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Research Article

Allogeneic gene-edited HIV-specific CAR-T cells secreting PD-1 blocking scFv enhance specific cytotoxic activity against HIV Env^{+} cells *in vivo*

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ABSTRACT

HIV-specific chimeric antigen receptor (CAR) T-cells have been developed to target HIV-1 infected CD4^{+} T-cells that express HIV Env proteins. However, T cell exhaustion and the patient-specific autologous paradigm of CAR-T cell hurdled clinical applications. Here, we created HIV-specific CAR-T cells using human peripheral blood mononuclear cells and a 3BNC117-E27 (3BE) CAR construct that enabled the expression of programmed cell death protein (PD-1) -blocking scFv E27 and the single-chain variable fragment of the HIV-1-specific broadly neutralizing antibody 3BNC117 to target native HIV Env. Compared with T cells expressing 3BNC117-CAR alone, 3BE CAR-T cells showed greater cytotoxic activity against HIV Env^{+} cells with stronger proliferation capability, higher killing efficiency, and enhanced cytokine secretion in the presence of HIV Env-expressing cells. Furthermore, we manufactured TCR-deficient 3BE CAR-T cells through gene editing and demonstrated that these CAR-T cells could effectively kill HIV Env $^{+}$ cells *in vivo* without the occurrence of severe graft-versus-host disease (GvHD) in NSG mice. These data suggest that we have provided a feasible approach to the generation of "off-the-shelf" anti-HIV CAR-T cells in combination with PD-1 checkpoint blockade immunotherapy, which can be a powerful therapeutic candidate for the functional cure of HIV.

1. Introduction

For individuals with acquired immunodeficiency syndrome (AIDS), combined antiretroviral therapy (cART) does not eliminate the viral reservoirs and requires lifelong adherence to expensive regimens with potential toxic effects (Barré-Sinoussi et al., 1983; Katlama et al., 2013). Recently, a functional cure for HIV infection has become a global research priority(Archin et al., 2014; Katlama et al., 2013; Liu et al., 2015), to boost the immune response in infected individuals so that antiviral drugs can be discontinued (Ruelas and Greene, 2013). A successful strategy for a functional cure will require potent and persistent cellular immune surveillance, and the adoptive transfer of effector T-cells modified with a chimeric antigen receptor (CAR-T) might be an applicable approach (Maldini et al., 2018). CAR-T therapy, which provides non-major histocompatibility complex-restricted recognition of cell surface components to kill target cells with high efficiency (Rosenberg and Restifo, 2015), has achieved tremendous successes in the treatment of hematological malignancies (Ma et al., 2019). Notably, one of the earliest clinical trials of CAR-T therapy was for the treatment of AIDS in 1994, using the "first generation" CD4-based CAR constructs (Roberts et al., 1994). Unfortunately, those CAR-T cells showed no clear benefits in HIV-infected individuals despite their safety (Deeks et al., 2002) and decade-long persistence *in vivo*

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(Scholler et al., 2012). In the second generation CAR containing costimulation modulates such as CD28 and 4-1BB (Hombach et al., 2013), the efficacy of anti-tumor CAR-T cells was significantly improved (Lee et al., 2015; Maude et al., 2014). The success of CD19 CAR-T cells has rekindled the interest in anti-HIV CAR-T therapy.

Since the CD4 domain might allow HIV-1 infection of CAR-T cells and the affinity could vary greatly between different HIV-1 envelopes (Johnston et al., 2009), several groups have tried to manufacture new anti-HIV CAR-T cells by replacing the extracellular $CD4^+$ domain of CAR with a broadly neutralizing antibody (bNAb) single-chain variable fragments (scFv), such as VRC01, 3BNC117, and 10E8 (Alfageme-Abello et al., 2021; Ali et al., 2016; Herzig et al., 2019; Liu et al., 2016). The safety, tolerability, and therapeutic efficacy of 3BNC117 in HIV-infected human individuals have been confirmed in phase I and phase IIa clinical trials (Caskey et al., 2015; Scheid et al., 2016). The potent antiviral activity of 3BNC117-based CAR *in vitro* (Ali et al., 2016) and *in vivo* (Jiang et al., 2021) has also been demonstrated. However, immunosuppression may be a significant obstacle to clinical application of bNAb-based CARs. The inhibitory receptor programmed cell death protein 1 (PD-1) is markedly upregulated on the surface of HIV-specific CD8 T-cells *in vivo*, resulting in a reduced capacity of cytotoxic T lymphocytes (CTLs) to produce cytokines as well as impaired proliferation (Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006). On the other hand, increasing evidence shows chronic HIV infection induces programmed cell death protein 1 ligand 1 (PD-L1) expression in a variety of cell types (Bushara et al., 2022; Grabmeier-Pfistershammer et al., 2011; Meier et al., 2008; Porichis and Kaufmann, 2012). Previous studies have demonstrated that the blockade of PD-1 using monoclonal antibodies enhances T-cell cycling and differentiation, resulting in rapid expansion of virus-specific CD8 T-cells with improved functional quality in simian immunodeficiency virus (SIV)-infected rhesus macaques (Harper et al., 2020; Velu et al., 2009). Recently, Liu et al. generated bNAb-derived CAR-T cells in which PD-1 expression was downregulated by using shRNA, and found that those CAR-T cells could reduce viral reservoir in HIV-1 infected individuals (Liu et al., 2021).

Another challenge in the development of anti-HIV CAR-T therapy for clinical application is the patient-specific autologous CAR-T paradigm. The majority of current CAR-T clinical trials utilize autologous T-cells from each patient, entailing unwarranted delays with prohibitive cost. For HIV-infected individuals, obtaining sufficient, good-quality T-cells for manufacturing therapeutic products proves difficult. Recently, many researchers have been paying attention to "off-the-shelf" CAR-T. Torikai et al. knocked out T-cell receptor (TCR) αβ expression in allogeneic CD19-CAR T cells using zinc finger nucleases (ZFNs) (Torikai et al., 2012). Similarly, Poirot et al. manufactured TCR-deficient CD19-CAR T-cells via the application of transcription activator-like effector nuclease (TALEN) gene-editing technology and avoided graft-versus-host disease (GvHD) in a xenograft mouse model (Poirot et al., 2015). Furthermore, Eyquem et al. directed a CD19-CAR to the TCR α constant (*TRAC*) locus using homology-directed recombination (HDR) together with CRISPR/Cas9 technology (Eyquem et al., 2017).

Here, we designed and produced anti-HIV CAR-T cells derived from 3BNC117 targeting HIV-1 envelope glycoprotein and secreting PD-1 blocking scFv E27 (Rafiq et al., 2018), and disrupted the expression of endogenous TCR in these CAR-T cells to avoid GvHD. We assessed the ability of 3BNC117-E27/TCR-deficient CAR-T cells to eliminate HIV Env^+ cells and found that the edited cells with enhanced T-cell potency vastly outperformed conventional 3BNC117-CAR T-cells and could be a potential candidate for the functional cure of HIV.

2. Materials and methods

2.1. Anti-HIV CARs

Second-generation 3BNC117 CAR was provided as a generous gift by Dr. Otto O. Yang (Ali et al., 2016). The PD-1 blocking scFv E27 gene was synthesized by GENEWIZ (GENEWIZ, Suzhou, China) as previously described (Rafiq et al., 2018), which adjoined the human IgG secretion signal (hIgGSS, DNA sequence: ATGGGCTGGAGCTGCATCATCCTGTTCCTGGTGGCAACCGCAACCGGAGTGCACAGC). 3BNC117 CAR and E27 were cloned into a lentiviral vector (pTRPE-GFP-T2A-mRFP, a generous gift from Professor James L. Riley) backbone downstream of the EF1a promoter.

