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ORIGINAL PAPER

Haematological Malignancy – Clinical

Development of novel humanized CD19/BAFFR bicistronic chimeric antigen receptor T cells with potent antitumor activity against B-cell lineage neoplasms

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Summary

Chimeric antigen receptor T cell (CAR-T) therapy has shown remarkable efficacy in treating advanced B-cell malignancies by targeting CD19, but antigen-negative relapses and immune responses triggered by murine-derived antibodies remain significant challenges, necessitating the development of novel humanized multitarget CAR-T therapies. Here, we engineered a second-generation 4-1BB-CD3ζ-based CAR construct incorporating humanized CD19 single-chain variable fragments (scFvs) and BAFFR single-variable domains on heavy chains (VHHs), also known as nanobodies. The resultant CAR-T cells, with different constructs, were functionally compared both in vitro and in vivo. We found that the optimal tandem and bicistronic (BI) structures retained respective antigen-binding abilities, and both demonstrated specific activation when stimulated with target cells. At the same time, BI CAR-T cells (BI CARs) exhibited stronger tumour-killing ability and better secretion of interleukin-2 and tumour necrosis factor-alpha than single-target CAR-T cells. Additionally, BI CARs showed less exhaustion phenotype upon repeated antigen stimulation and demonstrated more potent and persistent antitumor effects in mouse xenograft models. Overall, we developed a novel humanized CD19/BAFFR bicistronic CAR (BI CAR) based on a combination of scFv and VHH, which showed potent and sustained antitumor ability both in vitro and in vivo, including against tumours with CD19 or BAFFR deficiencies.

KEYWORDS
BAFFR, B-cell malignancies, CD19, chimeric antigen receptor, fully human antibody

INTRODUCTION

In recent years, novel therapeutic approaches based on chimeric antigen receptor T (CAR-T) cell therapy have emerged as a promising approach for the treatment of B-cell-derived haematological malignancies.[1–3] CD19 CAR-T cell therapy uses genetic engineering to modify the surface receptors of T cells, enabling them to recognize and target cancer cells that express the CD19 antigen.[4,5] CD19 CAR-T cell therapy has shown significant clinical efficacy in the treatment of

Abbreviations: ANOVA, analysis of variance; BAFFR, B-cell activating factor receptor; B-ALL, B-cell acute lymphoblastic leukaemia; CAR-T, chimeric antigen receptor T cell; E:T, effector-to-target; FBS, foetal bovine serum; IFN-γ, interferon-gamma; IL-2, interleukin-2; IV, intravenously injected; PBMC, peripheral blood mononuclear cell; scFv, single chain variable fragment; TNF-α, tumour necrosis factor-alpha; VH, variable region of heavy chain; VHH, single-variable domain on heavy chain, nanobody; VL, variable region of light chain.

Sungui Wu and Qian Luo have contributed equally.

B-cell-derived haematological malignancies.[6,7] However, in practical applications, there are still numerous challenges and limitations to the approach, one of which is the relatively high rate of post-treatment relapse.[8]

Emerging studies have indicated that approximately 30%–50% of patients treated with CD19 CAR-T cell therapy experience relapses.[9–11] This may be attributable to the short cell survival time, functional impairment of CD19 CAR-T cells, antigen loss, and immune escape by tumour cells.[12] Specifically, the effectiveness of CD19 CAR-T therapy is compromised in patients with unstable or lost CD19 antigen expression, which is a significant contributor to the high relapse rate.[13] To further enhance the therapeutic efficacy of CD19 CAR-T therapy and reduce relapse rates, researchers have explored multi-target combination treatment strategies. Recently, Ghorashian et al. reported the results of a phase I clinical trial (NCT02443831) in which patients with advanced B-cell acute lymphoblastic leukaemia (B-ALL) received CD19/CD22 bispecific CAR therapy.[14] Among these patients, 83% ($n = 10$) responded positively to CAR-T cell therapy, with no relapses observed as a result of antigen-negative escape within a median follow-up period of 8.7 months. A phase I clinical trial of CD19/CD20 bispecific CAR-T cell treatment for relapsed/refractory non-Hodgkin's lymphoma was reported by Larson et al.[15] In this trial, 90% ($n = 9$) of the patients responded to CAR-T cell therapy, and 70% ($n = 7$) of them achieved a complete response.

The B lymphocyte stimulator receptor (BAFFR) a receptor essential for B-cell maturation and survival,[16] is widely expressed in B-cell-derived haematological malignancies including mantle cell lymphomas, follicular lymphomas, marginal zone lymphomas, chronic lymphocytic leukaemia, hairy cell leukaemia, and diffuse large B-cell lymphomas.[17,18] The crucial functions of BAFFR in B cells and its expression pattern make it an attractive target for treating B-cell-derived haematological malignancies.

In this study, we combined humanized CD19 single-chain variable fragments (scFvs) and BAFFR single-variable domains on heavy chains (VHHs) to produce bispecific CARs targeting both CD19 and BAFFR. Our resultant bicistronic (BI) CAR-T cells demonstrated more sustained and effective cytotoxicity than single-target CAR-T cells against tumour cells both in vitro and in vivo, including tumour cells with CD19 or BAFFR loss, providing a novel, effective, and promising CAR-T therapy strategy that can overcome CD19 or BAFFR antigen loss and a pre-clinical basis for the potential clinical application of CD19/BAFFR bispecific CAR-T cells in the treatment of B-cell-derived haematological malignancies.

MATERIALS AND METHODS

Cell lines and culture

We used Jeko-1 (mantle cell lymphoma), Nalm-6 (pre-B acute lymphoblastic leukaemia), and Mec-1 (chronic lymphoblastic leukaemia) cell lines for this study—all of which were CD19+ and BAFFR+. Jeko-1-CD19 knockout (Jeko-1-CD19KO, CD19−/BAFFR+) and Jeko-1-BAFFR knockout (Jeko-1-BAFFRKO, CD19+/BAFFR−) cells were generated using Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9) technology, resulting in the deletion of CD19 or BAFFR genes. The NCI-H929 (multiple myeloma) cell line was used as a CD19−BAFFR− representative. Jeko-1, Jeko-1-CD19KO, Jeko-1-BAFFRKO, Nalm-6, and NCI-H929 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco). Mec-1 cells were cultured in IMDM (Gibco) supplemented with 10% FBS. Embryonic kidney 293T cells were cultured in DMEM (Gibco) supplemented with 10% FBS. To facilitate detection, Jeko-1, Jeko-1-CD19KO, Jeko-1-BAFFRKO, Nalm-6, Mec-1, and NCI-H929 cell lines were stably transduced with firefly luciferase via lentivirus, and monoclonal stable luciferase-expressing cell lines were generated by limiting dilution. All cell lines were verified before use.

Generation of CD19- and BAFFR-specific domains

The CD19-specific 78scFv domain was screened from the fully human scFv phage display antibody library (IMARS; Nanjing IASO Biotherapeutics, Nanjing, China) in a previous study,[19] and the BAFFR-specific 5VHH domain was screened from the fully human heavy-chain-only phage display antibody library (IMARS; Nanjing IASO Biotherapeutics) in a previous study.[20]

Plasmid construction

The fully human BAFFR-specific 5VHH and CD19-specific 78scFv domains were cloned into a second-generation CAR structure containing a CD8α hinge/transmembrane region, a 4-1BB costimulatory domain, and an intracellular CD3ζ domain. EGFRt was linked to the CAR fragment through a T2A sequence, in order to facilitate the detection of CAR-expressing cells. In the tandem CAR vector constructs, the two domains were linked by a flexible (GGGGS)×2 linker when BAFFR-5VH (a variable region of the heavy chain) was connected to the VH of CD19-78scFv, whereas the two domains were linked by a flexible (GGGS)×2 linker when BAFFR-5VH was connected to the variable region of the light chain (VL) of CD19-78scFv. The BI CAR constructs contained two individual CARs (CD19 CAR BBz and BAFFR CAR BBz) linked by the P2A sequence, enabling the simultaneous expression of CD19 CAR and BAFFR CAR on the surface of a single T cell.

