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ORIGINAL ARTICLE

Optimized T-cell receptor-mimic chimeric antigen receptor T cells directed toward the intracellular Wilms Tumor 1 antigen

S Rafiq[1], TJ Purdon[1], AF Daniyan[1], M Koneru[1], T Dao[2], C Liu[3], DA Scheinberg[1,2,4] and RJ Brentjens[1,4]

CD19-directed chimeric antigen receptor (CAR) T cells are clinically effective in a limited set of leukemia patients. However, CAR T-cell therapy thus far has been largely restricted to targeting extracellular tumor-associated antigens (TAA). Herein, we report a T-cell receptor-mimic (TCRm) CAR, termed WT1-28z, that is reactive to a peptide portion of the intracellular onco-protein Wilms Tumor 1(WT1), as it is expressed on the surface of the tumor cell in the context of HLA-A*02:01. T cells modified to express WT1-28z specifically targeted and lysed HLA-A*02:01+ WT1+ tumors and enhanced survival of mice engrafted with HLA-A*02:01+, WT1+ leukemia or ovarian tumors. This *in vivo* functional validation of TCRm CAR T cells provides the proof-of-concept necessary to expand the range of TAA that can be effectively targeted for immunotherapy to include attractive intracellular targets, and may hold great potential to expand on the success of CAR T-cell therapy.

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INTRODUCTION

CAR T cells directed to CD19 have demonstrated marked efficacy in patients with $CD19^+$ B-cell malignancies, most prominently those with relapsed or refractory B-cell acute lymphoblastic leukemia.[1–3] However, despite the clinical success seen with CAR T-cell therapy in the context of targeting CD19,[4–8] extension of CAR T-cell therapy to other cancers has been limited[9] due in part to a paucity of suitable tumor-associated antigen (TAA) expressed on most malignancies. Although this may be the case in the setting of extracellular antigens, there are a number of intracellular antigens overexpressed by tumor cells which to date have not been readily targetable.

One example of an intracellular TAA is Wilms Tumor 1 (WT1). WT1 is an oncogenic, zinc-finger transcription factor that is involved in proliferation, differentiation, organ development and apoptosis.[10–12] After birth, WT1 expression is limited to low levels in the gonads, kidney, spleen and bone marrow.[13] WT1 is overexpressed in numerous hematological malignancies, including acute myeloid leukemia (AML), as well as in many solid malignancies such as mesothelioma, gastrointestinal cancers, glioblastoma and ovarian cancer.[10,14] WT1 overexpression in malignant cells is correlated with a poor prognosis in both AML and lymphoid leukemia.[15,16] Multiple cancer vaccine studies have utilized WT1, most commonly peptides 126-134, RMFPNAPYL (RMF).[17] These vaccine strategies have induced cytotoxic CD8 T-cell responses against WT1-positive tumors.[14,18,19] However, the T-cell responses and prolonged remissions reported in AML patients treated with this approach have generally been reported in the setting of minimal residual disease, but not in the setting of overt disease.

One approach to making attractive intracellular TAA accessible is through the identification of scFv that recognize portions of intracellular TAA peptides in the context of human leukocyte antigens (HLAs) that are used to create TCR-mimic (TCRm) mAbs. Integration of these scFvs into CARs can generate TCRm CARs. We generated a TCRm CAR against WT1 utilizing a previously described scFv that recognizes the WT1 RMF peptide in the context of HLA-A*02:01 on the cell surface. The scFv was identified using phage display technology after screening with the recombinant WT1/HLA-A*02:01 complex and was used to create a fully human TCRm mAb termed ESK1.[20–23] The ESK1 antibody mediated clearance of established acute lymphocytic leukemia in mouse models.[20,21] Furthermore, the ESK1 bispecific T-cell engager antibody, ESK1-BiTE, effectively redirected T cells to kill tumor cells.[23]

The scFv specific for the WT1/HLA-A*02:01 complex allowed us to generate a novel CAR targeting an intracellular target expressed in the context of an HLA molecule. Although necessary, identification of an scFv to target an ideal TAA may not be sufficient to create a clinically effective CAR, as evident by the modest clinical responses seen with CD19-specific CAR T cells in patients with relapsed or refractory CLL.[24] Our group has previously described 'armored' CAR T cells that secrete IL-12 to enhance T-cell function.[25,26] IL-12 is a pleiotropic, pro-inflammatory cytokine that has a critical role in Th1-type immune responses.[27] IL-12 armored CAR T cells have longer persistence *in vivo*[28] and can eliminate the need for conditioning with lympho-depleting chemotherapy.[25]

Herein we report the generation and validation of a TCRm CAR construct capable of recognizing a peptide derived from intracellular WT1 in the context of HLA-A*02:01. This is the first work to describe optimized TCRm CAR T cells that demonstrate effective *in vivo* efficacy and serves a proof-of-principle that targeting of CAR T cells to tumor cells may be expanded beyond

[1]Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA; [2]Molecular Pharmacology Program, Sloan Kettering Institute; Memorial Sloan Kettering Cancer Center, New York, NY, USA; [3]Eureka Therapeutics, Emeryville, CA, USA and [4]Weill Cornell Medicine, New York, NY, USA. Correspondence: Dr RJ Brentjens, Department of Medicine, Memorial Sloan Kettering Cancer Center, 1250 1st Avenue, New York 10065, NY, USA.
E-mail: brentjer@mskcc.org

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surface expressed TAA to a new universe of additional promising intracellular TAA. Co-modification of TCRm CAR T cells with IL-12 augmented the anti-tumor efficacy of the T cells, additionally demonstrating potential for enhanced clinical anti-tumor responses. Collectively, the presented data represents promise for the evolution and expansion of CAR T-cell immunotherapy to a broader array of cancers.

MATERIALS AND METHODS

Primary cells and cell lines

Set2, OVCAR3, AML-14, BV173, Karpas, HL-60, SKLY-16, Nalm6, gpg29 fibroblast (H29) were obtained from the ATCC (Manassas, VA, USA) and cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10–20% heat-inactivated fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA), nonessential amino acids, HEPES (*N*-2-hydroxyethylpiperazin-*N*'-2-ethanesulfonic acid) buffer, pyruvate, L-glutamine, penicillin/streptomycin, and 2-Mercaptoethanol (Invitrogen). 293 gibbon ape leukemia virus envelope-pseudotyped (293Glv9) retroviral producing cell lines[29] were cultured in DMEM (Invitrogen) with 10% heat-inactivated fetal bovine serum.

Human T cells were isolated from healthy donors under Institutional Review Board approved protocol 095-054 using BD Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ, USA) as per the manufacturer's instructions or as a leukopak from the NYBC and separated using density gradient centrifugation with Ficoll (GE Healthcare, Little Chalfont, UK). T cells were cultured in RPMI-1640 supplemented with fetal bovine serum and 100 IU/ml IL-2 (Proluekin, Novartis, Basel, Switzerland). Primary AML samples were obtained from the MSKCC HOTB (Protocol 95-091).