2.2. Cell lines

HEK293T and Jurkat cells were purchased from ATCC (Manassas, VA). LHL2/3 and LEL6 cells were constructed in our laboratory and used elsewhere (Jiang et al., 2021). LHL2/3 cells were HL2/3 cells (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HL2/3 from Dr. Barbara K. Felber and Dr. George N. Pavlakis) encoded to highly express PD-L1 and the firefly luciferase gene. LEL6 cells are Jurkat cells that highly express Env, PD-L1, and the firefly luciferase gene. ACH-2 cells are HIV-1 latently infected CEM cells that contain a single copy of proviral DNA per cell (obtained from the NIH AIDS Reagent Program). HEK293T and LHL2/3 cells were maintained in DMEM (Gibco, Grand Island, NY) supplemented with 10% foetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). Jurkat and LEL6 and ACH-2 cells were maintained in RPMI1640 medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco).

2.3. Isolation and culture of primary human T lymphocytes

Primary human $CD3^+$ T cells were isolated from healthy donors from the Blood Center of Shanghai (Shanghai, China), and approved by the Ethics Committee of the School of Life Sciences, Fudan University, using Ficoll-Paque gradient separation (GE Healthcare, Boston, MA) and negative immunomagnetic bead selection according to the manufacturer's protocol (Miltenyi Biotec, Germany). Primary $CD3^+$ T cells were stimulated with CD3/CD28 T-cell activation Dynabeads (Gibco) for 48 h. These T-cells were expanded in Vivo15 (Lonza, Basel, Switzerland) medium supplemented with 10% FBS, 5 ng/mL IL-2 (R&D, St. Paul, MN), and 2 ng/mL IL-15 (R&D). The cells were maintained at 37 °C and 5% CO_2.

2.4. Production of pseudoviruses and generation of anti-HIV CAR-T cells

To produce pseudoviruses, HEK293T cells were seeded at a density of 5×10^6 cells per 10-cm dish. After 14–16 h, pseudoviruses were generated by co-transfecting HEK293T cells with plasmids pTRPE encoding various CAR moieties (10 μg), psPAX2 (6.8 μg), and pMD.2G (3.4 μg) using PEI following the manufacturer's instructions. Lentiviral supernatants were harvested at 48 and 72 h after transfection. Cell debris was removed by centrifugation at 2650×*g* for 20 min, followed by filtration through a 0.45 μm membrane filter (Millipore, Boston, MA). Pseudoviruses were concentrated by centrifugation at 125,000×*g* for 2 h at 4 °C, resuspended in the frozen stock solution, and stored at −80 °C. For infection, 1×10^6 stimulated $CD3^+$ cells were transduced with concentrated pseudovirus supernatant at specific MOIs plus polybrene (Yeason) at 7 μg/mL for 12 h. Pseudoviruses were then replaced by fresh culture media as described above.

2.5. Flow cytometry

The following monoclonal antibodies and reagents were used with the indicated specificity and appropriate isotype controls: FITC-conjugated goat anti-human $F(ab')_2$ antibody (Jackson, Pennsylvania), PE-conjugated mouse anti-human CD279 (PD-1) antibody (BioLegend, Santiago, CA), FITC mouse anti-human TCRαβ (BD Biosciences, San Jose, CA), FITC mouse anti-human CD25 (BD Biosciences), PE mouse anti-human CD69 (BD Biosciences), FITC mouse anti-human CD45RA (BD Biosciences), and PE mouse anti-human CD62L (BD Biosciences). All cells were washed, resuspended in 100 μL PBS containing specific antibodies, and incubated for 30 min at 4 °C. Data were acquired on a Beckman

Coulter Gallios flow cytometer and analysed using FlowJo version 10 (Tree Star, Ashland, OR).

2.6. Western blotting

The supernatants were harvested and the cells were lysed on ice for 30 min. The thermally denatured protein extracts were loaded onto a 10% polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and nitrocellulose membrane was blocked for 1 h. The antibodies and dilutions used in these experiments were as follows: rabbit-anti-HA (1:2500 dilution) (Abmart, Shanghai, China), rabbit anti-β-actin (1:2500 dilution) (Proteintech, Wuhan, China), and goat-anti-rabbit-secondary antibodies (1:5000 dilution) (Beyotime, Shanghai, China). Bands were visualized using an enhanced chemiluminescence (ECL) western blotting system (Santa Cruz Biotechnology).

2.7. Cytotoxicity assay

Lactate dehydrogenase (LDH) assay was performed to measure the specific killing activity of anti-HIV CAR-T cells toward LHL2/3 and LEL6 cells at different ratios from 1:1 to 10:1 using the CytoTox 96 non-radioactive cytotoxicity kit (Promega, Madison, WI) following the manufacturer's instructions. Briefly, anti-HIV CAR-T cells (effectors) and LHL2/3 cells or LEL6 cells (targets) were cultured together for 4–8 h at various effector-to-target (E:T) ratios in a 96-well U-bottom plate at 37 °C. And the effector cells were added at each concentration used in the experimental setup to obtain the effector cell spontaneous LDH release which would be taken into consideration in the final calculation to exclude self-lysis of the effector T cells. The supernatants were collected and incubated with 50 μL Cytotox 96 reagent for 30 min. A volume of 50 μL stop solution was added to each well, and absorbance values of the wells were measured at 490 nm using SYNERGY microplate reader (BioTek, Shoreline, WA, USA).

The expression level of luciferase was also detected after co-incubation at different E:T ratios to confirm the killing efficiency. Cells were harvested after co-incubation and resuspended in 50 μL PBS. Then, 50 μL of Glo reagent (Promega) was added to each well and incubated for 5 min. Luminescence was performed using an inspired plate reader (Biotek, Montpelier, VT).

2.8. Proliferation assay

The proliferation of anti-HIV CAR-T cells was measured using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD, USA). Briefly, the CAR-T cells were incubated with LHL2/3 cells at 1:1 for 24–96 h in a 96-well plate, then CAR-T cells were harvested into another 96-well plate and 10 μL of cell counting kit (CCK)-8 solution was added into each well of the 96-well plate and incubated for 1 h at 37 °C. Optical density (OD) values were measured at 450 nm using SYNERGY microplate reader (BioTek, Shoreline, WA, USA).

2.9. Construction of CRISPRs and cell electroporation

CRISPR-Cas9 crRNA and tracrRNA were chemically synthesized by IDT (Coralville, Iowa, United States) and mixed at a ratio of 1:1, followed by incubation at 95 °C for 5 min, and then allowed to slowly cool to room temperature to provide annealed single guide RNA (sgRNA). The following sgRNA targeting sequences were used in our study: *TRAC*-sgRNA-1, AGAGTCTCTCAGCTGGTACA; *TRAC*-sgRNA-2, TGTGCTAGACATGAGGTCTA; *TRAC*-sgRNA-3, ACAAAACTGTGCTAGACATG; *TRAC*-sgRNA-4, TCAGGGTTCTGGATATCTGT. S.p. HiFi Cas9 Nuclease V3 was purchased from IDT (Coralville, IA). Cas9 ribonucleoproteins (RNPs) were produced by complexing sgRNA and spCas9 for 10 min at 25 °C, and electroporated immediately after complexing.

Electroporation was performed using the Lonza 4D electroporation system (Basel, Switzerland) per the manufacturer's instructions. For 20 μL Nucleocuvette Strips, 2×10^6 cells resuspended in electroporation buffer were mixed with the RNP complex (containing 80 pmol Cas9 and 80 pmol sgRNA) and transferred to a cuvette for electroporation. After electroporation, pre-warmed 100 μL of media was added to the cuvette immediately, and the cells were incubated at 37 °C for 15 min. The electroporated cells were then resuspended in pre-warmed media containing FBS and cytokines, as described above, and transferred away from electroporation cuvettes.

