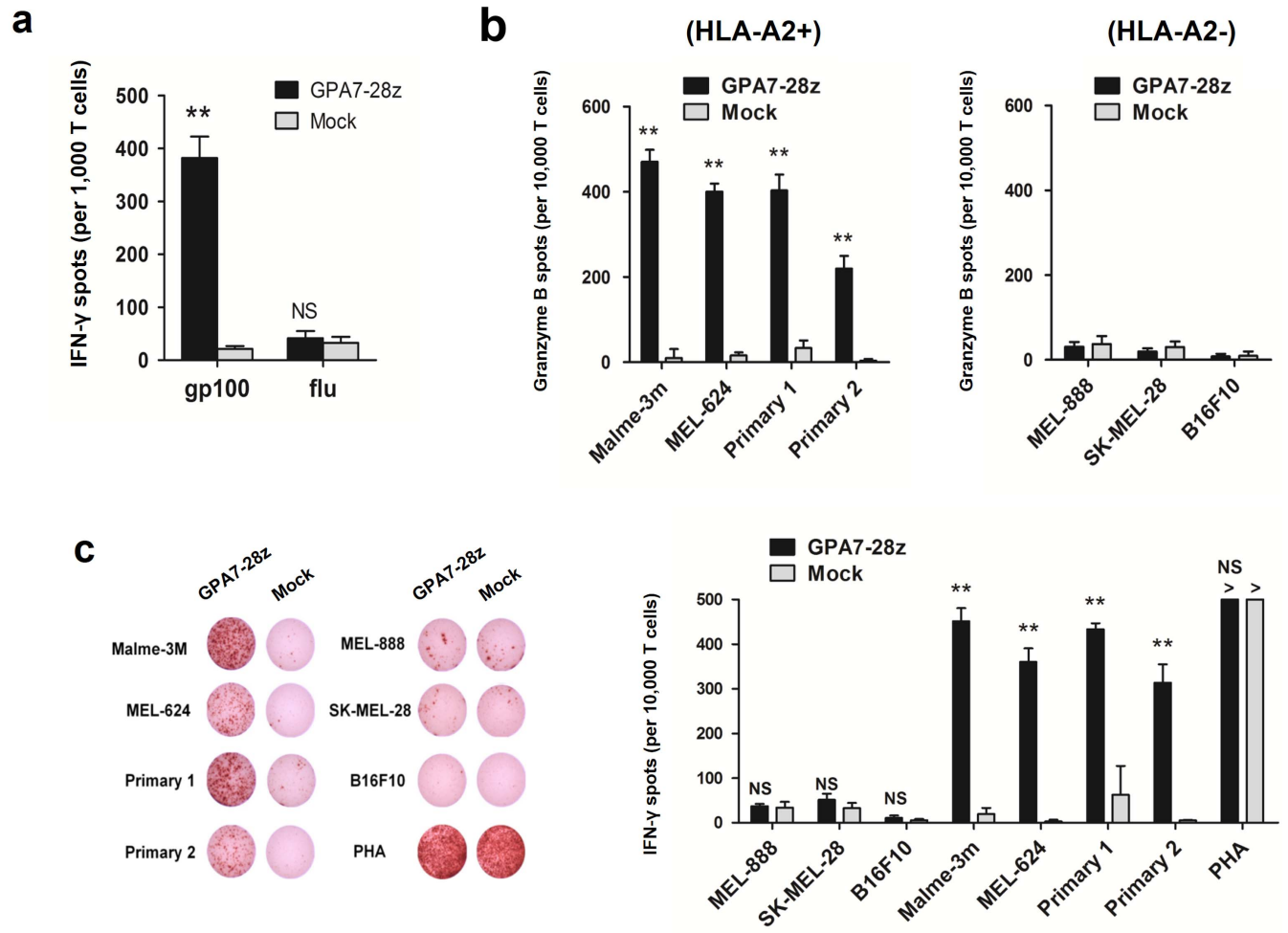

Figure 3 | GPA7-28z T cells exhibit a high functional activity in a peptide-specific and HLA-I restricted manner. (a) Cytokine secretions of transduced T cells were analyzed for antigen specificity in IFN-γ ELISpot. T2 cells were pulsed with $gp100_{209–217}$ or control $Flu_{58–66}$ peptide before incubation with GPA7-28z T cells. Mock-transduced T cells served as a negative control. IFN-γ release by GPA7-28z *vs* mock-transduced T cells was investigated. Data are presented as mean ± s.d. Asterisks indicate significant differences to control mock-transduced T cells (n = 4; Student's *t*-test; $^{**}P < 0.01$). (b) Granzyme B secretion by GPA7-28z and mock-transduced T cells in response to the stimulation with Malme-3m, MEL-624, Primary 1, Primary 2, MEL-888, SK-MEL-28 and B16F10. Data are presented as mean ± s.d. from four replicates ($^{**}P < 0.01$ versus T-Mock, Student's *t*-test). A representative experiment of three was shown. (c) IFN-γ release by GPA7-28z T cells compared with that by mock-transduced T cells (control) in response to the exposure to various melanoma cell lines, primary melanoma cell cultures and gp100-pulsed T2 cells. One representative well for each group (left panel) and calculated spot numbers (right panel) are shown. Asterisks indicate significant differences to control mock-transduced T cells (n = 4, $^{**}P < 0.01$, Student's *t*-test). A representative experiment of three was shown.

To monitor tumor growth *in vivo*, we expressed the firefly luciferase (*Luc*) gene in a HLA-A2-positive melanoma cell line Malme-3m. SCID-Beige mice were subcutaneously inoculated with 1×10^6 *Luc* expressing Malme-3m cells per mouse. On day 5, tumor-bearing mice were imaged by a bioluminescence imaging system for *Luc* expression, and the mice were divided into two groups randomly. Upon detection of palpable tumors, mice were received intratumoral injections of 5×10^6 GPA7-28z T cells or mock-transduced T cells on day 8 