Generation of retroviral constructs and transduction

The mammalian optimized ESK1 scFv sequence was obtained from Eureka Therapeutics Inc (Emeryville, CA, USA). The variable heavy and light chains were connected by a $(Gly_4Ser)_3$ spacer domain and a immunoglobulin κ-leader sequence was added. The gene was cloned into the SFG retroviral vector containing the CD28 transmembrane and cytoplasmic signaling domains along with the CD3ζ signaling domain. The fusion gene encoding the complete flexi human IL-12 was generously provided by Alan Houghton and Jedd Wolchok,[35] and modified to include a hCD8 leader peptide and an internal ribosome entry site.[24] All constructs were verified by sequencing.

Stable viral producing cell lines were created by calcium phosphate transfection first into H29 cells and subsequent retroviral transduction with viral supernatant into 293Galv9 cells. Retroviral transduction of primary human T cells has been previously described.[28]

Flow cytometry analyses

Flow cytometric analyses were performed using Gallios Flow Cytometer with Kaluza software (Beckman Coulter, Brea, CA, USA). Flow sorting of CAR-positive T cells was done using a BD Biosciences (San Jose, CA, USA) FACSARIA IIu Special Order System with BD FACSDIVA software.

CAR expression on T cells were detected with the following reagents: monoclonal goat anti-mouse phycoerythrin (PE) antibody (Invitrogen) for CD19-targeted CAR, Armenian hamster anti-4H11 CAR antibody conjugated to AlexaFluor-647 (MSKCC Monoclonal Antibody Facility), or WT1/HLA-A*02:01 Tetramer conjugated to Brilliant Violet-421 (NIH Tetramer Facility) to detect WT1-targeted CAR. WT1/HLA-A*02:01 epitope expression on cancer cells was detected with APC-labeled ESK1 antibody (Eureka). PerCP CMV/HLA-A*02:01 tetramer (MSKCC tetramer core facility) against the CMV peptides NLVPMVATV was utilized a control for WT1-targeted CAR expression.

Assays for CAR function

Cytokines were detected in the supernatant using the Luminex FlexMap3D system (Millipore Corporation, Billerica, MA, USA). Cytokine concentrations were assessed using Luminex Xponent 4.2 (Millipore Corporation).

T-cell cytotoxicity was assessed by 4 h chromium release assays, as previously described.[24]

To assess expansion, CAR T cells were co-cultured with Set2 or Nalm6 cells in the absence of supplemented cytokines. The cell count for each sample was assessed at indicated times by flow cytometry and cells were enumerated using 1,2,3 count ebeads (ebiosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Mouse tumor models

Experiments involving animals were conducted with the approval of an IACUC at MSKCC (00-05-065). In Set2 models, 8–12-week-old female CB17.Cg-PrkdcscidLystbg-J/Crl mice (SCID-Beige mice; Charles River Laboratories, Wilmington, MA, USA) were injected intravenously (i.v.) with 5×10^6 tumor cells. 2×10^7 CAR T cells were injected i.v. Mice were monitored clinically for disease progression.

In ovarian GFP-FFLuc$^+$ OVCAR3 tumor models, 8–12-week-old female SCID-Beige mice (Charles River Laboratories) were injected intraperitoneally (i.p.) with 1×10^7 tumor cells. Mice were monitored with bioluminescent imaging for established tumors at day 40, randomized and treated with 5×10^6 CAR$^+$ T cells on day 41. Mice were monitored clinically and with bioluminescent imaging for disease progression.

Bioluminescent imaging of GFP-FFLuc+ OVCAR3 cells in SCID/Beige mice was performed using Xenogen IVIS imaging system with Living Image software (Xenogen Biosciences, Cranbury, NJ, USA). Image acquisition was done on a 25 cm field of view at medium binning level at various exposure times.

nanoString technology gene expression analysis

CAR T cells were co-cultured with OVCAR3 tumor cells at a 1:1 ratio for 48 h. CAR T cells were flow sorted and total RNA was extracted with Ambion RNA extraction kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. RNA expression levels of 770 genes was detected using nanoString array (nCounter, Gene expression code set, PanCancer Immune Profiling Panel) and analyzed using nSolver Analysis system.

Statistical analysis

All analyses were calculated using Graphpad Prism 5.0 software (La Jolla, CA, USA). Survival data were assessed using a log-rank analysis and all other analyses were achieved with Mann–Whitney tests. Nanostring data was analyzed for statistical significance using nSolver analysis software.

RESULTS

Retrovirally transduced T cells express the WT1-28z CAR

The WT1-28z CAR was engineered using variable heavy and light chains sequences from the ESK1 scFv connected by a $(Gly_4Ser)_3$ spacer domain. The ESK1 scFv was ligated upstream to the CD28 transmembrane and signaling domain and CD3ζ signaling domain. The resulting WT1-28z gene was cloned into a SFG gamma-retroviral vector (Figure 1a). Following retroviral transduction, T cells expressed high levels of WT1-28z CAR, as assessed by flow cytometric analysis utilizing a WT1/HLA-A*02:01 tetramer (Figure 1b). The transduction efficiency of the CAR T cells was between 50 and 90% for all experiments. To confirm that the WT1-28z CAR was not artificially binding to WT1-HLA-A*02:01 tetramer due to high avidity for the antigen, a cytomegalovirus (CMV)-HLA-A*02:01 tetramer was used to stain CAR T cells. No binding of the CMV-HLA-A*02:01 tetramer was observed on WT1-28z CAR T cells (Supplementary Figure 1A).

WT1-28z CAR T cells are cytotoxic to HLA-A*02:01$^+$, WT1$^+$ cells

The cytotoxic capability of WT1-28z CAR T cells was assessed by ^{51}Cr release assays against a range of HLA-A*02:01$^+$, WT1$^+$ cancer cell lines and primary AML tumor samples. WT1-28z CAR T cells specifically and significantly lysed AML (AML-14), chronic myeloid leukemia (BV173), or ovarian cancer (OVCAR3) cell lines at a range of effector-to-target ratios, as compared with control T cells modified to express a CAR specific to either the ovarian cancer MUC16 antigen (4H11-28z)[30] or CD19(19-28z)[31] (Figure 1c). WT1-28z CAR T cells mediated lysis of AML primary samples as compared with control T cells ($P=0.009$, 0.02 and 0.02 at E:T ratios of 40:1, 20:1 and 10:1, respectively) (Figure 1e).