2.10. T7E1 assay and sequencing of PCR fragments

Cells were pelleted and lysed, and a sequence spanning the *TRAC* target site was PCR-amplified from cell lysates to determine the level of genomic disruption of *TRAC* using a T7E1 Surveyor Nuclease assay (NEB, Ipswich, MA). The PCR primers used for amplification of the target locus were as follows: *TRAC* forward, 5′-TTCCCATGCCTGCCTTTACTC-3'; *TRAC* reverse, 5′-GCTGTTGTTGAAGGCGTTTG-3′. The PCR products were ligated into the pMD18-T cloning vector (Takara Bio, Beijing, China) and transformed into *E. coli*. A single clone was selected and sequenced to calculate insertions or deletions (indels) and insertions.

2.11. ELISA assays

Anti-HIV CAR-T cells or untransduced $CD3^+$ T (UTD) cells were co-cultured with LEL6 cells (1×10^4 cells) at a 10:1 ratio for 24 h in 96-U bottom well plates. Supernatants were collected, and CAR-T cells cytokine release in response to stimulation with target cells was analysed using TNF-α and IFN-γ ELISA kits (Dakewe, Shenzhen, China).

Anti-HIV CAR-T cells or UTD cells were co-cultured with ACH-2 cells (2×10^4 cells) treated with 1 μmol/L JQ1 (an HIV latency-reversing agent) at different ratios from 1:1 to 10:1 for 24 h in 96-U bottom well plates, and HIV-1 production was measured via quantification of p24 using p24 ELISA kit (R&D System, Minnesota, USA). Supernatants of groups at 10:1 ratio were collected and the cytokine release was analysed using TNF-α ELISA kits (Dakewe, Shenzhen, China).

All assays were performed according to the manufacturers' instructions.

2.12. Luc-Env^+/PD-$L1^+$ NSG mouse model and CAR-T efficacy in vivo

Female NSG (NOD-*Prkdc*^{scid}*Il2rg*^{em1}/*Smoc*) mice aged 4–6 weeks, purchased from Shanghai Model Organisms, were maintained under specific pathogen-free (SPF) conditions. To generate the Luc-Env^+/PD-$L1^+$ mouse model, LEL6 cells were resuspended in PBS at 2×10^6 cells/mL and each NSG mouse was injected intravenously (i.v.) with 2×10^5 LEL6 cells on day 0. The mice were randomly divided into four groups (n = 4). Four days later, 2×10^6 UTD or CAR-T cells were injected into NSG mice via the tail vein. The second-round injections of 2×10^6 UTD or CAR-T cells were performed at Day 11 post LEL6 cell injection. The mice were subjected to weekly bioluminescence imaging using NightOWL LB983 (Berthold, Stuttgart, Germany), and the data were analysed and exported using IndiGO (Berthold).

2.13. GvHD studies

Mice were monitored for clinical GvHD 1–2 times per week. The degree of systemic GvHD was assessed using a scoring system described in previous studies (Cooke et al., 1996; Ghosh et al., 2017; Ren et al., 2017a) that incorporates five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity.

2.14. Statistics

Statistical analyses were performed using the GraphPad Prism software (GraphPad, San Diego, CA, United States). Experimental data are

presented as mean ± standard deviation (SD) and were analysed by *t*-test or one-way ANOVA. Statistical significance was set at $P < 0.05$.

3. Results

3.1. The genetic construction and expression of 3BNC117-based CARs against HIV-1

The composition of 3BNC117 CAR (3B CAR), which consists of the 3BNC117 scFv, IgG4 hinge, CD8 transmembrane (TM), and the 4-1BB signaling domain fused to the CD3 ζ signaling domain, was cloned to the pTRPE lentiviral vector to generate a "second generation" CAR vector (Fig. 1A). We additionally modified the CAR construct to include an HA-tagged anti-human PD-1 scFv E27 linked to the human IgG secretion signal (hIgGSS) peptide [3BNC117-E27 CAR (3BE CAR), Fig. 1A], which has been reported to block the PD-1/PD-L1 pathway by competitively binding to PD-1 and prevent CAR-T cells from exhausting (Rafiq et al., 2018) (Fig. 1B).

CAR genes were delivered by lentiviral vectors to primary $CD3^+$ T lymphocytes isolated from human peripheral blood mononuclear cells (PBMCs) to manufacture anti-HIV CAR-T cells. Flow cytometry using a goat antibody against human antigen-binding antibody fragment (Fab) was performed three days after transduction to demonstrate the expression of each CAR on the cell surface. At an MOI of ~5, the transduction of CAR lentiviral vectors produced approximately 7%–10% positive cells, while at a higher MOI (~10), the frequency of CAR-positive cells could increase to approximately 30%–50% (Fig. 1C).

3.2. Function of PD-1 blocking scFv E27 secreted by CAR-T cells in vitro

The expression and secretion of PD-1 blocking scFv E27 by 3BE CAR-T cells were demonstrated by Western blot analysis using an anti-HA-tag antibody (Fig. 2A). E27 was more abundant in the supernatant than in the cytoplasm, indicating the high efficiency of the secretion signal. To investigate the killing efficiency against HIV Env^+ cells *in vitro*, we co-cultured 3BE CAR-T cells with LHL2/3, which had PD-L1 overexpression by lentivirus based on HL2/3 (Ciminale et al., 1990) and constitutively expressed Env and PD-L1 at the cell surface (Jiang et al., 2021), at varying ratios. We then tested the specific cytotoxicity using an LDH release assay. The results suggested that both groups of 3BNC117 CAR-T cells efficiently eliminated Env^+ cells at a 10:1 ratio, whereas CAR-T cells secreting E27 scFv showed greater potency (Fig. 2B).

As E27 has been reported as a PD-1 blocking agent, we evaluated the binding of secreted E27 scFv to PD-1. The results of flow cytometry showed that 3BE CAR-T cells significantly decreased the surface detection of PD-1 compared to cells modified to express CAR alone (3B CAR-T cells)

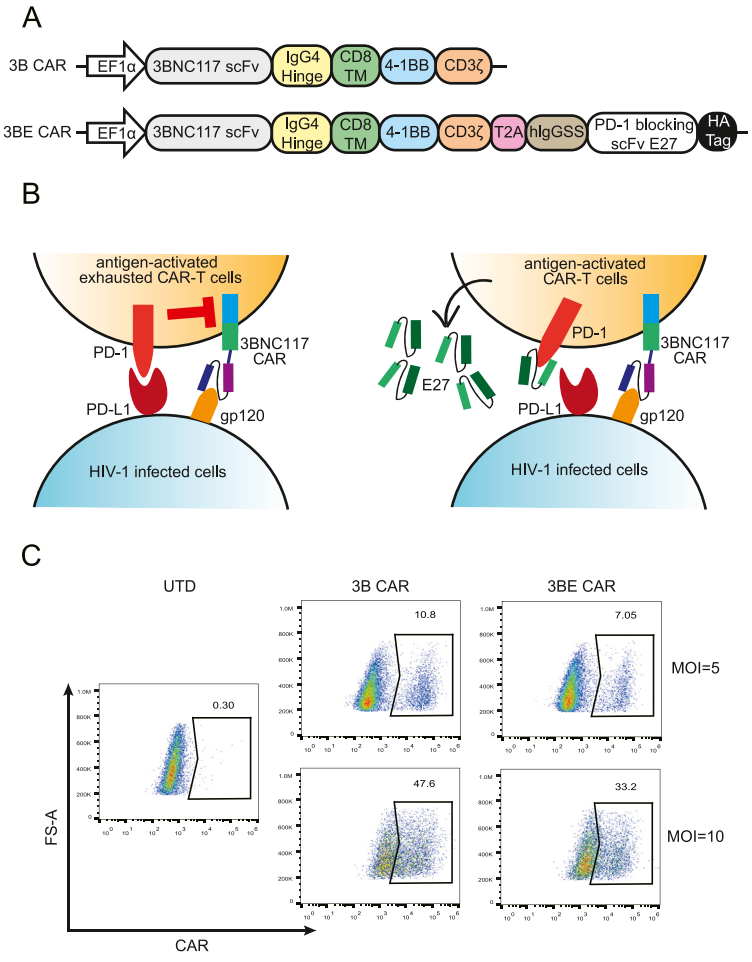

Fig. 1. Design and expression of 3BNC117-based CAR constructs. **A** Schematic representation of vectors encoding 3BNC117 CAR (3B CAR) and 3BNC117-E27 CAR (3BE CAR). EF1α, indicated in the diagram, represents the promoter sequences. **B** Schematic representation of the mechanism by which E27 blocks the binding of PD-1 to PD-L1 to prevent CAR-T cell exhaustion. **C** Representative flow plot showing surface CAR expression on $CD3^+$ T cells detected by human Fab using flow cytometry. UTD cells served as negative controls. TM, transmembrane; hIgGSS, human IgG secreting signal; UTD, untransduced.