Manufacture of CD19/BAFFR bispecific CAR-T cells

Lentivirus was packaged in a third-generation system containing a transfer plasmid, the GAG-POL plasmid pMDLg/pRRE, the rev plasmid pRSV-Rev, and the VSV-G plasmid

pMD2.G. After a 48 h transfection of 293 T cells, the supernatants were filtered through 0.45 μm filters (Millipore, USA) and concentrated using ultrafiltration tubes (Millipore). Subsequently, the lentivirus titres were measured and the lentivirus was stored at −80°C.

Human peripheral blood mononuclear cells from healthy donors were purchased from Milestone Biotechnologies (Beijing, China). Informed consent was obtained from all donors before specimen collection, following the principles of the Declaration of Helsinki. CD3+ T cells were isolated using CD3 microbeads (Miltenyi Biotec, Germany) according to the manufacturer's instructions, and cultured in CTS medium (Gibco) supplemented with 10% FBS (Gibco), 200 U/mL interleukin-2 (IL-2; Sigma-Aldrich, USA), and 1 × GlutaMax (Gibco). Dynabeads Human T-activator CD3/CD28 (Gibco) was used to activate the T cells. After 24 h of activation, the T cells were transduced with lentiviral vectors encoding CARs at multiplicities of infection of 2–5.

Flow cytometry

PE- or BV421-conjugated CD19 antibodies (clone HIB19; BioLegend), PE-conjugated BAFFR antibodies (clone11c1; BioLegend), and isotype antibody (clone MOPC-21; BioLegend) were used to determine the expression levels of CD19 and BAFFR in the different cell lines generated.

PE-labelled human CD19 protein (ACRO Biosystems, USA), PE-labelled human BAFFR protein (ACRO Biosystems), PE, PE/Cyanine7 or APC-conjugated EGFR antibody (clone: AY13; BioLegend) were used to detect CAR expression.

BV421-conjugated CD3 (clone: OKT3), FITC-conjugated CD4 (clone: RAP-T4), BV421 or FITC-conjugated CD8 (clone: SK1), FITC-conjugated CD69 (clone: FN50), PE/Cyanine7-conjugated CD25 (clone: BC96), PE/Cyanine7-conjugated CD107a (clone: H4A3), APC-conjugated LAG-3 (clone: 7H2C65), BV421-conjugated TIM-3 (clone: F38-2E2) antibodies were all purchased from BioLegend to evaluate CAR-T/T-cell states.

The degranulation of CAR-T cells was evaluated by staining with a PE/Cyanine7-conjugated CD107a antibody (clone H4A3) after co-incubation with different target cells for 4 h, and detected via flow cytometry.

The activation markers of CAR-T cells were stained with FITC-conjugated CD69 (clone FN50) and PE/Cyanine7-conjugated CD25 (clone BC96) after co-incubation with Jeko-1, Jeko-1-CD19KO, and Jeko-1-BAFFRKO cells for 24 h, then detected via flow cytometry.

Data were acquired using a magnet-activated cell sorting Quant Analyser 10 (Miltenyi Biotec) and analysed using FlowJo software version 10 (Tree Star, USA).

Luciferase-based cytolysis assay

To determine the cytotoxicity of CAR-T cells, CAR-T/T cells and target cells were seeded in white opaque plates in triplicate at the indicated ratios, then incubated at 37°C in 5% CO_2 for 24 h. Luminescence was detected using a Steady-Glo luciferase assay system (Promega, USA), according to the manufacturer's instructions.

Cytokine detection assay

To evaluate the cytokine release levels of CAR-T cells, CAR--T/T cells, and Jeko-1 cells or medium were co-incubated in U-bottom 96-well plates at an effector-to-target (E:T) ratio of 1:1 for 24 h. Homogeneous time-resolved fluorescence human cytokine detection kits (Cisbio; PerkinElmer) were used to detect the concentration of cytokines in the supernatant, following the manufacturer's protocol.

Repeat antigen stimulation assay

To perform a repeat antigen stimulation expansion assay, Jeko-1 cells were plated in a 6-well plate on day 0 and treated with mitomycin C to a final concentration of 1 μg/mL. On day 1, mitomycin-C-treated Jeko-1 cells (4×10^5) were washed three times with phosphate-buffered saline (PBS) and mixed with 8×10^5 viable CAR-T cells in a 24-well plate, in CTS medium supplemented with IL-2. On day 3, the new Jeko-1 cells were again treated as described on day 0. On day 4, viable CAR-T cells that were treated on day 1 were counted, and 8×10^5 CAR-T cells were once again mixed with 4×10^5 mitomycin-C-treated Jeko-1 cells in a new 24-well plate, as described for day 1. This process was repeated five times. The –fold expansion after each stimulation was calculated as (viable CAR-T cells on day 4)/(8×10^5), and the cumulative expansion was calculated using the following formula: cumulative-fold expansion = (–fold expansion 1) × (–fold expansion 2) × … × (–fold expansion *n*).

Mouse xenograft models

NKG mice, a type of severely immunodeficient mouse with the knockout of interleukin-2 receptor gamma based on the nonobese diabetic/severe combined immunodeficient (NOD/SCID) background strain, were purchased from Cyagen Bioscience (Suzhou, China) and maintained under specific pathogen-free conditions at the animal facility of Yunqiao Bioscience (Nanjing, China). All animal experiments were approved by the Institutional Animal Care and Use Protocol (AP-C210507.94). In the model established by Jeko-1, 6-week-old female NKG mice (five/group) were challenged, via intravenous injection (IV) with 1×10^6 luciferase-expressing tumour cells (Jeko-1), then treated with 4×10^6 CAR-T/T cells 3 days later. In the mixed model, 6-week-old female NKG mice (five/group) were challenged (IV) with 1.0×10^6 luciferase-expressing mixed tumour cells (Jeko-1-CD19KO:Jeko-1-BAFFRKO ≈ 1:1), then treated with 4×10^6 CAR-T/T cells 3 days

later. These experiments were controlled using the same dose of PBS, and the dose in the mock T group was calculated based on the CAR-T cells with the lowest transfection efficiency. Tumour burden was evaluated using a bioluminescence imaging system.

Graphs and statistical analysis

Graphs and data analyses were performed using GraphPad Prism Software version 9.0.0. A few of these graphs were obtained and modified from Figdraw. Unless otherwise stated, all data are representative of at least three independent experiments. All data are represented as means ± standard deviations—except for mouse tumour radiance quantification, body weight, and mouse peripheral blood data, which are shown as means ± standard errors of the mean. Significant differences were analysed using one-way analysis of variance (ANOVA), two-way ANOVA, the Mann–Whitney *U* test, or the log-rank test. *p*-Values are represented as either not significant (ns), $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, or $^{****}p<0.0001$.