In addition, following a 24-hour co-culture of CAR T cells with AML-14, BV173 or OVCAR3 cell lines (Figure 1d and Supplementary Figure 1B), WT1-28z CAR T cells secreted significantly more of the pro-inflammatory cytokines IFN-γ ($P\leqslant0.0001$, 0.03, and 0.0013, respectively) and IL-2 ($P=<0.0001$, 0.013 and

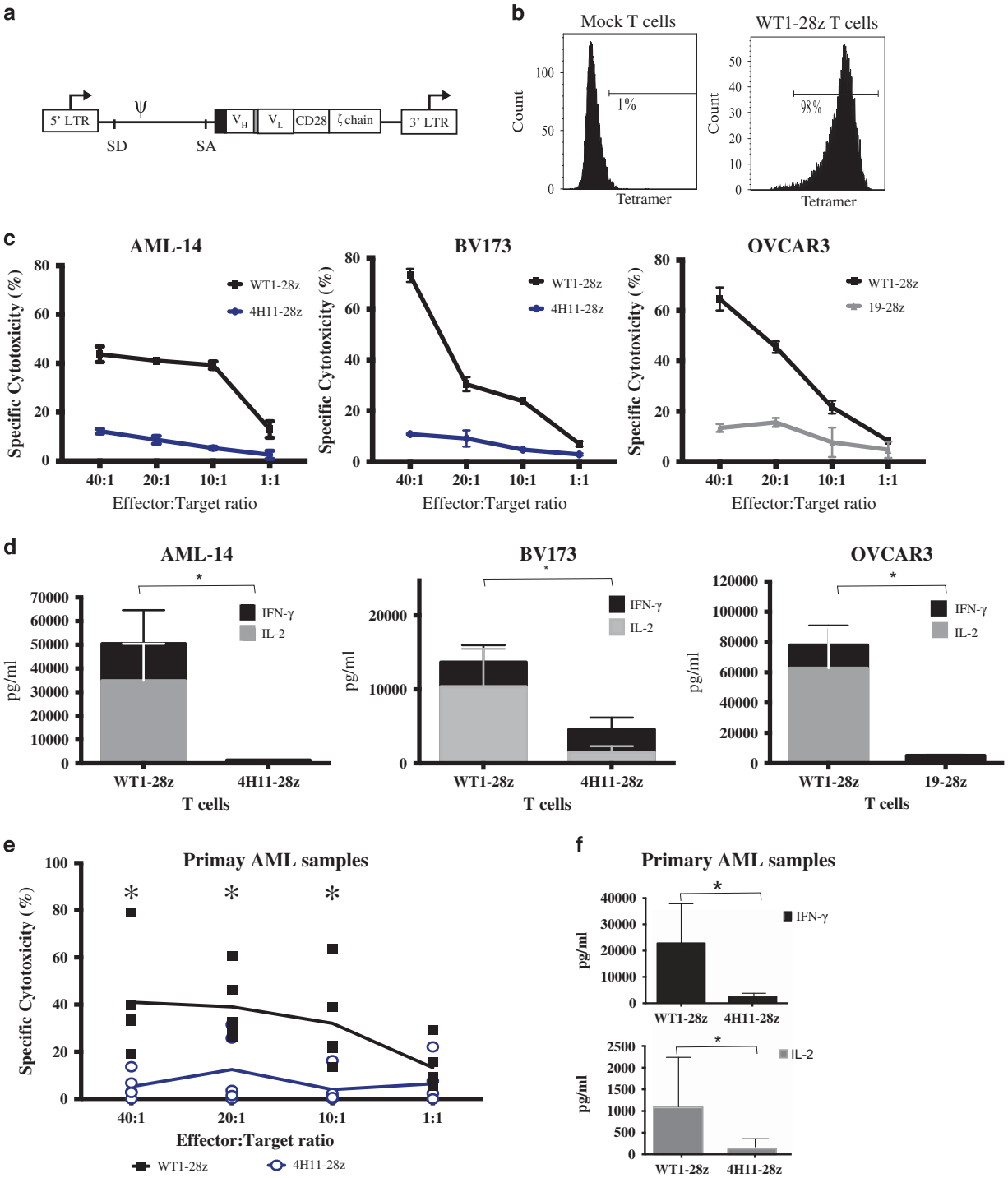

Figure 1. Generation and validation of WT1-28z CAR T cells. (**a**) Schematic representation of the WT1-28z constructs. WT1/HLA-A*02:01-specific scFv derived from the heavy (VH) and light (VL) chain variable regions of the ESK1 antibody; CD28: human CD28 transmembrane and cytoplasmic signaling domains; *z*-chain: human TCR zeta chain cytoplasmic signaling domain; black box: κ leader sequence; gray box: (Gly4Ser)3 linker. (**b**) Flow cytometry histograms depicting the expression of WT1-28z in retrovirally transduced into primary human T cells, as detected by binding to a fluorescently-labeled WT1/HLA-A*02:01 Tetramer (representative figure of $n > 10$). (**c**) WT1-28z CAR T cells are more toxic than control irrelevant antigen-specific control CAR T cells 4H11-28z or 19-28z CAR T cells against AML-14, BV173 and OVCAR3 in standard ^{51}Cr release assays (representative figures, $n = 3$ for each cell line). (**d**) WT1-28z CAR T cells when co-cultured with AML-14, BV173, or OVCAR3 cell lines for 24 h have significantly enhanced release of IFN-γ (*$P = < 0.0001$, 0.03, or 0.0013 respectively) and IL-2 (*$P \leqslant 0.0001$, 0.013, or < 0.0001 respectively), as compared with control 4H11-28z or 19-28z CAR T cells ($n = 3$ for each cell line). (**e**) Standard ^{51}Cr release assays with antigen-specific WT1-28z CAR T cells showed enhanced cytotoxicity against primary AML samples as compared with a control irrelevant antigen-specific 4H11-28z CAR T cells ($n = 5$) (*$P = 0.029$). (**f**) WT1-28z CAR T cells have significantly enhanced release of IFN-γ and IL-2 (*$P = 0.04$) when co-cultured with AML patient samples for 24 h, as compared with 4H11-28z CAR T cells ($n = 3$).

< 0.0001, respectively) than control CAR T cells, indicating specific stimulation through the WT1-specific CAR. Similarly, WT1-28z CAR T cells produced significantly more of IFN-γ ($P = 0.04$) and IL-2 ($P = 0.04$) when compared with control CAR T cells co-cultured with primary AML tumor cells (Figure 1f).

WT1-28z/IL-12 CAR T cells release functional IL-12

The function of the WT1-28z CAR T cells was augmented by creating the WT1-28z/IL-12 retroviral vector by insertion of an internal ribosomal entry site and the human flexi-IL-12 gene[25,32] (Figure 2a). IL-12 was only detected in supernatants from viral