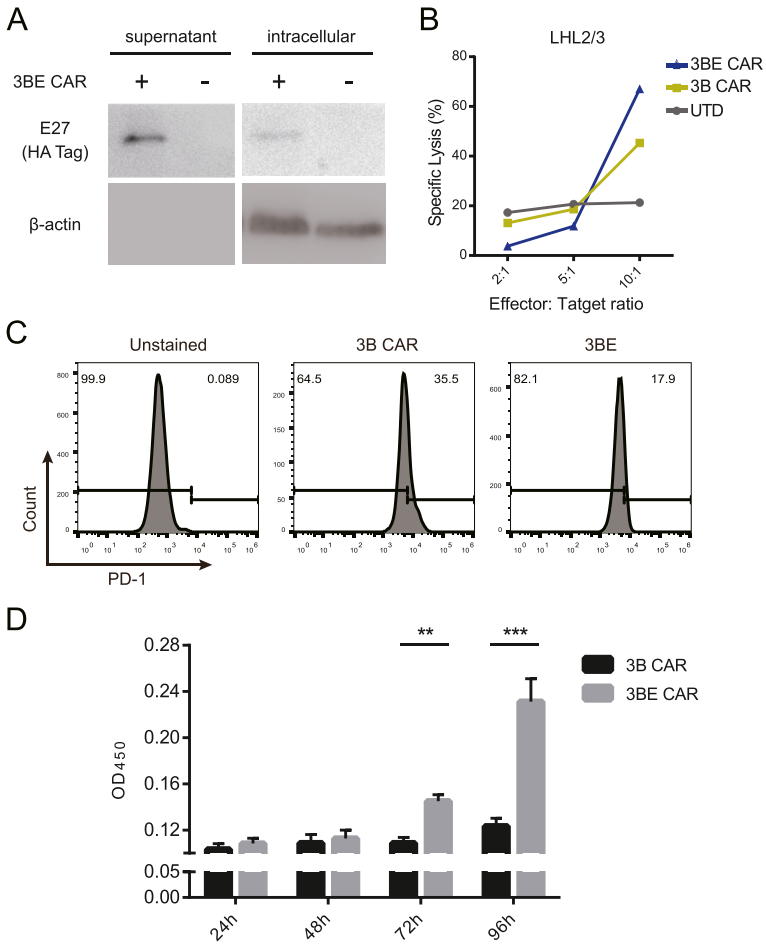

Fig. 2. The expression and secretion of PD-1 blocking antibody E27 enhances the proliferation and killing ability of CAR-T cells incubated with target cells. **A** Western blot of supernatant and intracellular material from cells transfected to express the secretable scFv with CAR, detected with anti-HA-tag antibody. **B** Anti-HIV CAR-T cells were incubated with LHL2/3 cells at different ratios (2:1, 5:1, and 10:1) for 4 h, and direct killing of LHL2/3 cells was performed using the LDH release assay. **C** Quantification of PD-1 detection by flow cytometry on 3BE CAR-T cells compared to 3B CAR-T cells. **D** Anti-HIV CAR-T cells were incubated with LHL2/3 cells at a ratio of 1:1 for 24–96 h, and CAR-T cell proliferation was detected by CCK-8. The data are shown as mean with standard deviation representative of three independent experiments. Statistical analysis was performed by *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$.

(Fig. 2C), which was consistent with previous reports (Rafiq et al., 2018) and suggested that E27 binds to CAR-T cells in an autocrine manner.

The PD-1/PD-L1 pathway plays an important role in T-cell exhaustion, anergy, and/or apoptosis (Dong et al., 2002; Freeman et al., 2000; Frey, 2015; Topalian et al., 2012); therefore, we hypothesized that PD-1-blocking scFv E27 could overcome the suppression to anti-HIV CAR-T cells. To verify this, 3B or 3BE CAR-T cells were co-cultured with LHL2/3 at a 1:1 ratio for 24–96 h, and proliferation of these CAR-T cells was detected using CCK-8. Ultimately, 3BE CAR-T cells showed significantly stronger proliferation capability than 3B CAR-T cells (Fig. 2D), demonstrating that E27 provides a proliferative advantage of anti-HIV CAR-T cells in the presence of target cells.

3.3. Disruption of human TRAC gene and functional inactivation of TCR in primary T cells with high efficiency by CRISPR/Cas9

To manufacture "off-the-shelf" anti-HIV CAR-T cells unable to initiate xenogeneic GvHD, efficient genomic editing to generate gene-disrupted T-cells that are deficient in TCR is key. Thus, we first selected four sgRNA-targeting sites on *TRAC* according to previously published results (Eyquem et al., 2017; Liu et al., 2017; Ren et al., 2017a; Ren et al., 2017b), all of which were within the first exon of the gene (Fig. 3A). Cas9 RNPs, complexes of spCas9 protein with the synthetic sgRNAs mentioned above, were electroporated into HEK293T cells, and their activity was validated by a mismatch-selective T7E1 surveyor nuclease assay. The results indicated that all the sgRNAs led to distinctly detectable cleavage at the genomic loci of *TRAC* except sgRNA-1; a higher cleavage rate with sgRNA-4 was confirmed, which we used in further experiments (Fig. 3B).

Next, electroporation of *TRAC*-4 RNP was performed on human primary $CD3^+$ T-cells, and disruption of the gene was confirmed by a T7E1 assay. We then amplified and subcloned the target region from the *TRAC*-4 RNP-treated T-cells. The genomic reading frame was confirmed to shift downstream of the *TRAC* target site by multiple peaks in Sanger sequencing data flanking the target site, as shown in Fig. 3C. Sanger sequencing data also confirmed that indels caused by NHEJ repair occurred in the majority (13/15, 86.7%) of *TRAC* PCR product clones, in which all mutations occurred precisely in the sgRNA-targeting region (Fig. 3D).