and day 13. On Figure 5a the results from three representative mice from the mock-transduced and GPA7-28z T cells cohorts were showed. Tumors grew consistently from day 5 in mice receiving mock-transduced T cells (n = 6) (Figure 5a, b). By contrast, mice receiving GPA7-28z T lymphocytes experienced a rapid tumor regression within 2 ~ 3 days of injection ($P < 0.01$), and the tumor growth in this group was significant delayed. Finally, a significant survival advantage was observed after the treatment with GPA7-28z T cells over that with mock-transduced T cells (Figure 5c). Median survival time of the mice receiving the mock-transduced T cells or GPA7-28z T cells was 28 d and 43.5 d, respectively ($P < 0.01$).

Discussion

Adoptive cell therapy (ACT) is one of the best available treatments for patients with metastatic melanoma[18,19]. Objective tumor regression was observed in 49% ~ 72% patients with metastatic melanoma after receiving tumor-infiltrating lymphocyte (TIL) transfer therapy[20]. Despite these successes, ACT based on TILs has some disadvantages. There is a difficulty to isolate or expand enough cells for adoptive transfer in many cases[21]. More importantly, anti-tumor efficacy of naturally occurring TILs appears to be restricted to melanoma, which precludes the application for TIL-based ACT[7]. The gene modification of T cells with TAA-specific TCRs could confer PBMCs from most patients with a desired anti-tumor activity *in vitro*. Theoretically, all types of tumor cells could be lysed by engineered T cells recognizing cognate TAA. However, only a few tumor-related high-affinity TCRs have been reported, due to the complicated procedure for selection and evolution. In addition, transgenic TCRs raise a safety concern about TCR mispairing. Pairing of one endogenous chain with one transgenic chain may generate a mixed TCR with unpredicted specificity. It has been documented *in vitro*[22] and in a murine model[23], in which mispaired TCRs were shown to drive 'off-