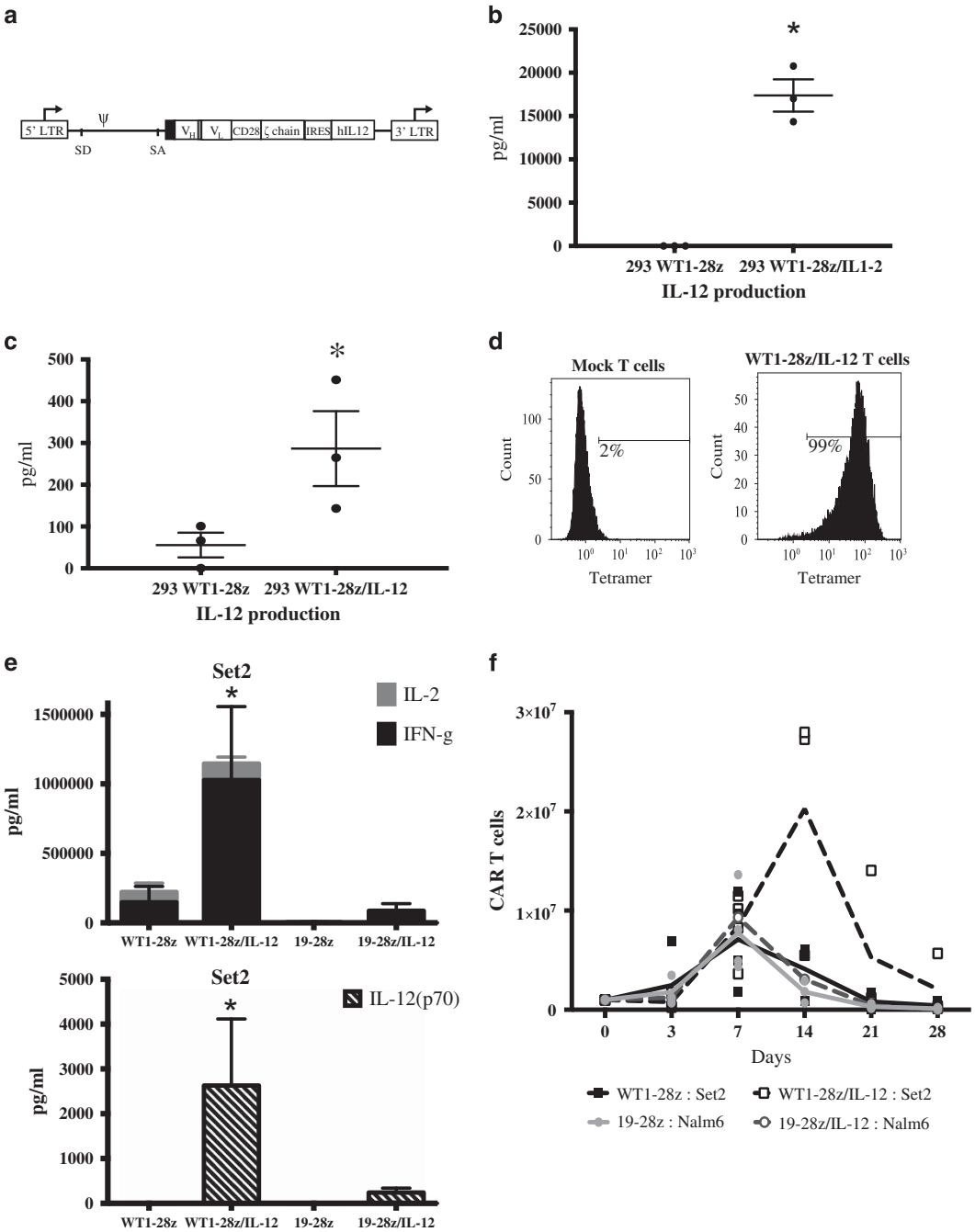

Figure 2. Generation and validation of WT1-28z/IL-12 CAR T cells. (**a**) Schematic representation of the WT1-28z/IL-12 CAR constructs. WT1/HLA-A*02:01-specific scFv derived from heavy (VH) and light (VL) chain variable regions of the ESK1 antibody; CD28: human CD28 transmembrane and cytoplasmic signaling domains; *z*-chain: human TCR zeta chain cytoplasmic signaling domain; flexi human IL-12 (hIL-12 f); LTR: 50 and 30 long terminal repeat; black box: κ leader sequence; gray box: (Gly4Ser)3 linker. (**b**) IL-12 expression by viral producer cell lines stably transduced with CAR constructs show that only 293Galv9 cells transduced with WT1-28z/IL-12 express IL-12 ($n = 3$ independent experiments) ($P = 0.01$). (**c**) The IL-12 produced by the WT1-28z/IL-12 CAR in 293Galv9 is functional, as determined by increases in levels of IFN-γ produced by peripheral blood mononuclear cell cultured in supernatant ($n = 3$ independent experiments) ($P = 0.03$). (**d**) Flow cytometry histograms depicting the expression of WT1-28z/IL-12 CARs in retrovirally transduced primary human T cells, as detected by binding to a fluorescently-labeled WT1/HLA-A*02:01 tetramer (representative figure of $n > 10$). (**e**) WT1-28z/IL-12 CAR T cells have significantly enhanced release of IFN-γ (*$P = 0.008$, 0.01, respectively) and IL-2 (*$P =$ 0.002, 0.002, respectively), when co-cultured with Set2 cells for 24 hours, as compared to 19-28z or 19-28z/IL-12 CAR T cells ($n = 4$). (**f**) WT1-28z/IL-12 CAR T cells proliferate after co-culture with Set2 cells ($n = 4$).