Even though we achieved high levels of TCR-negative knockout (TCR^-) T-cells (about 80% detected by flow cytometry) by utilizing *TRAC*-4 RNP, a higher proportion of T-cells deficient in TCR is necessary to avoid GvHD. Thus, a single step of CD3 negative selection was applied to enrich the TCR-disrupted population, and the frequency of TCR-cells could be increased to over 97% (Fig. 3E). To thoroughly test the functional inactivation of the TCR gene and the prevention of TCR stimulation-induced T-cells response after electroporation of *TRAC*-4

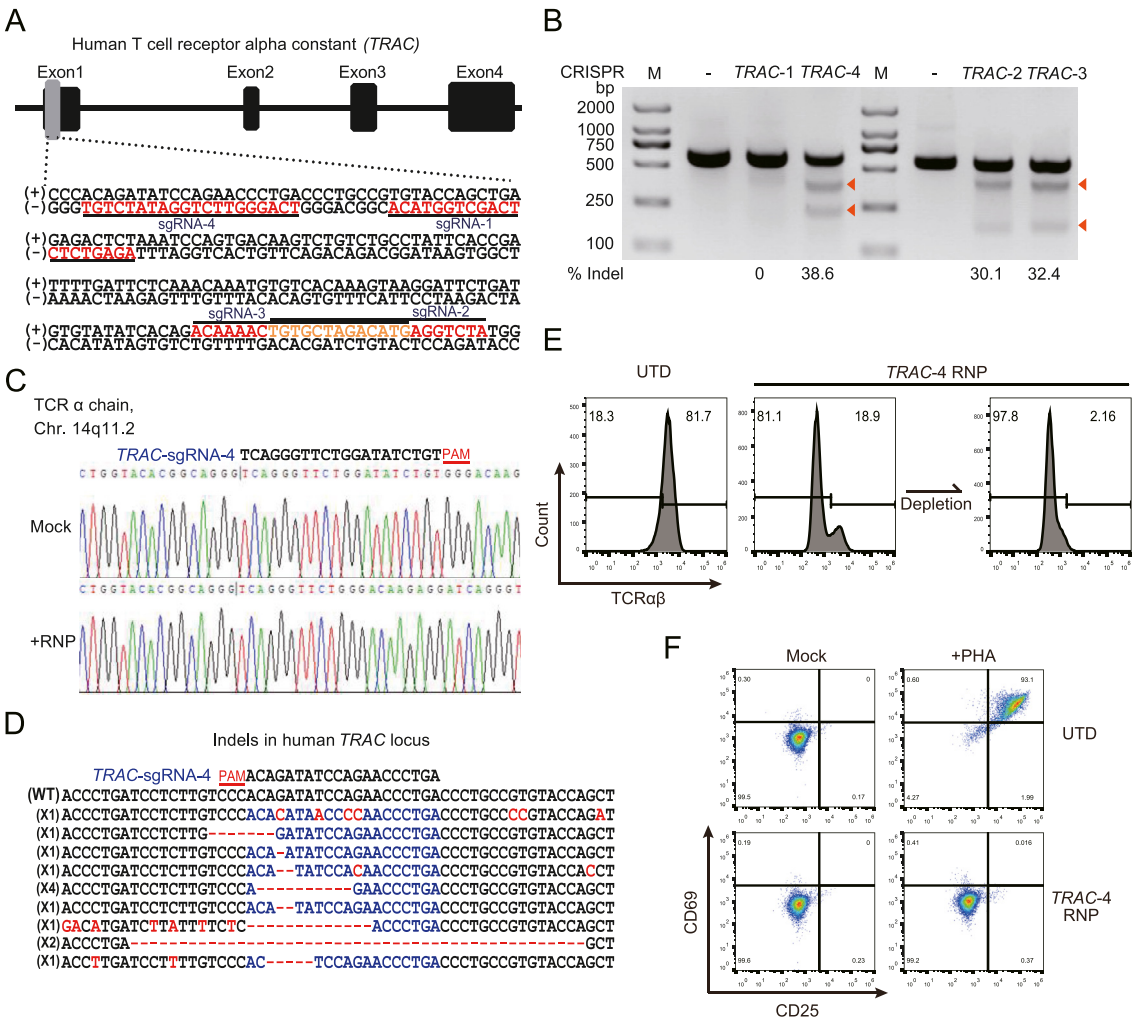

Fig. 3. CRISPR/Cas9 mediates efficient TCR disruption in T-cells. **A** Schematic diagram of sgRNA-targeting sites on *TRAC*. All targeting sites were within exon 1 of the gene. **B** RNPs targeting *TRAC* locus were electroporated into HEK293T cells. The amount of TCR-targeted gene disruption was measured using a mismatch-selective T7E1 surveyor nuclease assay on DNA amplified from the cells. The calculated amount of targeted gene disruption in *TRAC* (which was calculated using gradation analysis) is shown at the bottom. Arrows indicate the expected bands. **C** Multiple peaks in Sanger sequencing results showed CRISPR-mediated events of NHEJ at the *TRAC* genomic locus in $CD3^+$ T cells. **D** Indels and insertions observed by clonal sequence analysis of PCR amplicons from $CD3^+$ T cells after CRISPR-mediated recombination at the *TRAC* locus. **E** TCRαβ expression on CAR-T cells electroporated with *TRAC*-4 RNP and purified by negative selection using microbeads. **F** TCR-deficient cells are resistant to TCR stimulation. UTD or T cells electroporated with *TRAC*-4 RNP were treated for 36 h with 1 mg/mL PHA and analysed by flow cytometry for CD25/CD69 expression. NHEJ, non-homologous end joining; indels, insertions and deletions; RNP, ribonucleoprotein; UTD, untransduced; PHA, phytohemagglutinin.

RNP, the enriched cells were treated with phytohemagglutinin (PHA), and non-electroporated T-cells served as a positive control. The results showed that TCR-negative cells failed to upregulate the activation markers, CD69 and CD25, upon PHA-mediated TCR stimulation (Fig. 3F).

3.4. 3BE CAR-T cells have enhanced cytotoxic activity against HIV Env$^+$ cells regardless of the deficiency of TCR

TCR-deficient anti-HIV CAR-T cells secreting PD-1 blocking scFv E27 were manufactured by combining lentiviral transduction of 3BE CAR with electroporation of *TRAC*-4 RNP. Since the phenotype of CAR-T cells could affect their function and proliferation *in vivo* (Biasco et al., 2015; Gattinoni et al., 2011; Lugli et al., 2013; Sommermeyer et al., 2016), we measured the phenotype of our anti-HIV CAR-T cells 12 days after CD3/CD28 stimulation. The data showed that the $CD62L^+$ cells in all the groups of anti-HIV CAR-T cells were over 60%, which indicated that our CAR-T cells were mainly composed of naive and central memory cells, a phenotype associated with greater *in vivo* anti-HIV activity (Eyquem et al., 2017; Zhen et al., 2021) (Supplementary Fig. S1). The expression of CD45RA was also tested, and the percentage of $CD45RA^+$ cells was extremely low in all anti-HIV CAR-T cells (Supplementary Fig. S1), which indicated a smaller proportion of terminal effector cells and a potential advantage in persistence as well as efficacy *in vivo*.

To compare the specific cytotoxic activity against HIV Env^+ cells of these CAR-T cells, we co-cultured LHL2/3 with 3B, 3BE or 3BE/TCR^- CAR-T cells, or UTD cells as a control, at different ratios for 8 h and then assessed the specific killing of the Env^+ cells. All three groups of anti-HIV CAR-T cells displayed robust cytotoxicity against LHL2/3 compared to UTD cells, especially at 10:1 E:T ratio. Dose-dependent effects of these CAR-T cells on specific cytotoxic activity against Env^+ cells were observed in LHL2/3 at the indicated E:T ratios. The specific killing of LHL2/3 rose to 1.5- to 2-fold as the E:T ratio increased from 1:1 to 5:1, and continued to rise to approximately 2.6-fold at 10:1 E:T (Fig. 4A). Notably, 3BE CAR-T cells showed stronger killing activity than 3B CAR-T cells at each E:T ratio. In addition, TCR disruption had no discernible effect on the cytotoxicity of anti-HIV CAR-T cells against LHL2/3 (Fig. 4A). Furthermore, the expression level of luciferase, which was delivered into LHL2/3 as a reporter gene, was also detected after