RESULTS

Construction of CD19/BAFFR bispecific CAR-T cells

In a previous study, we successfully generated humanized single-chain antibody fragments targeting CD19 (78scFv) and a heavy-chain single-domain antibody against BAFFR (5VHH) using phage display technology.[19,20] Prior to constructing bispecific CARs, we confirmed that the sequence order of VH and VL (78HL or 78LH) did not affect the function of the CD19 CAR (Figure S1). Subsequently, we designed various combinations of bispecific CARs, including tandem and BI CARs. Among the BI CARs, we selected the construct with the highest transduction efficiency to compare with the tandem CARs, owing to their minimal functional differences (Figure S2).

To prevent erroneous cross-linking between 5VH and 78scFv in the tandem CARs, we used a truncated linker, (GGGS) × 2, when 5VH was close to VL, and a (GGGGS) × 2 linker when 5VH was close to VH (Figure 1A). These tandem CAR-T cells were referred to as Tan 5H-78HL, Tan

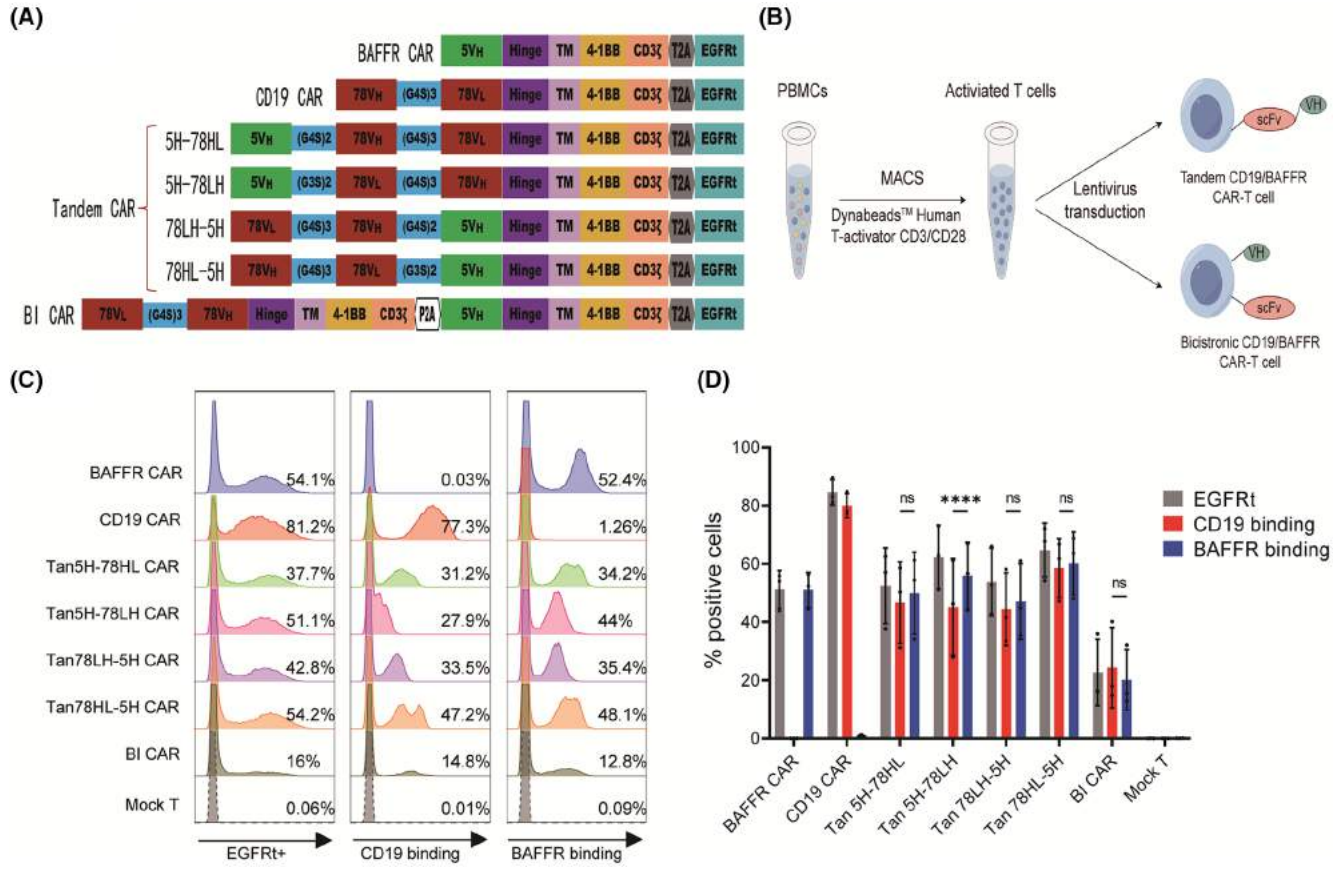

FIGURE 1 Structure of the CARs, schematic representation of CAR preparation, and tests of transduction efficiency of the CARs. (A) Structure schematic diagram of BAFFR CAR, CD19 CAR, four tandem CARs, and BI CAR. (B) Schematic diagram of manufacturing CD19/BAFFR bispecific CAR-T cells in this study. The isolation of T cells from PBMC used magnet-activated cell sorting technology. (C) Representative flow cytometry analysis demonstrating transduction efficiency and binding capabilities of CD19/BAFFR CAR-T cells on day 7. (D) Quantitative analysis of the data presented in (C). The results are presented as mean ± SD ($n=3$), two-way analysis of variance. $^{****}p<0.0001$, ns, no significance. BAFFR, B-cell activating factor receptor; BI CAR, bicistronic CAR-T cells; CAR-T, chimeric antigen receptor T cell; PBMC, peripheral blood mononuclear cells.

5H-78LH, Tan 78LH-5H, and Tan 78HL-5H cells. They were transduced into T cells via lentiviral vectors, 24 h after T cell activation (Figure 1B). On day 7, the transduction efficiency and corresponding antigen-binding abilities of the bispecific CARs were evaluated using an EGFRt antibody and recombinant CD19 and BAFFR proteins (Figure 1C,D). The results showed that different arrangements of the structures had significant impacts on the antigen-binding abilities of the CARs and that the CD19 binding ability of Tan 5H-78LH was strongly compromised and abolished. Therefore, we performed subsequent experiments using the remaining three tandem CARs and BI CARs.

Specific degranulation and activation of CD19/BAFFR bispecific CAR-T cells in vitro

The expression levels of CD19 and BAFFR in the target tumour cell lines were assessed using flow cytometry (Figure 2A,B). NCI-H929 cells, being CD19−/BAFFR−, served as negative controls. Jeko-1, Nalm-6, and Mec-1 are tumour cell lines that naturally express CD19 and BAFFR. In contrast, Jeko-1-CD19KO and Jeko-1-BAFFRKO cells served as BAFFR and CD19 single-positive target cells, respectively. Neither CD19 nor BAFFR knockout affected the proliferation, viability, or in vivo tumourigenicity of Jeko-1 cells (Figure S3). This could be because tumour cells can compensate for the loss of CD19 or BAFFR by adapting to alternate survival signalling pathways, such as NOTCH, B-cell maturation antigen, inflammatory cytokines, B-cell receptors, and toll-like receptors.[21,22]

Degranulation is a crucial step in the perforin-mediated killing of tumour cells by CD19/BAFFR bispecific CAR-T cells. Initially, we co-cultured bispecific CAR-T cells with Jeko-1, Nalm-6, MEC-1, Jeko-1-CD19KO, Jeko-1-BAFFRKO, and NCI-H929 cells, then evaluated their degranulation capacity by assessing CD107a surface expression levels via flow cytometry. After 4 h of stimulation with Jeko-1, Mec-1, or Jeko-1-BAFFRKO cells, both Tan 5H-78HL and BI CAR exhibited higher levels of CD107a than did Tan 78LH-5H and Tan 78HL-5H. There was