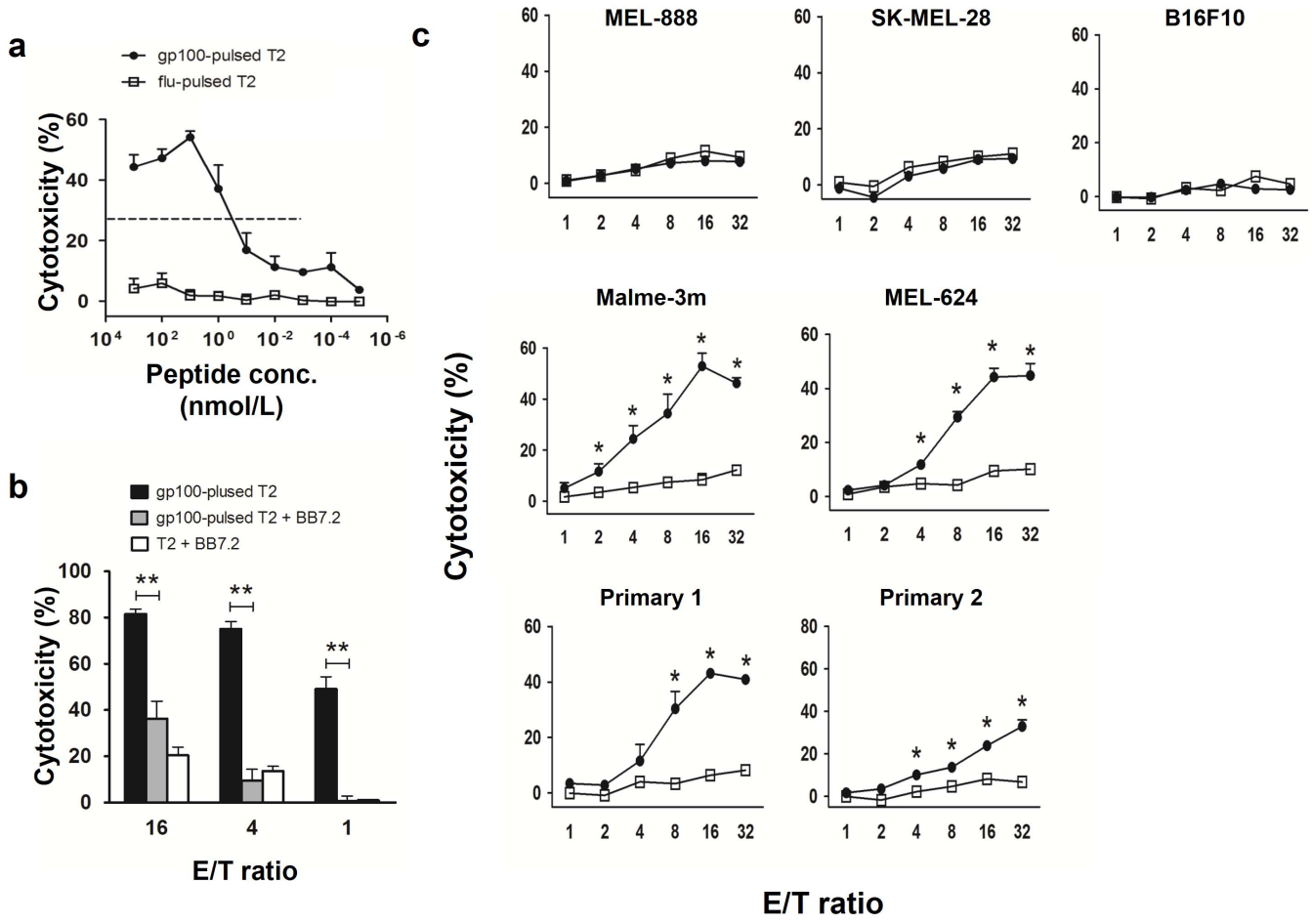

Figure 4 | CAR transduced, GPA7-28z T cells efficiently lyse T2 cells pulsed with gp100 peptides as well as HLA-A2 positive melanoma cells. (a) Specificity and functional avidity assessment of GPA7-28z T cells. GPA7-28z T cells were incubated with calcein-AM-labeled T2 cells at E/T ratio of 1, in the presence of different concentrations of gp100 peptide (●) or flu peptide (□). Functional avidity was defined as the peptide concentration generating 50% of the maximum specific response. Data are presented as mean ± s.d. (n = 3; Student's *t*-test). (b) GPA7-28z-mediated cytotoxicity toward gp100 was blocked by a specific antibody to HLA-A2. T2 target cells incubated with gp100 peptide (black), with gp100 peptide and BB7.2 antibody (gray), or T2 cells without peptide, but plus antibody (white) were plated with GPA7-28z T cells in a 4-hour cytotoxicity assay at E/T ratios from 16 to 1. Four replicates were used for each dilution of effector cells. Data are presented as mean ± s.d. (n = 3; one-way ANOVA followed by Tukey's post-hoc test was used for statistic analysis of the data; **P < 0.01). (c) GPA7-28z T cells exert enhanced cytotoxic lysis against HLA-A2-positive melanoma cell lines and primary melanoma cells. Cytotoxic ability of GPA7-28z (●) and mock-transduced (□) T cells towards Malme-3m, MEL-624, Primary 1, Primary 2, MEL-888, SK-MEL-28 or B16F10 was compared at different E/T ratio between 1 to 32. Data are presented as mean ± s.d. calculated from three repeated experiments (*$P < 0.05$ versus T-Mock, Student's *t*-test).