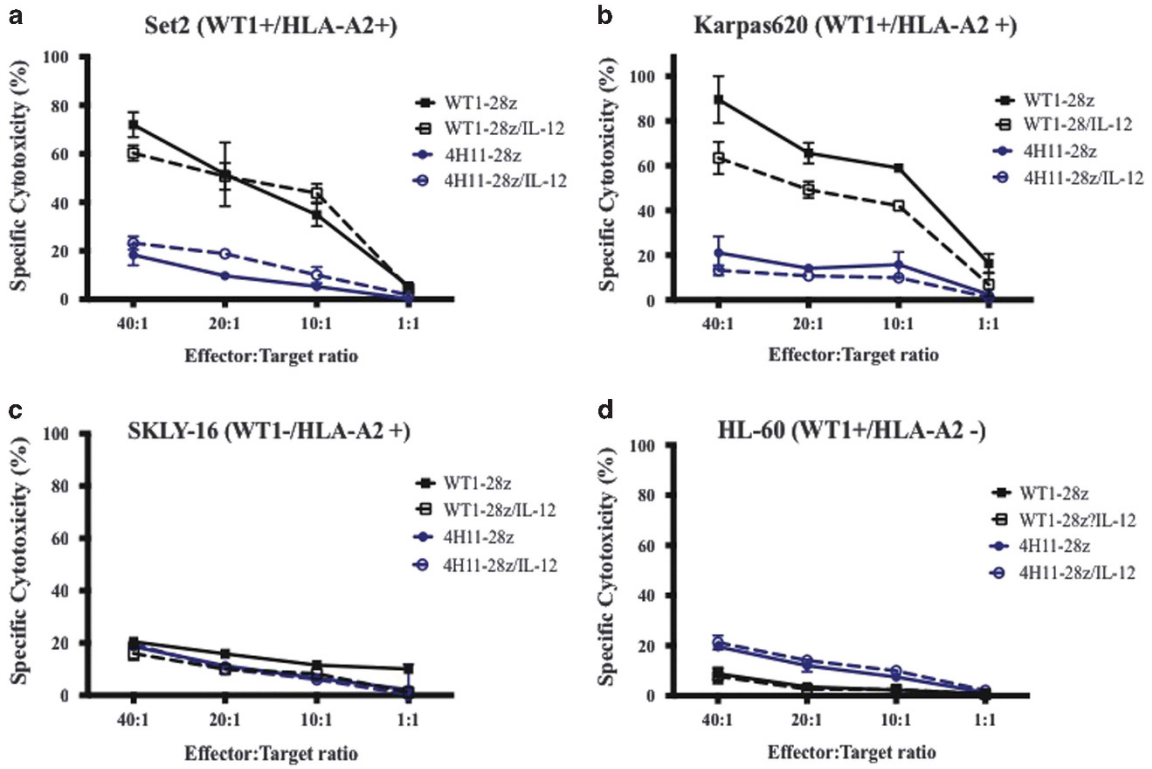

Figure 3. WT1-28z and WT1-28z/IL-12 CAR T cells are specific to WT1/HLA-A*02:01. (**a** and **b**) WT1-28z or WT1-28z/IL-12 CAR T cells are significantly more toxic than control irrelevant antigen-specific CAR T cells 4H11-28z and 4H11-28z/IL-12 CAR T cells against Set2 and KARPAS-620 in standard ^{51}Cr release assays (representative figures, $n=3$ for each cell line). WT1-28z significantly more lysed Set2 cells than 4H11-28z CAR T cells at all the effector to target ratios tested ($P \leqslant 0.005$). WT1-28z/IL-12 significantly more lysed Set2 cells than 4H11-28z/IL-12 CAR T cells at 40:1, 20:1 and 10:1 effector to target ratios tested ($P < 0.0006$). WT1-28z significantly more lysed KARPAS-620 than 4H11-28z CAR T cells at all the effector to target ratios tested ($P < 0.007$). WT1-28z/IL-12 significantly more lysed KARPAS-620 than 4H11-28z/IL-12 CAR T cells at 40:1, 20:1, and 10:1 effector to target ratios tested ($P < 0.0003$). (**c**) WT1-28z and WT1-28z/IL-12 CAR T cells show no toxicity toward the HLA-A*02:01-positive, WT1-negative cell lines SKLY-16 compared with control 4H11-28z or 4H11-28z/IL-12 CAR T cells in standard ^{51}Cr release assays ($n=3$). (**d**) WT1-28z and WT1-28z/IL-12 CAR T cells show no toxicity toward the WT1-positive, HLA-A*02:01-negative cell line HL-60 as compared with control 4H11-28z or 4H11-28z/IL-12 CAR T cells in standard ^{51}Cr release assays ($n=3$).

producer cells transduced with the WT1-28z/IL-12 but not the WT1-28z construct (Figure 2b). Supernatant from WT1-28z/IL-12 viral producer cells containing IL-12 stimulated significant levels of IFN-γ secretion from peripheral blood mononuclear cell compared with supernatant from producer cells expressing the WT1-28z CAR alone (Figure 2c), validating the biological function of the secreted IL-12.

T cells transduced with the WT1-28z/IL-12 construct exhibited high levels of WT1-28z/IL-12 CAR expression as detected by flow cytometry (Figure 2d). The CD8/CD4 ratio of both WT1-28z and WT1-28z/IL transduced CAR T cells was similar (Supplementary Figure 1C). WT1-28z/IL-12 CAR T cells co-cultured with the megakaryoblastic cell line Set2 cells secreted IL-12 and significantly more IFN-γ as compared WT1-28z, 19-28z or 19-28z/IL-12 CAR T cells ($P=0.02$, 0.008, 0.01, respectively). WT1-28z/IL-12 CAR T cells secreted significantly more IL-2 as compared 19-28z or 19-28z/IL-12 CAR T cells ($P=0.002$, 0.002, respectively) (Figure 2e). Finally, WT1-28z/IL-12 CAR T cells had greater proliferation than WT1-28z, 19-28z or 19-28z/IL-12 CAR T cells at 14 days (Figure 2f).

WT1-directed CAR T cells specifically recognize the WT1/HLA-A*02:01 complex

The specific lysis function of WT1-28z and WT1-28z/IL-12 CAR T cells was determined by ^{51}Cr release cytotoxicity assays targeting a variety of HLA-A*02:01^{+}, WT1^{+} cancer cell lines (Figure 3). Both WT1-28 and WT1-28/IL-12 CAR T cells mediated significant lysis of Set2 as well as the multiple myeloma cell line KARPAS-620 (Figures 3a and b) when compared with control CAR T cells.

To further validate WT1-28z CAR specificity to WT1 peptide presented by HLA-A*02:01, T cells were co-cultured with cancer cells that express HLA-A*02:01 but not WT1. As expected, neither WT1-28z, WT1-28z/IL-12, nor control CAR T cells lysed the WT1^{-}, HLA-A*02:01^{+} B-cell lymphoma cell line SKLY-16 (Figure 3c). Conversely, neither WT1-28z, WT1-28z/IL-12 nor control CAR T cells lysed the WT1^{+} but HLA-A*02:01^{-} acute promyleocytic leukemia cell line HL-60 (Figure 3d). In addition, the WT1-directed CAR T cells did not lyse primary AML samples that were negative for HLA-A*02:01 (Supplementary Figure 1E). Collectively, these data supports the conclusion that the WT1-28z CAR, when expressed in human T cells, mediates specific targeting of the WT1/HLA-A*02:01 complex and not WT1 or HLA-A*02:01 individually.

WT1-28z and WT1-28z/IL-12 CAR T cells prolong the survival of mice bearing xenograft acute leukemia

The *in vivo* anti-tumor efficacy of WT1-directed CAR T cells was tested in a xenograft model of acute leukemia. Set2 AML cells were inoculated i.v. into SCID/Beige mice. Mice were treated with a single i.v. dose of CAR T cells 7 days post-tumor injection. The MUC-16 antigen-specific 4H11-28z and 4H11-28z/IL-12 CAR

T cells were used as controls in these experiments. WT1-28z CAR T cells significantly improved survival of tumor-bearing mice compared with mice treated with control CAR T cells ($P=0.01$) or untreated mice ($P=0.006$) (Figure 4a). Mice treated with WT1-28z/IL-12 CAR T cells demonstrated significantly enhanced survival over mice treated with control CAR T cells ($P=0.004$) (Figure 4a). Overall, 29% of mice treated with WT1-28z/IL-12 exhibited long-term survival compared with 6% of mice treated with WT1-28z although this difference failed to reach statistical significance ($P=0.06$).

We subsequently compared the *in vivo* efficacy of WT-28z and WT1-28z/IL-12 CAR T cells in a more delayed treatment model. Set2 AML cells were inoculated i.v. into SCID/Beige mice. Mice were treated with a single dose of CAR T cells via i.v. infusion 14 days post-tumor inoculation. As seen in Figure 4b, treatment with WT1-28z/IL-12 CAR T cells significantly enhanced survival of mice in this delayed treatment model compared to treatment with WT1-28z CAR T cells ($P=0.006$). Significantly, in this delayed treatment model, tumor-bearing mice treated with T cells modified with the WT1-28z CAR alone demonstrated no enhanced survival when compared with mice treated with control CAR T cells. Collectively, these data demonstrate the ability WT1-28z CAR T cells to eradicate systemic HLA-A*02:01$^+$, WT1$^+$ AML tumor cells, consistent with our previous *in vitro* studies, and further demonstrate the enhanced ability of WT1-28z CAR T cells secreting IL-12 to enhance survival in mice with more advanced disease.