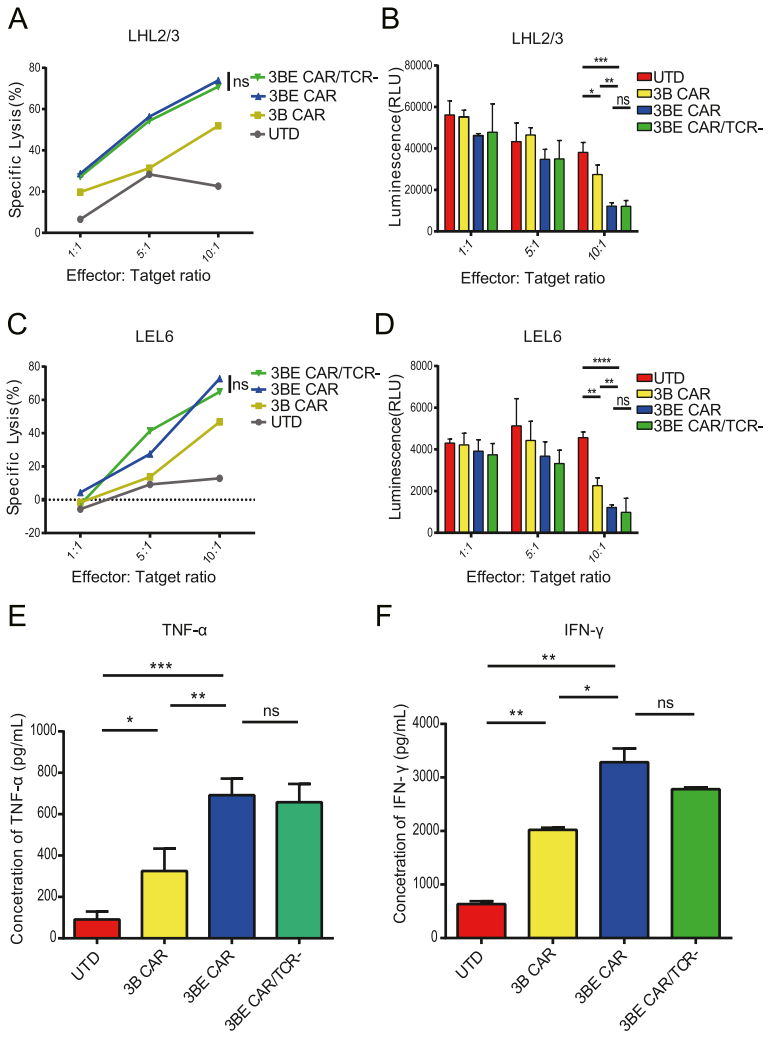

Fig. 4. 3BE CAR-T cells show enhanced cytolytic and cytokine secreted function on target cells regardless of the deficiency of TCR. Anti-HIV CAR-T cells were incubated with the target cells at different ratios (1:1, 5:1, and 10:1) for 8 h. Direct killing of LHL2/3 (**A**) or LEL6 cells (**B**) was performed using the LDH release assay. Detection of luminescence (RLU) in co-cultures reflects the lysis of LHL2/3 (**C**) or LEL6 cells (**D**). Anti-HIV CAR-T cells were co-cultured with LEL6 cells (1×10^4 cells) at 10:1 for 24 h, and supernatants were collected for ELISA to show the production of TNF-α (**E**) and IFN-γ (**F**) in the co-cultures. The data are shown as mean with standard deviation representative of three independent experiments. Statistcal analysis was performed by *t*-test or one-way ANOVA. ns $P > 0.05$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. UTD, untransduced; ELISA, enzyme linked immunosorbent assay; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.

co-incubation at different E:T ratios to confirm the killing efficiency. These results were consistent with those mentioned above. At a 10:1 E:T ratio, the reduction in luciferase expression caused by 3BE CAR-T cells was more remarkable than that caused by 3B CAR-T cells, but was equivalent to that caused by 3BE/TCR$^-$ CAR-T cells (Fig. 4B).

Since LHL2/3 was derived from HeLa cells rather than human T-cells, the LEL6 cell line, a luciferase-expressing Env$^+$/PD-L1$^+$ clonal derived from Jurkat cells previously constructed in our laboratory (Jiang et al., 2021), was used to examine whether similar results could be obtained. The results from these cells also indicated that 3BE CAR-T cells had greater cytotoxic activity than 3B CAR-T cells, even with the deficiency of TCR (Fig. 4C and D).

To further analyse the effect of the secretion of E27 and the disruption of *TRAC* on CAR-T cell function, the cytokine secretion ability of each type of anti-HIV CAR-T cell was tested when cultured in the presence of antigens. After co-culture with LEL6 at a 10:1 E:T ratio, all three groups of anti-HIV CAR-T cells produced and released much more TNF-α than UTD cells (Fig. 4E), and 3BE CAR-T cells showed enhanced cytokine secretion compared to 3B CAR-T cells. Importantly, 3BE/TCR$^-$ CAR-T cells did not demonstrate significantly reduced TNF-α release compared to 3BE CAR-T cells (Fig. 4E), indicating that the lack of endogenous TCR expression does not impair effector function. Similar results were observed for IFN-γ secretion (Fig. 4F).

To further examine the HIV-1-specific functional activity of anti-HIV CAR-T cells in virus-replication suppression, HIV latently infected cells ACH-2, which were used to test anti-HIV CAR-T cell functions in many published studies (Hale et al., 2017; Jiang et al., 2021; Zhen et al., 2021), were treated with JQ1 (one of bromodomain and extraterminal domain inhibitors which could successfully reactivate HIV-1 in different latency models (Banerjee et al., 2012; Li et al., 2013) to reverse latency and produce HIV-1. And then, those cells were co-cultured with 3B, 3BE, 3BE/TCR$^-$ CAR-T cells, or UTD cells at different ratios. After 24 h, HIV-1 production was measured via quantification of p24. The results of p24-ELSA assays showed that the intracellular p24 concentrations in all the CAR-T groups were significantly lower than that in UTD group, and the intracellular p24 concentrations in 3BE and 3BE/TCR$^-$ CAR-T group were significantly lower than that in 3B CAR-T group both at 5:1 and 10:1 E:T ratio (Fig. 5A). The cytokine secretion ability of each type of anti-HIV CAR-T cells was also tested when co-cultured with ACH-2 cells treated with JQ1. At a 10:1 E:T ratio, all three groups of anti-HIV CAR-T

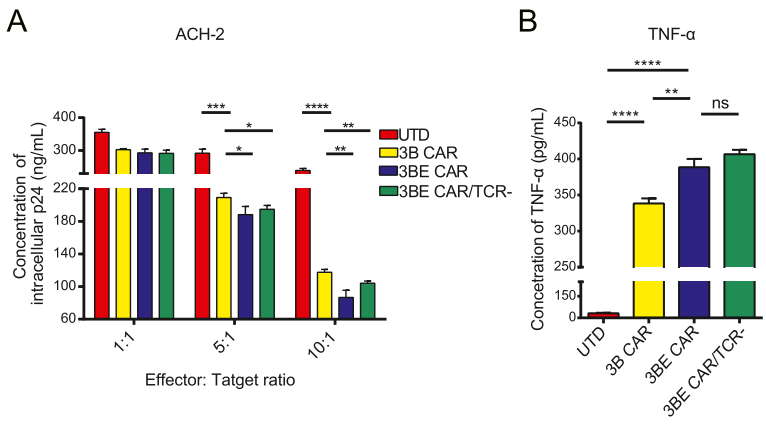

Fig. 5. 3BE CAR-T cells show greater functional activity to suppress HIV-1 replication *in vitro* regardless of the deficiency of TCR. **A** Anti-HIV CAR-T cells were incubated with HIV-1 latently infected cells ACH-2 (treated with HIV latency-reversing agent JQ1) at different ratios (1:1, 5:1, and 10:1) for 24 h. Cell pellets were collected, and intracellular p24 was detected by ELISA. **B** Anti-HIV CAR-T cells were co-cultured with HIV-1 latently infected cells ACH-2 (treated with HIV latency-reversing agent JQ1) at a 10:1 ratio for 24 h, and supernatants were collected for ELISA to show the production of TNF-α in the co-cultures. The data are shown as mean with standard deviation representative of three independent experiments. Statistcal analysis was performed by *t*-test. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. UTD, untransduced; ELISA, enzyme linked immunosorbent assay; TNF-α, tumor necrosis factor-α.

cells released much more TNF-α than UTD cells, and both 3BE and 3BE/TCR$^-$ CAR-T cells showed enhanced cytokine secretion compared to 3B CAR-T cells (Fig. 5B). These results suggested the greater potency of 3BE and 3BE/TCR$^-$ CAR-T cells compared to 3B CAR-T cells to control HIV-1 replication in the reactived HIV-1 latently infected cell line.