target' effects and cause an autoimmune syndrome. In this study, we developed a chimeric receptor that mimicked the specificity of TCRs while bypassing these limitations. Compared with the scarce clinical samples with which TCRs were selected from, the antigen-binding domains of TCR-like CARs were generated using a phage-display library with a large diversity of molecules (greater than 10^9). This diversity enables us to obtain antibodies specific for tumor-related peptide/HLA complex within a short period of time. Besides, TCR-like CARs are not subjected to mispairing, because they are single molecules that do not interact with endogenous TCR chains.

Furthermore, TCR-like CAR also broadened the spectrum of CAR surveillance. Infusions of CAR-redirected T cells have yielded therapeutic responses in patients with hematopoietic tumors[24,25]. Traditional CARs are mainly derived from mouse monoclonal antibodies (mAbs). Antigens recognized by mAbs are required to be expressed on cell surface of tumor, thus intracellular melanoma-differentiation antigens such as gp100 and MART-1 could not be targeted by previous CAR structures. In this aspect, TCR-like CARs are superior to traditional CARs, which could recognize both extracellular and intracellular antigens that are processed and presented extra-cellularly in the complex formed with HLA molecules. Therefore, TCR-like CARs are able to recognise both surface and cytoplasmic TAA in tumor as peptide/HLA complex. In addition, the presence of soluble antigen (e.g. CD30 or carcinoembryonic antigen) shed by malignant or normal cells might compete with tumor recognition and/or function by traditional CARs[26].

In melanoma, gp100 is a most commonly recognized TAA, which is strongly expressed in more than 90% of melanoma tumors, regardless of stage[4]. Recognition of gp100 by natural existing TILs[27,28] and TCR-transduced T[6] cells has shown strong correlations with clinical responses to adoptive therapies. Based on these clinical efficacies, TCR-like antibodies are developed against gp100/HLA complex to target melanoma cells. Previous studies have reported that TCR-like antibodies against gp100/HLA could be expressed in *E. coli* as naked antibodies used for antigen-presentation study[10] or as immunotoxins to kill melanoma[9]. Here, we first demonstrated a chimeric receptor based on TCR-like antibody could redirect primary human T cells to specifically recognize and lyse melanoma cells. T cells expressing GPA7-28z construct showed a selective recognition to gp100/HLA-A2 complex presented on cell surface. GPA7-28z-transduced