WT1-28z and WT1-28z/IL-12 CAR T cells prolong the survival of mice bearing xenograft ovarian tumors

To assess efficacy in solid tumor models, the *in vivo* anti-tumor function of TCRm CAR T cells was investigated utilizing a xenograft ovarian tumor model. The HLA-A*02:01$^+$ WT1$^+$ human OVCAR3 cell line was injected i.p. into SCID/Beige mice. Mice were imaged to determine tumor burden and randomized before being treated with CAR T cells via i.p. injection 6 weeks post-tumor inoculation. Given that the 4H11-28z CAR T cells used as control CAR T cells in previous experiments are targeted to ovarian tumors, we utilized CD19-specific 19-28z and 19-28z/IL-12 CAR T cells as controls in these experiments.[25] A single dose of WT1-28z CAR T cells mediated significantly enhanced survival compared to control CAR T-cell-treated mice ($P=0.009$) as well as untreated mice ($P=0.0034$) (Figure 4c). In addition, a single i.p. infusion of WT1-28z/IL-12 CAR T cells significantly enhanced the survival of tumor-bearing mice compared with treatment with control CAR T cells ($P\leqslant 0.0001$) (Figure 4c). Mice treated with WT1-28z/IL-12 exhibited 88% survival at 250 days compared with 67% survival with WT1-28z CAR T cells but this difference was not statistically significant ($P=0.23$). As early as 2 weeks after CAR T-cell injection (day 77 post-tumor cell inoculation) mice treated with WT1-28z or WT1-28z/IL-12 CAR T cells had significantly lower cancer burden as assessed by bioluminescent imaging (ROI) (Figure 4d). These studies support the potential of utilizing WT1-28z CAR T cells in both WT1+ hematological as well as solid tumor malignancies.

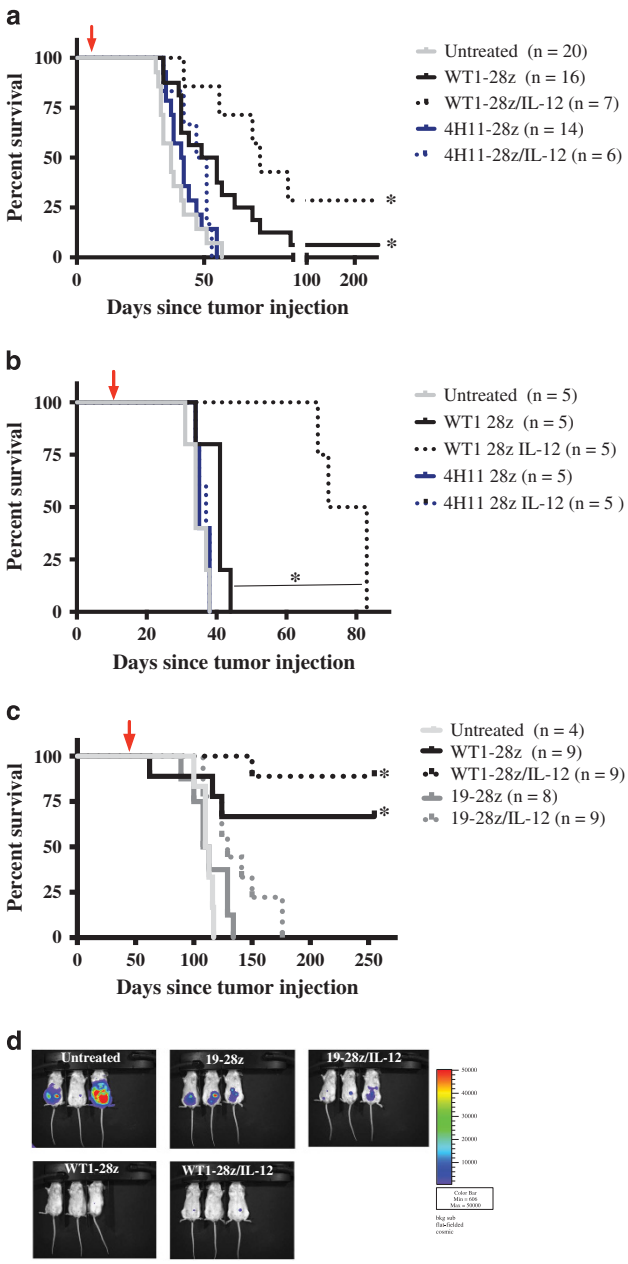

Figure 4. WT1-28z/IL-12 CAR T cells eradicates established tumors in a human xenograft mouse models. (**a**) SCID/Beige mice were injected i.v. with 5E6 Set2 cells and treated with i.v. injection of 2E7 CAR-positive primary human T cells 7 days post-tumor injection. WT1-28z CAR T cells significantly improved survival of mice compared to untreated mice (*$P=0.006$) or mice treated with control 4H11-28z CAR T cells (*$P=0.01$). WT1-28z/IL-12 CAR T cells had significant survival over mice treated with 4H11-28z/IL-12 CAR T cells (*$P=0.004$). ($n=3$ separate experiments). (**b**) SCID/Beige mice were i.v. injected with 5E6 Set2 cells and treated with i.v. injection of 2E7 CAR-positive primary human T cells 14 days post-tumor injection. WT1-28z/IL-12 CAR T cells significantly enhanced survival of mice in a more established disease model compared with WT1-28z CAR T cells (*$P=0.006$). (**c**) SCID/Beige mice were i.p. injected with 1E7 OVCAR3 cells and treated 41 days post-tumor injection with i.p. injection of 5E6 CAR-positive primary human T cells. WT1-28z CAR T cells mediated significantly enhanced survival compared with treatment with control 19-28z CAR T cells (*$P=0.009$) or untreated mice (*$P=0.0034$). A single dose of WT1-28z/IL-12 CAR T cells mediated significantly enhanced survival compared with treatment with control 19-28z/IL-12 CAR T cells (*$P\leqslant 0.0001$). Mice treated with WT1-28z/IL-12 CAR T cells exhibited enhanced survival as compared with WT1-28z CAR T cells, but this difference was not statistically significant ($P=0.23$) ($n=2$ independent experiments). (**d**) Bioluminescent imaging of mice on day 58 after OVCAR3 tumor injection show eradication of tumor in mice treated with WT1-28z or WT1-28z/IL-12 CAR T cells.