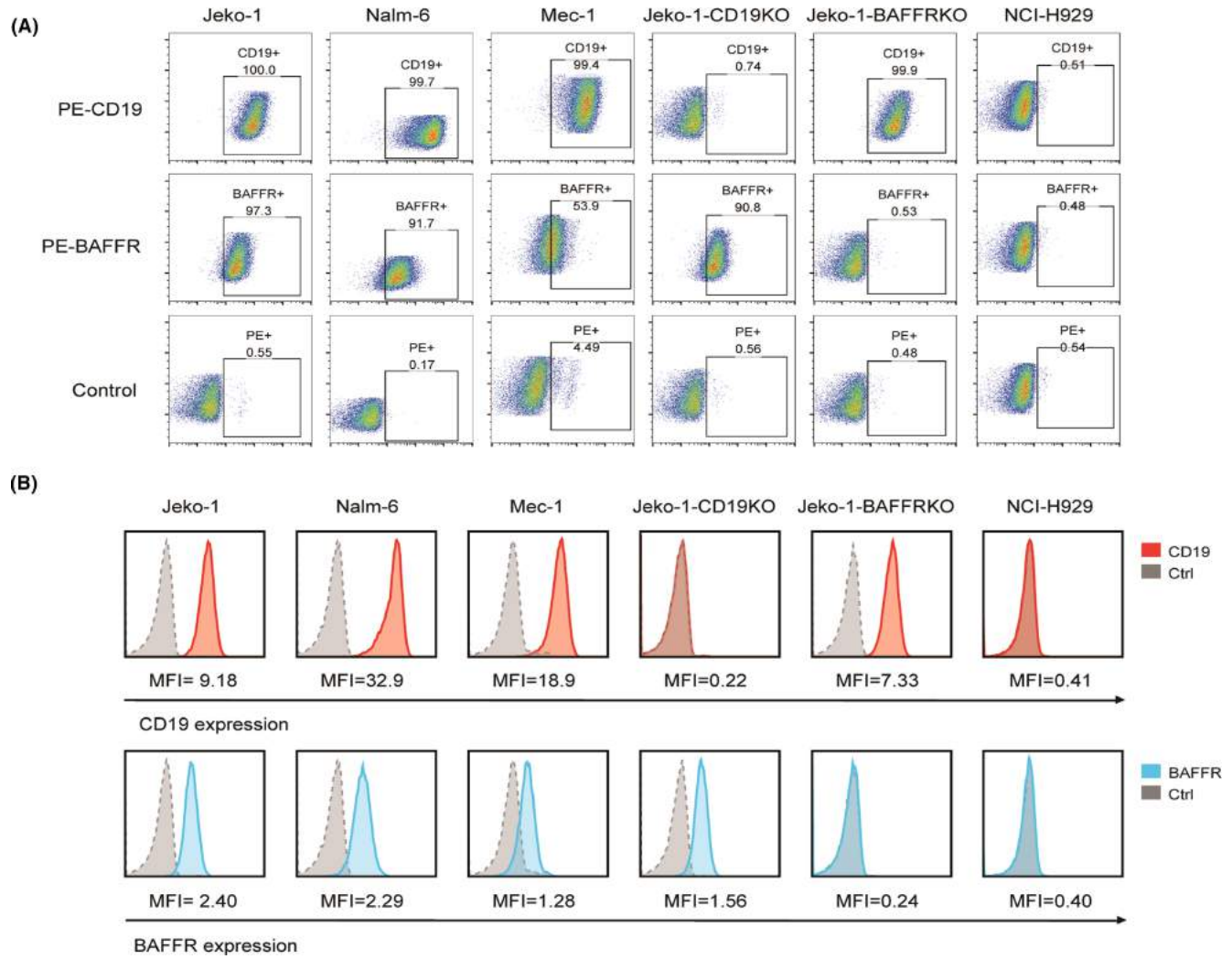

FIGURE 2 The expression levels of CD19 and BAFFR in the target tumour cell lines. (A, B) The expression levels of CD19 and BAFFR in the target tumour cell lines were assessed using flow cytometry. BAFFR, B-cell activating factor receptor.

no significant difference observed upon stimulation with Jeko-1-CD19KO cells (Figure 3A,B), indicating that the positioning of 78scFv on the outer region affects its degranulation release capacity, while the position of 5VHH was unaffected. We therefore proceeded with a functional assessment of the Tan 5H-78HL and BI CAR-T cells. Compared to those in unstimulated CAR-T cells, CD19/BAFFR CAR-T cells exhibited higher levels of the T-cell activation markers CD25 and CD69 after stimulation with Jeko-1, Jeko-1-CD19KO, or Jeko-1-BAFFRKO cells (Figure 4A,B).

Potent specific cytotoxicity and significant expansion of CD19/BAFFR bispecific CAR-T cells against tumour cells in vitro

To assess the cytotoxicity of the CD19/BAFFR bispecific CARs, we conducted a luciferase-based assay. The CD19/BAFFR bispecific CARs exhibited dose-dependent cytotoxicity against CD19+ or BAFFR+ tumour cells, but no cytotoxicity against NCI-H929 cells (CD19−BAFFR−).