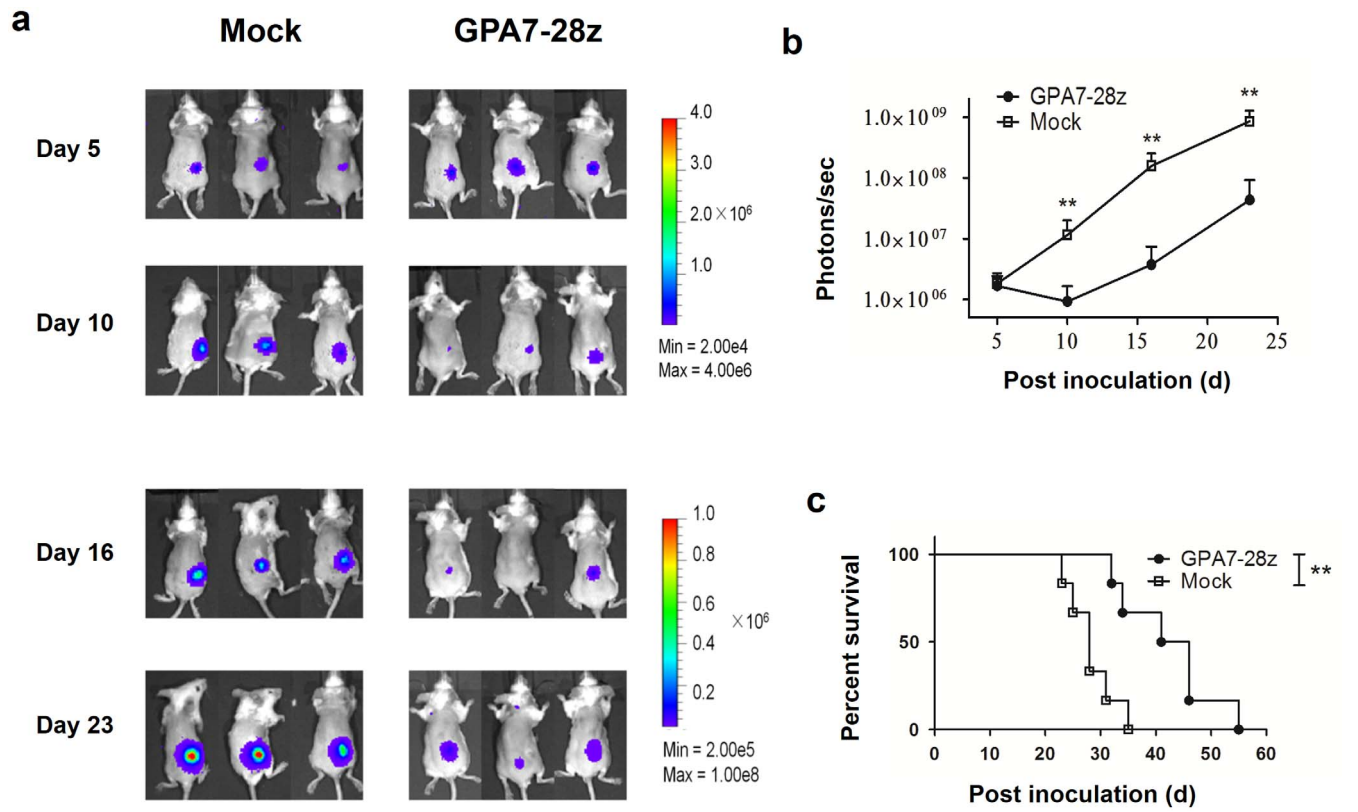

Figure 5 | Anti-tumor efficacy of GPA7-28z-transduced human primary T cells *in vivo*. SCID-Beige mice were inoculated subcutaneously with 1×10^6 Malme-3m-luc melanoma cells. On day 8 and 13, tumor-bearing mice were locally treated with 5×10^6 GPA7-28z-transduced (n = 6) or mock-transduced (n = 6) human primary T cells. Tumor growth was monitored using an *in vivo* imaging system (Xenogen IVIS imaging system). (a) Tumor growth was measured as photon emission in a representative cohort of 3 mice from each group on day 5, 10, 16 and 23. (b) Photon emission from luc^+ tumor cells was quantified and intensity of bioluminescent signal was presented as mean ± s.d. from 6 mice each for both two cohorts. (c) Survival curves of mice engrafted with Malme-3m-luc tumor cells receiving GPA7-28z or mock-transduced T cells. For intensity of bioluminescent signal, the significant difference between that of GPA7-28z and that of mock-transduced T cell-treated groups was compared using a two-tailed Student's *t*-test (n = 6; $^{**}P < 0.01$). For survival curves, data were plotted using the Kaplan–Meier method and statistically analysed by log-rank analysis (n = 6; $^{**}P < 0.01$).

T cells rapidly proliferated and released IFN-γ in response to T2 cells loaded with gp100 peptide. Specific *in vitro* lysis of gp100-pulsed T2 cells by redirected T cells was observed even in the presence of very low concentration of peptides. It has been demonstrated that, compared with bivalent antibodies in solution, avidity of CARs could be improved when multiple antibody-derived binding domains were arrayed on a cell surface[29]. In this regard, functional avidity of 0.3 nM was caculated, which quantitatively reflected the activation threshold of GPA7-28z T cells.