IL-12 secreting TCRm CAR T cells exhibit an enhanced pro-inflammatory gene expression profile

To define the mechanism associated with enhanced *in vivo* anti-tumor efficacy of IL-12 secreting CAR T cells, we next investigated the molecular pathways that may lead to superior T-cell cytotoxic function of the WT1-28z/IL-12 CAR T cells compared with WT1-28z CAR T cells by gene expression analysis. CAR T cells were stimulated by co-culturing with a three cells. FACs sorting was used to isolate CAR T cells and genomic RNA was extracted and analyzed for expression of 770 genes in the nanoString technology PanCancer Immune Profiling Panel. We focused on genes with statistically significant ($P = \leqslant 0.05$) differential expression between WT1-28z and WT1-28z/IL-12 CAR T cells. Overall, a distinct transcriptional profile of gene expression was seen differentiating WT1-28z/IL-12 from WT1-28z CAR T cells (Figures 5a–c), including differential expression of genes encoding cytokines (Figure 5a), chemokine receptors, and molecules involved in chemotaxis (Figure 5b). WT1-28z/IL-12 CAR T cells

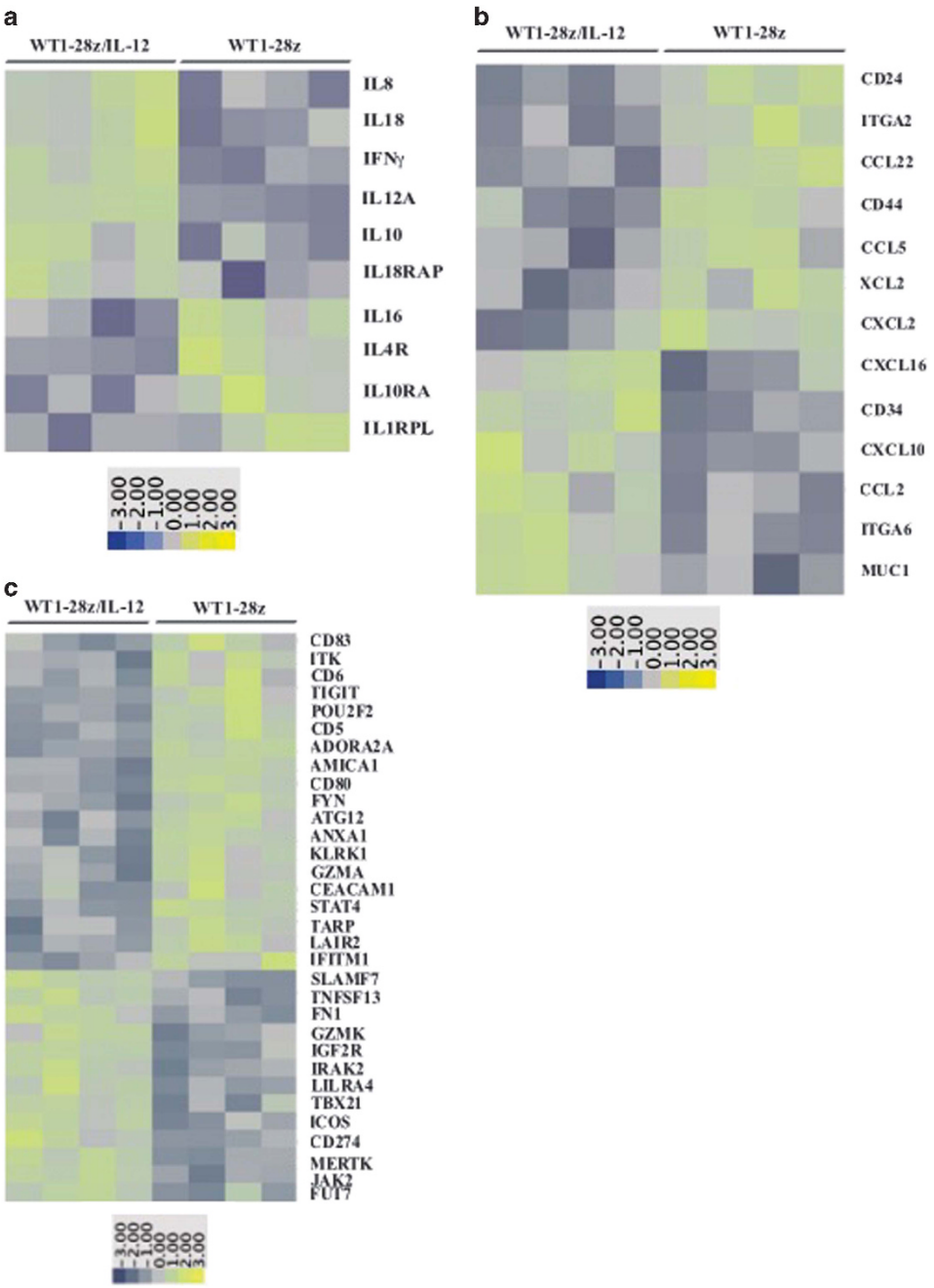

Figure 5. Differential gene expression of WT1-28z/IL-12 CAR T cells compared with WT1-28z CAR T cells when stimulated with OVCAR3 tumor cells for 48 hs. (**a**) Heat map of statistically significant ($P \leqslant 0.05$) differential gene expression of cytokines between four normal donor T cells expressing WT1-28z/IL-12 CAR or WT1-28z CAR. (**b**) Heat map of statistically significant ($P \leqslant 0.05$) differential expression of genes involved in chemokine or chemotaxis functions between four normal donor T cells expressing WT1-28z/IL-12 CAR or WT1-28z CAR. (**c**) Heat map of statistically significant ($P \leqslant 0.05$) genes showing a distinct transcriptional profile between four normal donor T cells expressing WT1-28z/IL-12 CAR or WT1-28z CAR.

expressed higher levels of IL-12 A and IFN-γ compared with WT1-28z CAR T cells (Figure 5a). Furthermore, WT1-28z/IL-12 CAR T cells demonstrated increased expression of genes involved in Th1 polarizing of T cells such as TBX21, SLAMF7, IL18, IL18RAP and a decrease in genes involved in Th2 polarizing such as AMICA1, IL4R and ITK.[33,34]

DISCUSSION

Identification of suitable surface exposed TAA that are ubiquitously expressed on all tumor cells but are absent or only minimally expressed on normal tissues is a major obstacle to cancer immunotherapy in general and particularly with respect to traditional CAR T-cell therapy. Although there may be a paucity of identified surface exposed TAA suitable for targeting by the immune system, there are a number of intracellular TAA masked from recognition by antibodies, and consequently antibody-derived CAR T cells, that would otherwise be attractive targets for immune-based therapies of cancer. One approach to target these otherwise mAb and CAR T-cell 'untargetable' intracellular antigens is through the generation of TCRm antibodies that specifically recognize peptides from these intracellular antigens expressed on the surface of the tumor cell in the context of HLA molecules. With the advent of highly diverse human scFv phage display libraries, it is now possible to generate scFvs and consequent mAbs capable of recognizing these peptide/HLA complexes on the tumor cell surface. ESK1 is a previously described mAb that recognizes the WT1 RMF peptide in the context of the HLA-A*02:01 and has demonstrated significant anti-tumor efficacy in preclinical models of disease. WT1 is a highly attractive target for immune-based therapies of cancer given that the antigen is markedly overexpressed on a variety of both solid tumor and hematological malignancies with minimal or low level expression on normal tissues. Whereas mAb therapy can be effective, cellular therapy with CAR T cells has its advantages. CAR T cells are a living drug that can co-modified to deliver immune-modulatory molecules to the tumor microenvironment. Herein we demonstrate the successful conversion of the WT1-targeted ESK1 mAb into a CAR, WT1-28z, with specificity to the RMF peptide in the context of HLA-A*02:01 expressed on the tumor cell surface.