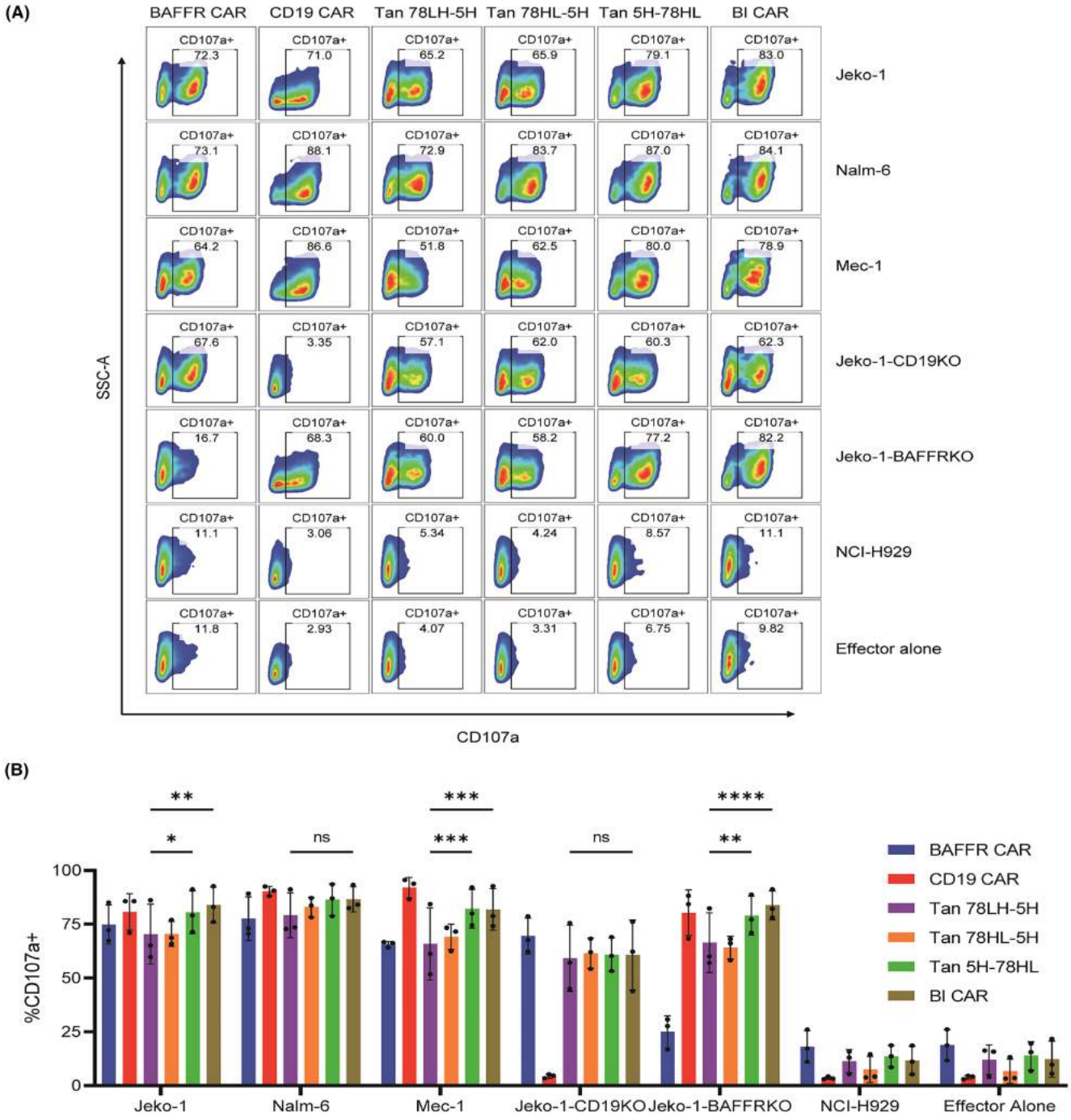

FIGURE 3 Degranulation capacity of CAR-T cells. (A) Representative flow cytometry analysis of CAR-T degranulation capacity. (B) Quantitative analysis of the degranulation data. The results are displayed as mean ± SD for three donors, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, ns, no significance, two-way analysis of variance. CAR-T, chimeric antigen receptor T cell.

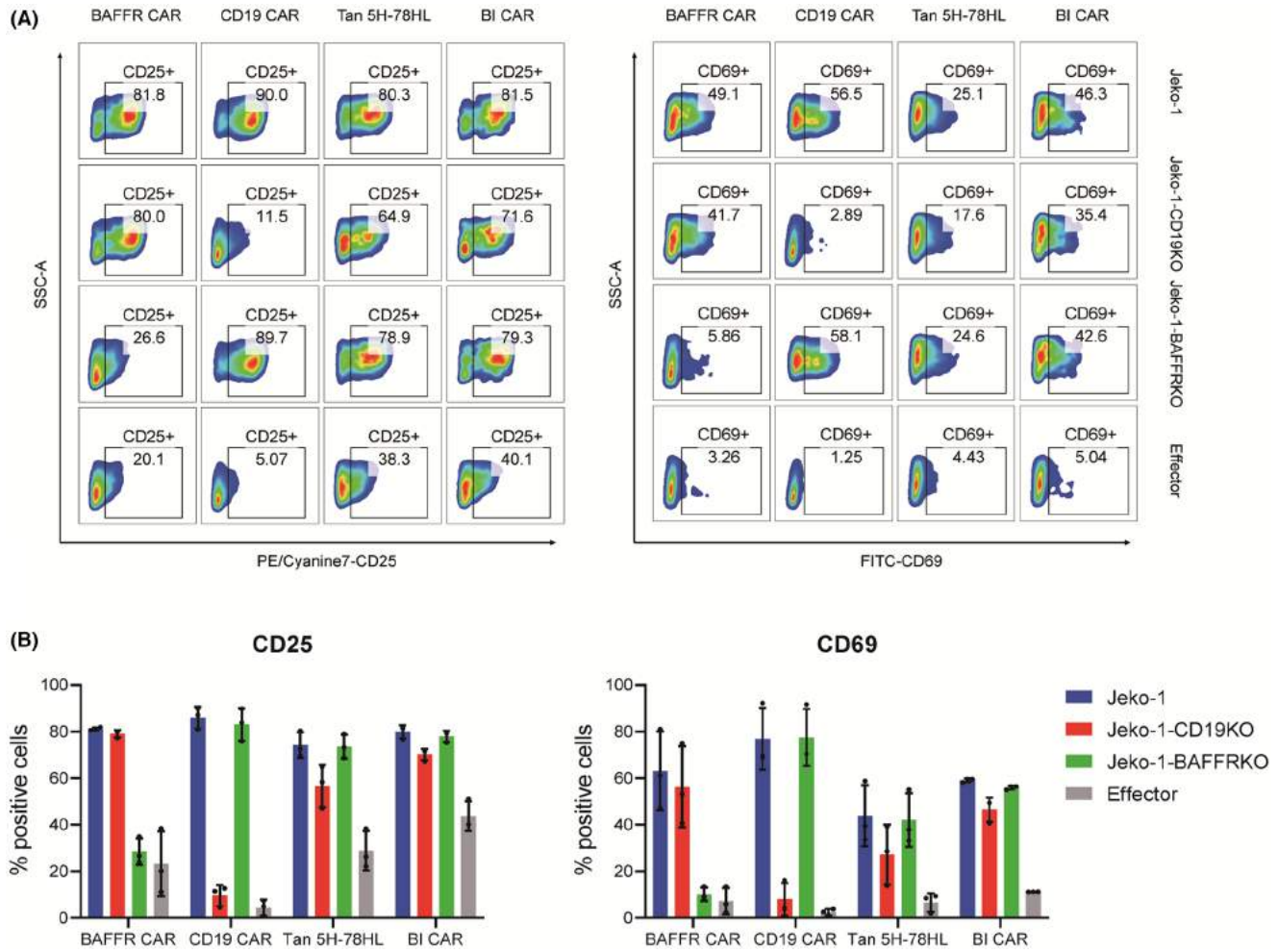

FIGURE 4 Activation capacity of CAR-T cells. (A) Representative flow cytometry analysis of CD25 and CD69 expressions of CAR-T cells after 24-h coincubation with tumour cells. (B) Quantitative analysis of the CD25 and CD69 expressions. The results are displayed as mean ± SD for three donors. CAR-T, chimeric antigen receptor T cell.

The cytotoxicity of the bispecific CARs was observed to be superior to that of the single CARs (Figure 5A). Concurrently, we evaluated the levels of IL-2, tumour necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) in the cell culture supernatant after co-culturing the bispecific CARs with Jeko-1 cells at an E:T ratio of 1:1 for 24 h. The release levels of IL-2 and TNF-α were observed to be higher in the BI CAR-T cells than those in the Tan 5H-78HL CAR-T cells, while there was no significant difference in terms of how much IFN-γ was released (Figure 5B).

The proliferative capacity of CAR-T cells is a crucial factor that influences therapeutic efficacy. To test this hypothesis, we simulated in vivo antigen stimulation and evaluated the proliferative capacity and expression of exhaustion markers in CAR-T cells after multiple rounds of in vitro antigen stimulation. After five rounds of stimulation with mitomycin C-treated Jeko-1 cells, the proliferation of CAR-T cells was significantly higher than that of mock T cells (Figure 5C). We measured the expression levels of the exhaustion markers LAG-3 and TIM-3 in the CAR-T cells before and after two rounds of stimulation. Their expression levels on CD4+ BI CARs were lower than those on CD19 CARs after the second stimulation with Jeko-1 cells, and the expression level of LAG-3 on CD8+ BI CARs was lower than that on CD19 CARs (Figure 5D).