Another novelty of our strategy is to employ a single-domain antibody (sdAb)[30] instead of traditional single-chain variable fragment (scFv) as the targeting moiety of CAR. sdAb, derived from VHH domain of llama heavy chain antibody, contains only one antigen-binding domain, thus it stands for the smallest targeting unit ever exist[31]. Most scFvs are derived from the V_H and V_L fragments of murine antibodies, which may induce host immunogenic responses. A case report showed that three patients treated with T cells expressing CAIX-CAR had developed anti-scFv antibody responses[32]. Compared with murine monoclonal antibodies, VHH sequences shows much higher degree of homology with human VH sequences. In preclinical and clinical settings, no immunogenic rejection has been reported concerning on sdAbs. In addition, sdAbs possess longer and more flexible antigen-binding sites (or CDRs). This unique structure enables sdAbs to access into small cavities and clefts, thus being suitable for binding to a wide range of protein epitopes. Our findings indicated that high functional avidity would not decrease the selectivity of TCR-like CAR based on a sdAb. Cross-activity represents a general feature of TCR recognition, especially in high affinity TCRs, while it has not been observed in GPA7.

GPA7-28z T cells not only recognized exogenous gp100 peptide loaded on T2 cells, but also functionally interacted with HLA-A2-positive melanoma expressing gp100. Because most malignancies tumor cells lack the expression of costimulatory ligands, we coupled the chimeric receptor to the endodomain of CD28, which enhanced the activation, proliferation and cytotoxic ability of engineered T cells after the CAR-antigen engagement. GPA7-28z allowed T cells to specifically secret IFN-γ when contacting HLA-A2 positive melanoma. IFN-γ, released by mature differentiated CD8+ T cells and some types of CD4+ T cells, could enhance the immune response by up-regulating the expression of HLA class I molecules on both tumor cells and tumor-resident antigen-presenting cells. Direct lytic activity was subsequently verified *in vitro* by analysis of cytotoxicities of GPA7-28z T cells on a series of HLA-A2-positive melanoma cell lines as well as HLA-A2-negative melanoma.

To additionally evaluate the clinical relevance of our findings, we validated specific lysis of primary human melanoma cells collected from melanoma patients by GPA7-28z retargeted T cells *in vitro*. Moreover, significant anti-tumor activity of retargeted T cells against an established melanoma was demonstrated in a xenograft mouse model. Administration of GPA7-28z T cells could suppress an established tumor growth *in vivo*, although not completely eradicate the tumor cells. This might be due to insufficient persistence of engineered T cells. Besides, function sustaining and trafficking of human

T cells in a xenotransplant murine tumor models would be more difficult than that of autologous T cells in patients' immune system[33]. Further co-expression with other costimulatory motif (e.g. 4-1BB)[25], cytokines (e.g. IL-21)[34] or chemokine receptors (e.g. CCR4)[35] might improve the *in vivo* efficacy of engineered T cells.

In conclusion, we have shown that GPA7-28z, a TCR-like CAR, could redirect T cells to target melanoma *in vitro* and *in vivo*. These findings have proved a new principle of concept and could be reproduced for a variety of tumor antigens and applicable in clinical applications with great potential.

Methods

Cell lines and peptides. T2 cells are deficient in TAP function whose HLA-I protein could be loaded with exogenous peptides. T2 cells and the mouse melanoma cell line B16F10 were maintained in RPMI-1640 medium (Invitrogen, Grandland, N.Y.) supplemented with 10% (vol/vol) fetal calf serum (FBS; Hyclone, Australia). HLA-A2-positive (Malme-3m and MEL-624) and HLA-A2-negative (SK-MEL-28 and MEL-888) human melanoma cell lines were cultured in DMEM medium supplemented with 10% FBS. Human melanoma cell line MEL-624 was a kind gift from Dr. Y. Kawakami[14]. The other cell lines were obtained from American Type Culture Collection (ATCC).

Primary human melanoma cells Primary 1 were isolated after surgery from a subcutaneous metastasis in a patient (HLA-A2 positive) with uveal melanoma. Primary 2 cells were obtained from a primary melanoma lesion in a HLA-A2 positive patient. Informed consent was obtained from patients. Primary tumor cells were maintained in F12 medium containing 20% FBS. After 5 days in culture, non-adherent cells were removed, retaining the adherent fraction for further study (passage number < 4).

HLA-A2-restricted peptide $gp100_{209-217}$ (ITDQVPFSV) and Flu_{58-66} (GILGFVFTL) were synthesized by SBS Genetech Co. (Beijing, China).

All investigations were conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines and have been approved by the Biomedical Research Ethics Committee of CAS Key Laboratory of Pathogenic Microbiology and Immunology.