3.5. *TCR-disrupted 3BE CAR-T cells retain cytotoxic activity against HIV Env$^+$ cells in an HIV NSG mouse model with diminished GvHD*

TCR-deficient 3BE CAR-T cells showed robust *in vitro* cytotoxic activities against HIV Env $^+$ cells in our initial assays, such as lytic capacity, cytokine secretion, and potency to control HIV-1 replication. Through optimisation of our protocol, we developed a process to generate 3BE/TCR$^-$ CAR-T cells (Supplementary Fig. S2). Briefly, primary human T-cells were transduced with lentiviral 3BE-CAR on day 1 after stimulation, and *TRAC*-4 RNP was electroporated in the T-cells on day 5. The TCR-negative cell population was further enriched, and finally, the 3BE/TCR$^-$ CAR-T cells were harvested. To further study the cytotoxic activity against HIV Env$^+$ cells of anti-HIV CAR-T cells *in vivo*, 3B/3BE or 3BE/TCR$^-$ CAR-T cells were infused into NSG mice 4 and 11 days after the intravenous engraftment of LEL6 cells, and UTD cells were infused into control NSG mice. The signals of LEL6 cells were monitored via luminescence imaging once a week for approximately one month, representing HIV/AIDS progression (Fig. 6A). Engraftment and growth of LEL6 cells were evident in all mice in the UTD group by day 18 and increased sharply by days 18–30; whereas the bioluminescence intensity and area in the mice treated with the three groups of anti-HIV CAR-T cells were significantly smaller than that in the UTD mice (Fig. 6B and C). *In vivo*, 3B CAR-T cells exhibited limited efficacy to kill HIV Env$^+$ cells as the bioluminescence intensity in the mice in the 3B group increased by days 18–25. In contrast, the bioluminescence intensity in mice treated with 3BE or 3BE/TCR$^-$ CAR-T cells maintained a fairly low level (Fig. 6C), suggesting enhanced cytotoxic activities against HIV Env$^+$ cells of 3BE and 3BE/TCR$^-$ CAR-T cells for the restraint of HIV Env$^+$ cells in NSG mice compared to 3B CAR-T cells. The persistence of CAR-T cells in the peripheral blood of each mouse was detected 29 days post-HIV Env$^+$ cell implantation by amplifying the genomic DNA of cells in the peripheral blood with specific primers designed for 3BNC117 scFv. The expected DNA bands were detected in the 3B, 3BE, and 3BE/TCR$^-$ CAR-T-treated groups instead of the UTD group (Supplementary Fig. S3), indicating the persistence of our CAR-T cells in the peripheral blood of NSG mice.

Although 3BE CAR-T cells showed robust cytotoxicity against HIV Env$^+$ cells *in vivo*, treatment with 3BE CAR-T cells did not prolong the survival of HIV Env$^+$ NSG mice, while mice treated with 3BE/TCR$^-$ CAR-T cells exhibited significantly higher survival rates 37 days post-transplantation (Fig. 6D). We also found decreased weight loss rates in mice treated with 3BE/TCR$^-$ CAR-T cells compared with mice treated with 3BE CAR-T cells (Fig. 6E). Furthermore, we evaluated clinical GvHD scores of mice according to previously reported studies (Cooke et al., 1996; Ghosh et al., 2017; Ren et al., 2017a) by monitoring five clinical parameters after CAR-T cell injection, including weight loss, posture (hunching), activity, fur texture, and skin integrity. The GvHD scores were significantly lower in recipients of 3BE/TCR$^-$ CAR-T cells than in recipients of 3BE CAR-T cells in our NSG mouse model (Fig. 6F). These results suggested that TCR-deficient anti-HIV CAR-T cells could be advantageous in terms of *in vivo* security.

4. Discussion

To achieve a functional cure for HIV-1, it is essential to enhance specific immune responses to control viral replication and eliminate virus-infected cells (Katlama et al., 2013). We chose to revisit anti-HIV CARs given the success of CARs in cancer immunotherapy, and many studies have taken advantage of novel CARs based on the new generation of bNAbs and demonstrated their HIV-1-specific functional activity (Ali et al., 2016; Herzig et al., 2019; Liu et al., 2016).

However, several immune checkpoint molecules have shown upregulated expression in chronic HIV infection, such as PD-1 and Tim3, associated with the decline in function and exhaustion of specific CTLs in HIV-infected individuals (Day et al., 2006; Porichis and Kaufmann, 2011; Scholler et al., 2012; Trautmann et al., 2006). Therefore, we first generated 3BNC117-E27 CAR-T cells to both eliminate HIV-infected cells and secrete PD-1 blocking scFv E27 to overcome the immune inhibition caused by the PD-1/PD-L1 pathway. Our study demonstrated that E27 could specifically bind to PD-1 on the surface of 3BE CAR-T cells to block PD-1 signaling, thus enhancing the HIV Env-specific functional activities of CAR-T cells with stronger proliferation capability, greater killing activity, better capability for virus-replication suppression and enhanced cytokine secretion. Distinguished from the PD-1 dominant negative receptor (DNR), which can only block the PD-1/PD-L1 pathway in CAR-T cells expressing DNR, E27 secreted from our 3BE CAR-T cells may affect many other cells with high expression of PD-1 during HIV infection, such as HIV-infected CD4$^+$ T cells (Chew et al., 2016; Fromentin