CD19/BAFFR bispecific CAR-T cells exhibit potent antitumour activity in vivo

To evaluate the in vivo function of these bispecific CAR-T cells, we constructed xenograft models in 6-week-old NKG mice via tail vein injection of 1×10^6 Jeko-1-luciferase cells, and administered 4×10^6 CAR-T/T cells 3 days later. All CAR-T cell treatment groups showed delayed tumour progression in tumour-bearing mice, and bispecific CAR-T cells demonstrated potent tumour suppression capability when compared with that in the mock T-cell group. However, the BI CARs showed better antitumor efficacy than that in the Tan 5H-78HL ones (Figure 6A,B). Moreover, compared to those in the PBS and mock T groups, the survival times of mice treated

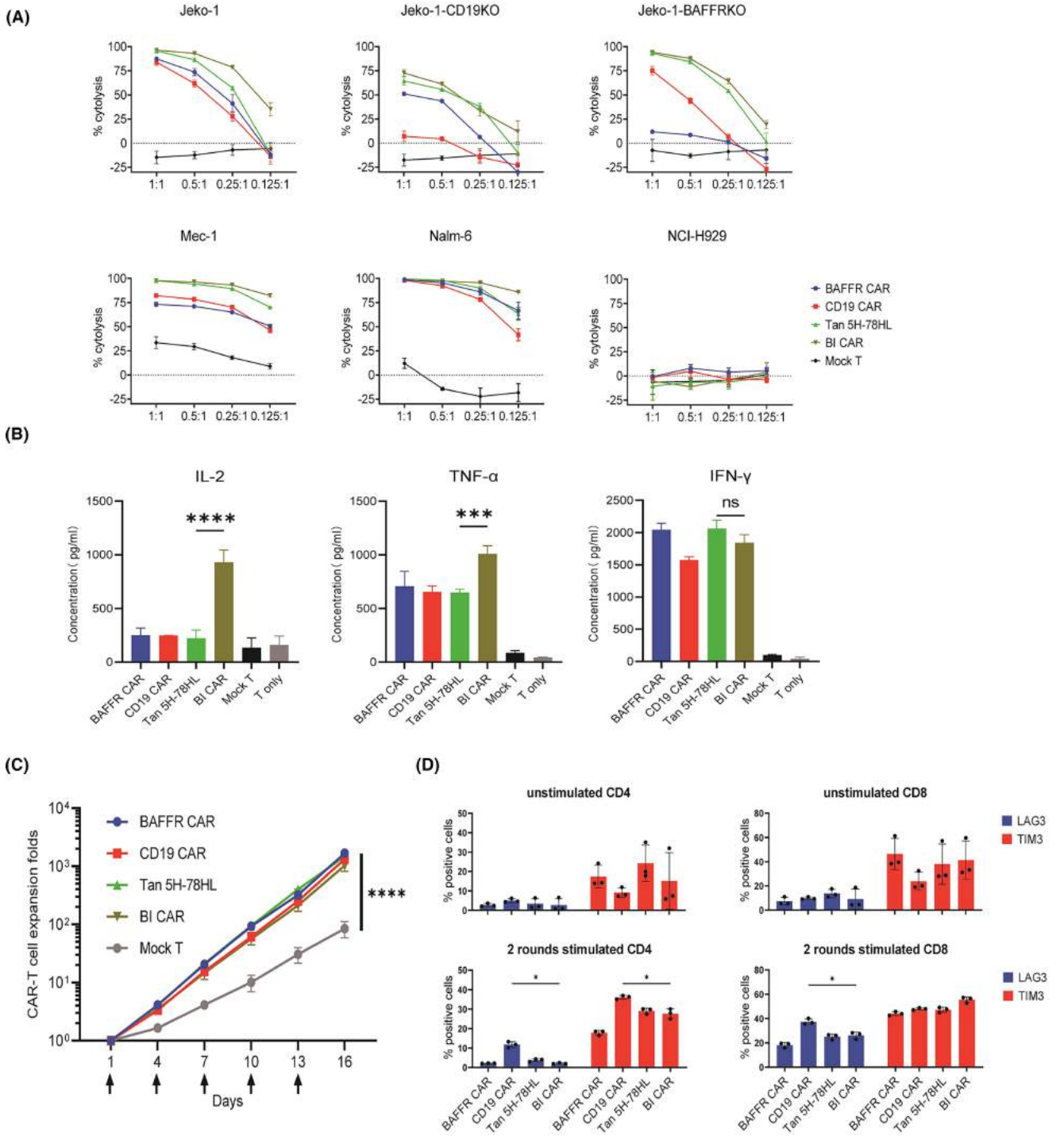

FIGURE 5 In vitro functional comparison of CD19/BAFFR bispecific CAR-T cells with single-target CAR-T and tandem CAR-T cells. (A) Cytotoxicity of CAR-T cells was evaluated by luciferase detection after 24-h co-incubation with target cells at different ratios for 24 h. The data represent mean ± SD ($n = 3$). (B) CAR-T/T cells were incubated with Jeko-1 cells (CD19+ BAFFR+) at a 1:1 ratio for 24 h. Cytokines released in the culture supernatant by CAR-T/T cells were quantified using homogeneous time-resolved fluorescence technology. The results are displayed as mean ± SD ($n = 3$), ***$p < 0.001$, ****$p < 0.0001$, ns, no significance, one-way analysis of variance (ANOVA). (C) The expansion fold of CAR-T/T cells after multiple rounds of stimulation with tumour cells was calculated. The *X*-axis represents the days of coculture, and the arrow indicates the addition of mitomycin-C pre-treated Jeko-1 cells. The data represent mean ± SD ($n = 3$), ****$p < 0.0001$, one-way ANOVA. (D) Surface expression of LAG-3 and TIM-3 on CAR-T cells before and after two rounds of stimulation with mitomycin-C-treated Jeko-1 cells. The data represent mean ± SD ($n = 3$), *$p < 0.05$, two-way ANOVA. BAFFR, B-cell activating factor receptor; CAR-T, chimeric antigen receptor T cell.

with CAR-T cells were significantly longer (Figure 6C). No sudden decreases in body weight were observed in the mice that were treated with CAR-T cells (Figure 6D). Flow cytometry analysis of peripheral blood revealed that the BI CARs exhibited the highest relative expansion ratio on day 31 (Figure 6E; Figure S4A shows this finding in detail).