Construction of lentiviral vector. The coding sequence of CAR frame was synthesized by Generay Biotech Co., Ltd. (Shanghai, China), which contains human IgG1-CH2CH3 hinge region[15], followed by the CD28 transmembrane-intracellular domain (135 ~ 202 aa) and endodomain from CD3-ζ chain (29 ~ 142 aa). The sdAb GPA7 targeting $gp100_{209-217}$/HLA-A2 complex has been described previously[11]. GPA7 sequence was cloned in pCDH lentiviral vector (System Biosciences, CA) in frame with Fc hinge region, CD28 and CD3-ζ, to form the GPA7-28z construct.

Lentivirus production and PBMC transduction. High-titer lentiviral vectors were produced and concentrated as previously described[16]. Briefly, 20×10^6 293T cells were seeded onto 150-mm^2 gelatin-coated plates 24 hours before transfection. Cells were transfected with 22.5 μg GPA7-28z vector, 7.5 μg Plp/VSVG, 15 μg Plp1 and 10 μg Plp2, using Fugene HD transfection reagent (Roche Diagnostics, IN). The viral supernatant was harvested at 48 hours post-transfection and cell debris was removed by centrifugation. Viral particles were subsequently concentrated by ultracentrifugation for 3 hours at 25,000 rpm and resuspended in PBS.

Peripheral blood obtained from healthy donors with informed consent was purified by centrifugation over Ficoll gradients. Peripheral blood mononuclear cells (PBMCs) and T-cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS and 100 IU/mL IL-2. Isolated PBMCs were activated using 5 μg/mL anti-CD3 antibody OKT3 and 2 μg/mL anti-CD28 antibody (eBioscience, San Diego, CA) on day 0. On day 2 and day 3, activated lymphocytes were transduced with concentrated lentiviral vectors at MOI 5, in the presence of 10 μg/mL protamine sulfate (Sigma-Aldrich, St. Louis, MO). Six hours after transduction, the medium was replaced with RPMI-1640 supplemented with 10% heat-inactivated FBS and 100 IU/mL IL-2.

Antigen-specific expansion of transduced T cells. After two-rounded transduction, PBMC culture were harvested and washed with PBS for three times. Irradiated (60 Gy) T2 cells loaded with $gp100_{209-217}$ peptide (5 μmol/mL) were used as feeder cells to restimulate transduced T cells. Therefore, transduced T cells were co-cultured with gp100-pulsed T2 cells at a ratio of 1 : 5. After a total of 14 days in culture, the gp100-stimulated T cells were obtained and tested for functional activities toward T2 cells, melanoma cell lines, and fresh human melanoma cells *in vitro* and *in vivo*.

Flow cytometry. PE-conjugated gp100/HLA-A2 tetramer (Epigen Biotec, Beijing, China) and FITC-labeled anti-hIgG1 antibody (Sigma-Aldrich) were used to evaluate GPA7-28z expression on T cells 72 hours after lentiviral transduction. Mock-transduced T cells served as control. Immunofluorescence was mesured using a FACScan instrument (Millipore Guava Easycyte mini, Billerica, MA) and analyzed with Flowjo software.

After restimulated with gp100-pulsed T2 cells, GPA7-28z-transduced T cells were stained using anti-CD3-PE, anti-CD4-FITC, anti-CD8-FITC and gp100/HLA-A2 tetramer-PE on day 14. All above antibodies were purchased from BD Pharmingen (San Diego,CA).

Enzyme-linked immunospot assay. For IFN-γ Enzyme-linked immunospot (ELISpot) analysis against peptide-loaded T2 cells, T2 cells were pre-incubated with 20 μM $gp100_{209-217}$ or Flu_{58-66} peptide for 3 hours at 37°C. 1,000 GPA7-28z-transduced or mock-transduced T cells (per well) were plated in triplicate and stimulated overnight with 5,000 peptide-loaded T2 cells (per well) on a 96-well ELISpot plate coated with anti-IFN-γ antibody (Mabtech AB, Hamburg, Germany). After 16 hours of incubation, plates were incubated with the respective capture antibodies and developed following the instructions of the human IFN-γ ELISpot kits (Mabtech AB). Tumor-specific activity was measured by incubating 10,000 T cells with the HLA-A2-positive melanoma cell lines (Malme-3m and MEL-624), HLA-A2-positive primary melanoma cells (Primary 1 and Parimary 2), and HLA-A2-negative melanoma cell lines (MEL-888, SK-MEL-28 and B16F10). All target cells were plated at 50,000 cells/well. IFN-γ and granzyme B releasing were detected using human IFN-γ and granzyme B ELISpot kits (both by Mabtech AB). Spots were counted using an automated reader (CTL ImmunoSpot S5 UV Analyser, CTL Europe, Bonn, Germany).