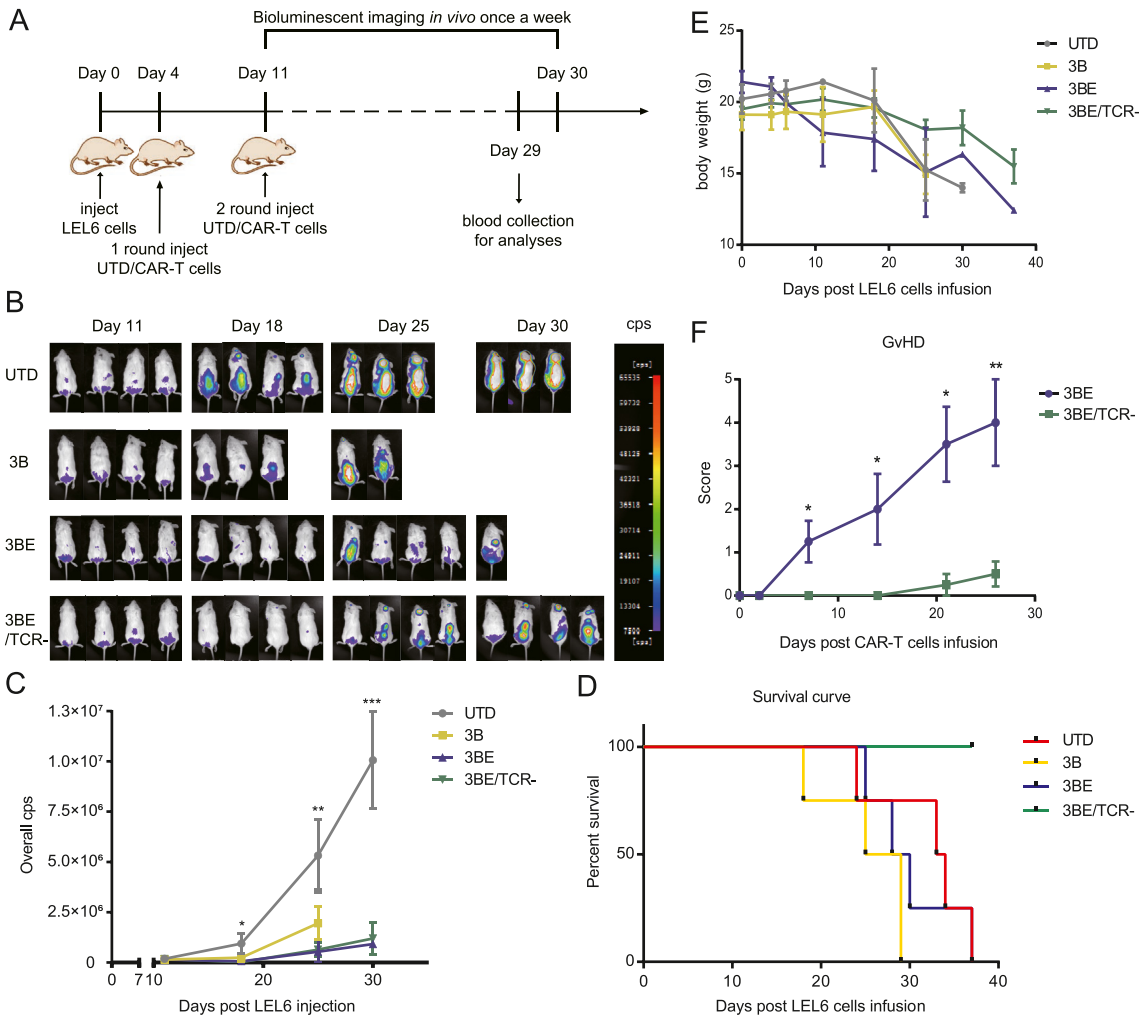

Fig. 6. 3BE/TCR$^-$ CAR-T cells retain cytotoxic activity against HIV Env $^+$ cells in an HIV NSG mouse model with diminished GvHD. **A** Illustration of the experimental design. An HIV-1 Env$^+$/Luc$^+$/PD-L1$^+$ NSG mouse model was established in NSG mice by inoculating 2×10^5 LEL6 cells/mouse (i.v. by tail, n = 4). Four days and 11 days post-inoculation, the mice were treated with 3B CAR-T cells, 3BE CAR-T cells, or 3BE/TCR$^-$ CAR-T cells. Mice treated with UTD cells served as controls. Bioluminescence imaging was conducted weekly after the mice were treated with the last single T cell injection (day 11). **B** LEL6 progression and distribution were evaluated using serial bioluminescence imaging. **C** Bioluminescence values of mice receiving different treatments. **D** Survival of the mice was monitored. **E** Sequential body weights were analysed after LEL6 cell infusion. **F** GvHD scores of mice receiving 3BE CAR-T cells or 3BE/TCR$^-$ CAR-T cells. The data are shown as mean with standard deviation. Statistcal analysis was performed by *t*-test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. GvHD, graft-versus-host disease; Luc, luciferase; UTD, untransduced; cps, counts per second.

et al., 2016), and play a broader role in the treatment of HIV. Furthermore, 3BE CAR-T cells can provide long-lasting expression and secretion of PD-1-specific antibodies, which is superior to the one-dose injection of antibodies with a short half-life. However, further investigation is necessary to determine whether secreted E27 can influence the proliferation, differentiation, and exhaustion states of 3BE CAR-T cells *in vivo*. Evaluation of the prevention of *in vivo* exhaustion provided by secreted E27 in 3BE CAR-T-cells could be essential for advancing our strategy for clinical application. In addition, blockers targeting other immune checkpoint molecules, such as CTLA-4 and Tim3, can be taken into consideration in our platform as a potential strategy toward HIV functional cure.

Although the current CAR-T therapy showed promising results against HIV *in vitro* and in animal models (Namdari et al., 2020), the autologous paradigm could be an obstacle to the clinical application of CAR-T therapy. Therefore, development of processes for manufacturing "off-the-shelf" anti-HIV CAR-T cells from third-party donors is necessary. Since the key barrier to the adoptive transfer of third-party CAR-T cells is the occurrence of GvHD, we took the first step to eliminate endogenous TCR using CRISPR/Cas9 technology to achieve high levels of TCR-deficient anti-HIV CAR-T cells to avoid GvHD. Our study showed that the *in vitro* functional activities of TCR-deficient anti-HIV CAR-T cells, such as specific killing activity, HIV-1 replication suppression function and cytokine secretion, were equivalent to those of conventional anti-HIV CAR-T cells. Furthermore, these TCR-deficient 3BE CAR-T cells powerfully killed HIV Env$^+$ cells *in vivo* without the occurrence of severe GvHD in NSG mice. These results suggest that TCR deficiency could reduce graft-versus-host activity of anti-HIV CAR-T cells without compromising their cytotoxic activity relative to standard CAR-T cells, potentially laying a solid foundation for the development of universal allogeneic anti-HIV CAR-T therapy.

Nevertheless, our study had some limitations. The mouse model we established did not recapitulate an HIV infection model; therefore, the potency of virus replication suppression of our CAR-T cells *in vivo* has not been confirmed. Considering the complex mechanisms of HIV-1 infection and latency, subsequent studies could include HIV-1 true virus-infected cells and latently infected cells.

5. Conclusions

In summary, we have provided a feasible approach for the large-scale generation of "off-the-shelf" anti-HIV CAR-T cells with potent cytotoxic activity against HIV Env$^+$ cells and diminished graft-versus-host activity by combining lentiviral transduction of 3BE CAR with electroporation of *TRAC*-4 RNP. This therapeutic strategy not only achieved a rational combination of CAR-T therapy and antibody therapy of PD-1 blockade for the treatment of HIV but also provided universal anti-HIV CAR-T cells for adoptive transfer without compromising cytotoxic activity. It will be necessary to extend our observations to an HIV-infected humanized mouse model and further to HIV-infected individuals to confirm the potential of our strategy as a powerful therapeutic candidate for the functional cure of HIV.

Data availability

All data generated or analysed during this study are available from the corresponding author upon reasonable request.

Ethics statement

Primary human CD3$^+$ T cells were isolated from healthy donors from the Blood Center of Shanghai (Shanghai, China), and approved by the Ethics Committee of the School of Life Sciences, Fudan University. All immunodeficient mouse experiments were approved by the Institutional Animal Care and Use Committee of Fudan University and were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Fudan University.

Author contributions

Hanyu Pan: methodology, formal analysis, investigation, writing-original draft, writing-review and editing. Xinyi Yang: investigation and writing-review. Jing Wang: investigation. Huitong Liang: investigation and writing-review. Zhengtao Jiang: writing-review. Lin Zhao: writing-review. Yanan Wang: writing-review. Zhiming Liang: writing-review. Xiaoting Shen: writing-review. Qinru Lin: writing-review. Yue Liang: writing-review. Jinglong Yang: writing-review. Panpan Lu: writing-review. Yuqi Zhu: writing-review. Min Li: writing-review. Pengfei Wang: writing-review and editing. Jianqing Xu: writing-review. Hongzhou Lu: writing-review. Huanzhang Zhu: conceptualization, funding acquisition, resources, supervision, writing-review and editing. All authors have read and approved the submitted manuscript.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2023.01.003.

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