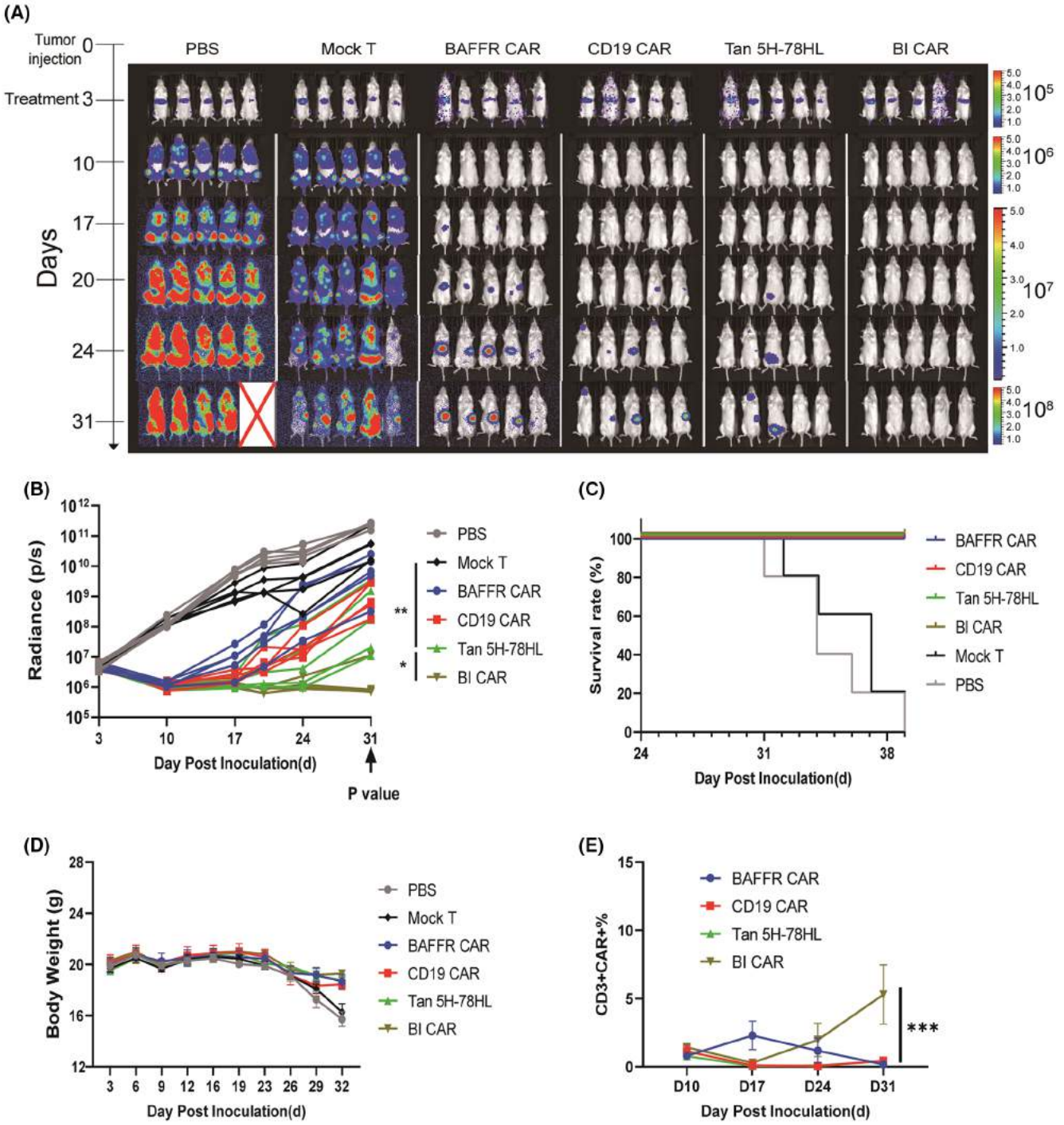

FIGURE 6 The antitumor activity of CD19/BAFFR bispecific CAR-T cells in vivo. (A) Tumour growth was monitored using bioluminescence imaging. (B) Quantitative analysis of photon counts in (A). The date are displayed as mean ± SEM ($n = 5$), *$p < 0.05$, **$p < 0.01$, two-tailed unpaired Mann–Whitney test. (C) Overall Kaplan–Meier survival curve. (D) Body weight curve. The results are presented as mean ± SEM ($n = 5$). (E) Flow cytometry analysis of CAR+ T cells in peripheral blood of mice on day 10, 17, 24, and 31. Gated on CD3+ EGFRt+ cells. The data represent mean ± SEM ($n = 5$), ***$p < 0.001$, two-way analysis of variance. BAFFR, B-cell activating factor receptor; CAR-T, chimeric antigen receptor T cell.

Bicistronic rather than tandem CAR-T cells overcome antigen escape in vivo

To assess the in vivo efficacy of bispecific CAR-T cells in combating antigen escape, a mixed tumour model was established in NKG mice by co-injecting Jeko-1-CD19KO and Jeko-1-BAFFRKO cells at a 1:1 ratio (Figure 7A). Mice in the CAR-T group received an infusion of 4×10^6 CAR-T cells via the tail vein on day 3. A significant difference in the ability of Tan 5H-78HLs and BI-CARs to combat antigen escape tumour cells was observed in vivo. The BI CARs exhibited a pronounced inhibitory effect in