Cytotoxicity assay. The cytotoxic activity of GPA7-28z T cells and mock-transduced T cells was evaluated in a Calcein-AM release assay[17]. In brief, target cells including peptide-pulsed T2 cells and melanoma cells were labeled with 15 μM calcein-AM for 30 min at 37°C. T2 cells were pre-incubated with a series for ten-folded diluted peptide ($10^{-5} \sim 10^3$ nmol/mL) for 3 h at 37°C. After washes in complete medium twice, target cells were adjusted to 10^5/ml and 100 μL added per well of a 96-well V-bottom plate. Subsequently, effector cells were added to wells at the given ratios. The test was performed in four replicates for each E/T ratio, with at least six replicate wells for spontaneous (only target cells in complete medium) and maximum release (only target cells in medium plus 2% Triton X-100). After incubated at 37°C for 4 hours, supernatants were harvested and transferred into a black 96-well plate. Fluorescence was measured using a Synergy TM H4 Multi-Mode Microplate Reader (BioTek instruments, Winooski, VT) (excitation filter: 485 ± 9 nm; band-pass filter: 530 ± 9 nm). Percent specific lysis was calculated as (experimental release - spontaneous release)/(maximum release – spontaneous release) × 100. For antibody blocking assay, gp100-pulsed T2 cells were pre-incubated with 10 μg/mL mouse anti-HLA-A2 antibody BB7.2 for 30 min before mixing with GPA7-28z T cells.

Mouse tumor model and quantitative bioluminescence. All animal studies were carried out under the standard protocol approved by the Institutional Animal Care and Use Committee of IMCAS. Female eight-week-old SCID-Beige mice were purchased from Peking University Health Science Center (Beijing, China). Firefly-luciferase (Luc) expressing HLA-A2-positive Malme-3m cells (1×10^6 in 100 μL) were inoculated subcutaneously on the flank of each SCID-Beige mouse. On day 8 and 13, 5×10^6 mock-transduced T cells or GPA7-28z-transduced T cells were injected locally. Six mice bearing established tumor were treated for either group. The animals were imaged by Xenogen IVIS 100 imaging system (Caliper Life Sciences, Hopkinton, MA) to monitor the tumor growth. Photo emission from luciferase-expressing tumor cells was quantified using Living Image software (Caliper Life Sciences). The signal intensity of tumor burdens was expressed as total photons/s/cm^2 (p/s/cm^2/sr).

Statistical analysis. All data are presented as the means ± s.d. Student's *t-test* was used to evaluate differences in cytokine secretion, specific cytolysis, and photon counts of tumor burdens. For antibody blocking assay, data were compared using one-way ANOVA followed by Tukey's *post-hoc* test. Kaplan-Meier survival curves were compared using the log-rank test. Statistical analyses were carried out with GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

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Acknowledgments

We thank Dr. Y. Kawakami (National Cancer Institute, NIH, US) for kindly provision of cell lines. This work was supported by grants from the Ministry of Science and Technology of China (S & T Major Program: No. 2012ZX10004701-001-002), 973 program (2013CB531502) and the National Natural Science Foundation of China(No. 31370889, 31070783, 31170829, 81021003), Beijing Natural Science Foundation (5112022).

Author contributions

G.Z. and B.G. conceived and designed the experiments. G.Z., L.W., H.L.C., X.M.W., G.L.Z., J.M., H.M.H., W.H., W.W. and Y.F.Z. carried out the experiments and analyzed the data. G.Z., C.Z.L., M.Y.S. and B.G. supervised the project. G.Z. and B.G. wrote the manuscript with inputs from all authors. All authors reviewed the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhang, G. *et al.* Anti-melanoma activity of T cells redirected with a TCR-like chimeric antigen receptor. *Sci. Rep.* **4**, 3571; DOI:10.1038/srep03571 (2014).