Having generated the WT1-28z CAR from the ESK1 mAb, we demonstrate that T cells modified to express this CAR specifically induced T-cell activation and consequent cytotoxic activity against both hematological and solid tumor HLA-A*02:01$^+$, WT1$^+$ cell lines. Significantly, WT1-28z CAR T cells only recognized and lysed WT1$^+$/HLA-A*02:01$^+$ primary tumor cells or cell lines, failing to recognize or lyse WT1$^+$/HLA-A*02:01$^-$ or WT1$^-$/HLA-A*02:01$^+$ tumor cell lines. *In vivo* both WT1-directed CAR T cells significantly enhanced the survival of both leukemic and ovarian tumor-bearing mice. WT1-28z CAR T cells optimized to secrete IL-12 demonstrated further enhanced *in vivo* anti-tumor efficacy and showed a distinct T-cell gene expression profile consistent with the observed enhanced in *in vivo* anti-tumor efficacies.

The work presented here is the first to demonstrate the *in vivo* therapeutic efficacy of TCRm CAR T cells in both hematological and solid tumors and significantly expands the work from previously published reports. Zhao *et al.* previously described second-generation 4-1BB containing TCRm CARs directed toward WT1 in the context of HLA-A*02:01 (ref. 35) similarly utilizing a scFv isolated from a human scFv phage library. Although the authors demonstrate that T cells and NK-92 cells transduced with this CAR-mediated lysis of WT1$^+$/HLA-A*02:01$^+$ human tumor cell lines, significantly these studies were restricted to *in vitro* studies. In addition, another second-generation WT1-targeted CAR was tested in *in vitro* comparison assays to a high-affinity WT1 TCR,[36] although these studies were limited in demonstrating the function of the CD28-FcγRI containing CAR. While both these studies both generated WT1-targeting CAR T cells, their lack of *in vivo* analysis may be inadequate for evaluating CAR T-cell function, as CAR T cells can have discrepant *in vitro* and *in vivo* activities.[37] Herein we have significantly expanded upon both these *in vitro* analyses of WT1-targeted TCRm CAR T cells, as well as optimized our studies to more clinically meaningful *in vivo* murine tumor models.

To this end, we further expanded on our *in vivo* results of WT1 TCRm CAR T cells by demonstrating enhanced anti-tumor efficacy when TCRm CAR T cells are further modified to secrete the pro-inflammatory IL-12 cytokine. We and others have previously demonstrated enhanced function of IL-12-secreting engineered T cells with respect to *in vitro* proliferation, cytotoxicity, and a retained central memory-effector phenotype.[26,38–40] Consequently, we have reported markedly augmented *in vivo* anti-tumor efficacy and persistence of IL-12 armored CAR T cells when compared with T cells modified to express the CAR alone.[26,28] This work also adds to the mechanistic insight of enhanced *in vivo* anti-tumor efficacy of IL-12 secreting CARs by demonstrating differential gene expression profiles between activated WT1-28z and WT1-28z/IL12 T cells. Significantly, we found that WT1-28z/IL-12 CAR T cells exhibited an upregulated expression of a gene signature associated downstream of IFN-γ signaling, as well as Th1 polarization when compared with activated T cells modified to express the WT1-28z CAR alone.

Clinical trials with WT1 peptide vaccines have reported promising clinical success,[14,18,19] although efficacy in some studies was highly correlated and dependent on the presence and subsequent vaccine mediated induction of pre-existing WT1-specific T cells in patients.[14] The WT1 TCRm CAR T-cell approach overcomes this limitation associated with WT1 vaccine-based immuno-therapies. The TCRm CAR strategy described herein directly results *de novo,* in the *ex vivo* generation WT1-specific T cells. Consequently, our TCRm CAR T-cell immunotherapy approach bypasses the reliance of a pre-existing endogenous T-cell WT1-specific immune response, likely further limited or abrogated by prior chemotherapy, that is requisite for clinical efficacy in the setting of WT1 vaccines. The limited success of WT1 vaccine therapy, while validating WT1 as an attractive target for immune-based therapies of cancer, may be markedly enhanced in the clinical setting utilizing WT1-targeted TCRm CAR T cells. However, a limitation of the TCRm CAR T-cell approach, as compared with a polyvalent vaccine, is the restriction to a specific HLA type and a single presented epitope.

Our novel targeting approach involves gene transfer of CARs specific for internal TAA into T cells as a substitute for *ex vivo* expansion of tumor infiltrating lymphocytes with an endogenous cancer-specific TCR. *Ex vivo* expansion of WT1-reactive T cells and TCR gene therapy have had limited success in the clinic.[41–44] However, our TCRm CAR T-cell approach may have benefits over these approaches. T cells require co-stimulation to be optimally activated and lyse targets. Although our TCRm CAR requires HLA specificity, this technology allows for additional modification to include co-stimulation. This is a benefit over TCR therapy as immune evasion by cancer cells includes lack of or down-regulation of co-stimulatory molecules.

The data presented here provides the first proof-of-concept that CAR T cells can successfully enhance survival in mice by targeting intracellular antigens. Significantly, unlike current CAR T-cell clinical trials wherein the modified T cells have recognition restricted to TAA expressed on the tumor cell surface and in turn with application to only a limited number of closely related malignancies, the presented WT1 TCRm CAR T-cell approach allows for the application of a single CAR to a wider array of malignancies including both hematological and solid tumor malignancies. We further, and uniquely, demonstrate that the *in vivo* efficacy of TCRm CAR$^+$ T cells could be markedly enhanced when genetically modified to secrete IL-12. This finding will significantly impact on the design of planned future clinical trials

wherein we plan to treat patients with relapsed and refractory WT1$^+$/HLA-A*02:01$^+$ tumors with WT1-28z/IL-12-armored CAR T cells. Finally, we conclude that the presented data not only serves as a rationale for clinical translation of WT1 TCRm CAR T-cell technology, but further serves as a proof-of-principle for TCRm CAR T-cell technology that in turn can be readily expanded to numerous attractive intracellular TAA including telomerase, PRAME, MAGE, and mutated RAS, each relatively ubiquitously expressed on a broad array of tumor cell types.

CONFLICT OF INTEREST

RJB is a co-founder, stockholder and consultant for Juno Therapeutics Inc. DAS and TD are inventors of TCRm technology owned by MSKCC, licensed to Novartis.

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