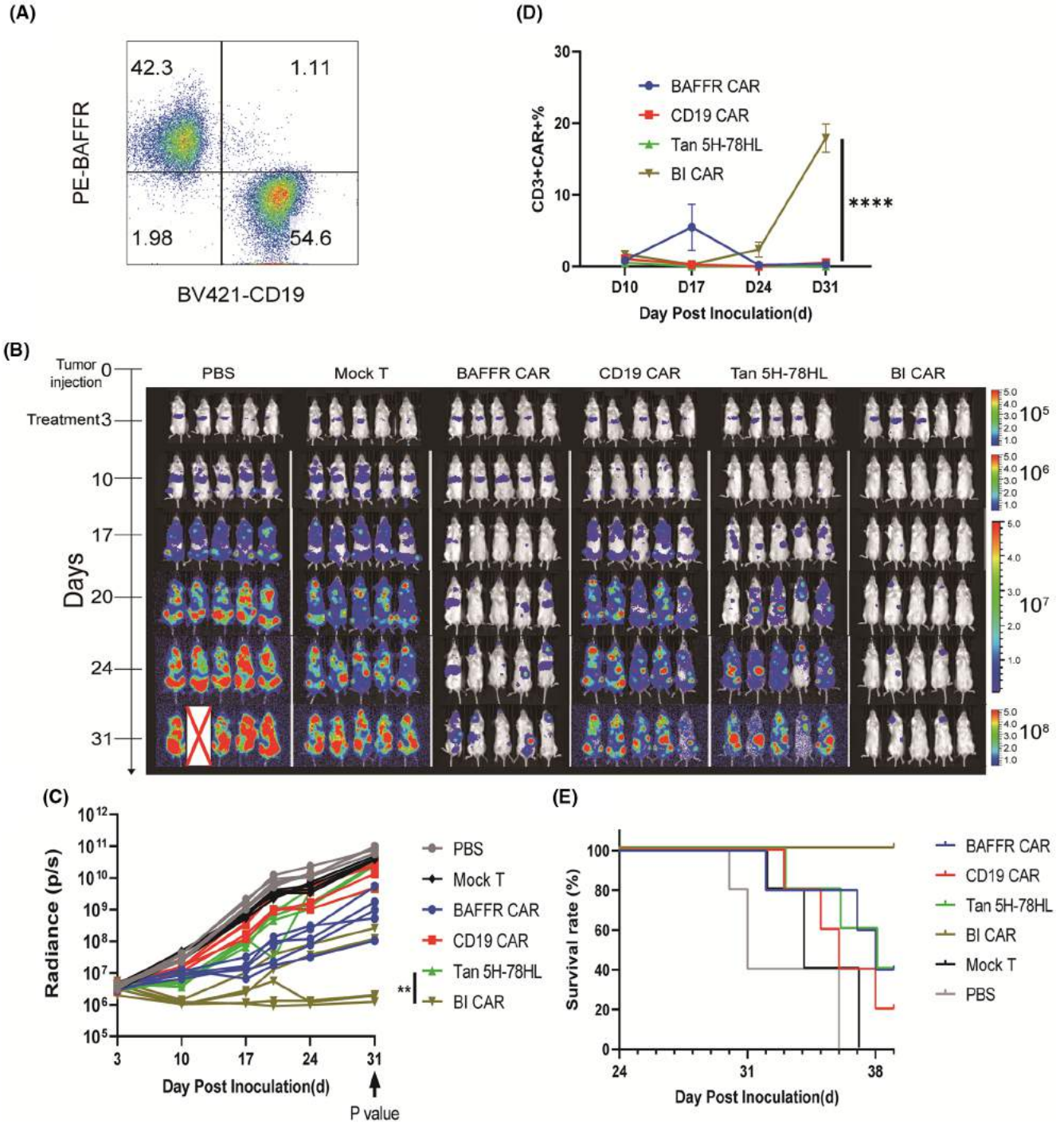

FIGURE 7 CD19/BAFFR bicistronic CAR-T cells overcome single-antigen escape. (A) Flow cytometry analysis showing the percentage of Jeko-1-CD19KO and Jeko-1-BAFFRKO cells in the mixed tumour cells used to inject into mice. (B) Tumour growth was monitored using bioluminescence imaging. (C) Quantitative analysis of photon counts in (B). Mean ± SEM ($n = 5$), $^{**}p < 0.01$, two-tailed unpaired Mann–Whitney test. (D) Flow cytometry analysis of CAR+ T cells in peripheral blood of mice on day 10, 17, 24, and 31. Gated on CD3+ EGFRt+ cells. The data represent mean ± SEM ($n = 5$), $^{****}p < 0.0001$, two-way analysis of variance. (E) Overall Kaplan–Meier survival curve. BAFFR, B-cell activating factor receptor; CAR-T, chimeric antigen receptor T cell.

the mixed tumour model, whereas Tan 5H-78HLs showed limited efficacy (Figure 7B,C). To assess the expansion of CAR-T cells in mice, flow cytometry was used to determine the percentage of CD3+EGFR+ CAR-T cells in the peripheral blood on days 10, 17, 24, and 31. The results revealed a significantly greater proportion of CAR+ cells in the peripheral blood samples of mice treated with BI CARs compared to those treated with Tan 5H-78HLs on day 31 (Figure 7D; Figure S4B shows this in greater detail). These findings indicated that compared to that in Tan 5H-78HLs, the BI CARs showed superior in vivo sustained expansion. Compared to that in Tan 5H-78HL cells, BI CARs

significantly prolonged the survival times of mice in the mixed tumour model (Figure 7E).

DISCUSSION

Although CD19 CAR-T therapy has shown promising efficacy against B-cell malignancies, antigen escape, and relapse pose significant challenges to patient survival. Gardner et al. reported two cases of CD19-negative relapse following CD19 CAR-T cell therapy for B-cell acute lymphoblastic leukaemia.[23] Similarly, Park et al. reported four cases of CD19-negative relapse among 53 adults with relapsed/refractory B-cell acute lymphoblastic leukaemia following treatment with CD19 CAR-T cells.[24] These studies highlight the limitations of using CD19 single-target CAR-T cells. Therefore, we explored the use of bispecific CAR-T cells expressing both CD19 and BAFFR.

A number of previous studies have explored the incorporation of CD19 and other targets into bispecific CAR-T cells,[15,25–28] including BAFFR.[29] However, most of these bispecific CAR constructs used various combinations of two scFvs and murine-derived antibodies. Murine-derived antibodies can trigger immune responses resulting in production of anti-drug antibodies. These can compromise the efficacy of CAR-T cells.[30,31] Additionally, tandem scFvs may lead to incorrect protein folding, affecting antigen-binding capacity.[32] In this study, we applied a combination of humanized scFv and VHH to CD19/BAFFR CAR-T cells for the first time. Compared to traditional murine-derived scFvs, our humanized scFvs and VHHs resulted in minimal generation of anti-drug antibodies,[33] thereby enhancing the in vivo persistence of CAR-T cells. Moreover, the compact size and long complementarity-determining region 3 (CDR3) of VHH facilitate access to hidden antigen epitopes, which are important for targeting tumours with concealed antigen expression.[34,35] Furthermore, substituting scFv with VHH significantly reduced the misfolding of antibody fragments, thereby improving protein stability. This approach shortens the length of CAR fragments to a certain extent, thereby improving transduction efficiency and simplifying manufacturing processes.[36]

In this study, to prevent misfolding and the potential loss of antigen affinity in the tandem structure, we shortened the linker between scFv and VHH in advance. Our strategy proved successful, as only the CD19 affinity of the Tan 5H-78LH tandem structure was significantly compromised. All of the other structures retained their corresponding affinities (Figure 1C,D). In our degranulation assay, we observed that 5VHH maintained a stable level of degranulation regardless of its position in the construct, whereas the degranulation level corresponding to 78scFv significantly decreased when 78scFv was placed in the front position (Figure 3). This may be attributable to an alteration in the distance between the scFv and the membrane upon tandem combination, which could affect their respective functions.[37] However, VHH, with its higher stability and affinity,[38] was less affected.

Our cytokine detection assays showed that, although there was no significant difference in IFN-γ secretion levels, the BI CARs exhibited higher levels of IL-2 and TNF-α secretion compared to those in Tan 5H-78HLs (Figure 5B). IL-2 is a potent growth factor that promotes the expansion and enhances the cytotoxic activity of T cells.[39,40] TNF-α promotes T-cell activation, proliferation, and cytotoxic activity, while regulating survival and apoptosis.[41] Therefore, we hypothesized that BI CARs would show improved persistence. Our subsequent animal experiments confirmed this hypothesis. The antitumour effect of Tan 5H-78HLs in the Jeko-1 model was inferior to that of the BI CARs (Figure 6A,B), and the detection of peripheral blood in mice showed that the BI CARs exhibited a more sustained expansion (Figure 6E). This was further highlighted in a more challenging mixed tumour model, in which BI CARs with better persistence consistently maintained effective tumour suppression – whereas Tan 5H-78HLs quickly lost their levels of antitumor activity (Figure 7B,C). A flow cytometry analysis of peripheral blood samples obtained from the mice once again demonstrated the superior in vivo persistence of our BI CAR-T cells (Figure 7D).

Although BI CARs exhibit excellent therapeutic efficacy, their transduction efficiency (15%–35%) is lower than that of tandem CARs, largely because of their larger DNA size. BI CARs are approximately 22% longer than the tandem CARs. To improve the transduction efficiency of BI CARs, the EGFRt fragment can be removed, reducing the length by 1071 bp (~28% of the original BI CAR DNA length). Furthermore, although we observed that the BI CARs had more durable in vivo antitumor effects compared to that in Tan 5H-78HLs, along with higher levels of IL-2 and TNF-α release in vitro, the underlying mechanisms behind this merit further exploration. Moreover, the fully humanized BI CARs did not induce anti-drug immune responses; however, owing to limitations in animal models, this advantage could not be demonstrated directly. Finally, BI CARs exhibit good safety in cells that do not express the corresponding antigen and no lethal toxicity has been observed in mice; however, their safety and efficacy in humans remain unknown.

In summary, we demonstrated the feasibility of constructing CAR-T cells using a combination of scFvs and VHHs. Our CD19/BAFFR BI CARs represent an effective strategy for addressing clinical CD19 antigen loss-driven relapses, offering the potential for long-term relief to patients.

AUTHOR CONTRIBUTIONS

BC, XD, TT, and QL contributed to study concept and design. SW, QL, CZ, SZ, FL, JL, BS, and YH performed the experiments, collected and analysed the data. SW wrote the first draft of the manuscript. BC, XD, and TT reviewed and revised this manuscript. All the authors have read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. Data are available upon reasonable request.

ETHICS STATEMENT

The PBMCs from healthy donors were obtained from Shanghai Liquan Hospital by Milestone Biotechnologies (China), and the collection of PBMCs was approved by the ethical committee of Shanghai Liquan Hospital. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Yunqiao Bioscience (approval No. AP-C210507.94).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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