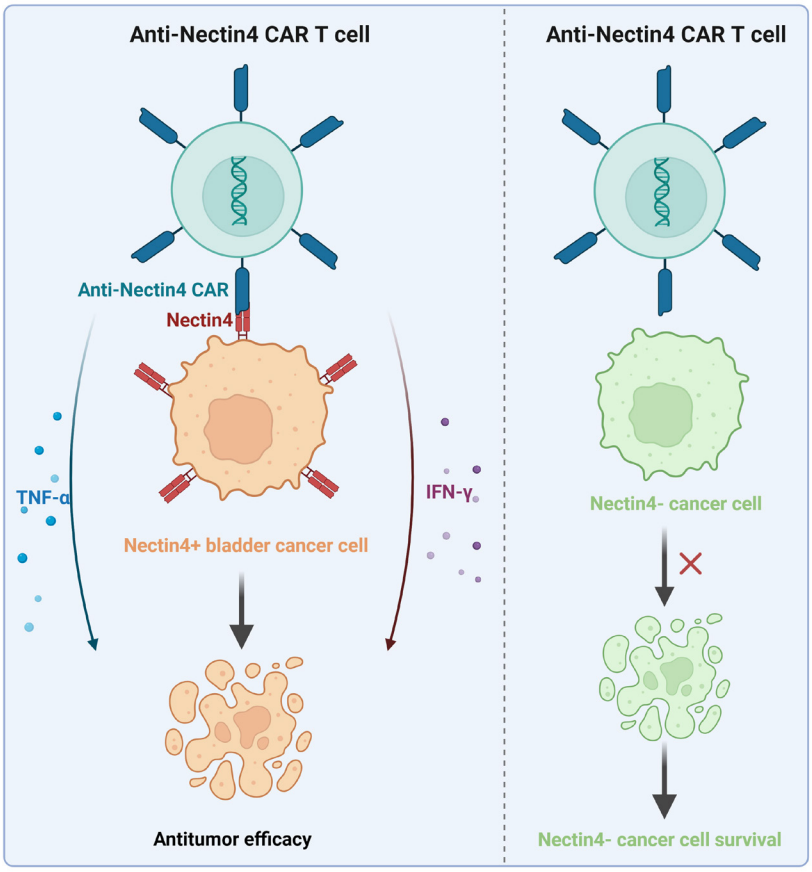
BIO Integration

Novel CAR-T Cells Specifically Targeting Nectin4 Exhibit Effective Anti-Tumor Efficacy in Bladder Cancer Cell Lines

Graphical abstract



Highlights

- Engineered novel Nectin4 targeted VHH-CAR-T cells.
- VHH^{Nectin4}-CAR-T cells could specifically recognize Nectin4^{+} BLCA cells, exhibit strong specific killing ability, secrete cytokines, and mediate cell apoptosis.
- VHH^{Nectin4}-CAR-T had no effect on the growth of Nectin4^{-} U87-MG cells.

Authors

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In brief

A novel Nectin4-targeted VHH-CAR-T cell was established. It effectively killed Nectin4^{+} bladder cancer cells *in vitro*, with enhanced specific killing and immune-related effects.

Original Article

BIO Integration

Novel CAR-T Cells Specifically Targeting Nectin4 Exhibit Effective Anti-Tumor Efficacy in Bladder Cancer Cell Lines

Jinping Niu[1], Guodi Liu[1,2], Sai Zhang[3], Chengnan Xu[3], Ziwen Xu[1], Xingyuan Ma[1,*], Xiaoli Tian[1,2,*] and Wenyun Zheng[3,*]

Abstract

Background: Bladder cancer (BLCA) is one of the most common malignancies and the second most frequent urogenital tract tumor. Cell and gene therapy, which offer many advantages in treating BLCA, are urgently needed. However, there is an important limitation of the currently reported single chain antibody used as a chimeric antigen receptor (CAR) targeting domain. Specifically, CAR aggregation leads to CAR-T depletion, which may originate from the linker peptide and folding stability between the variable domains (VH and VL) of the single chain antibody of the CAR. Humanized, small size, strong affinity single domain antibodies (variable domain of heavy chain of heavy-chain antibody [VHH]) derived from camelids are promising alternatives.

Methods: Second-generation Nectin4-targeted VHH-CAR-T cells were constructed and the specific killing efficacy was determined against BLCA cells *in vitro*. VHH Nectin4-CAR lentivirus was transduced into human T cells and CAR-T cell phenotypes were analyzed by flow cytometry. Cell killing efficacy was assessed using Nectin4-positive BLCA cells (SW780 and RT4) and Nectin4-negative U87-MG cells as controls using the xCELLigence Real Time Cell Analysis system. Cytokine secretion expression (IFN-γ and IL-2) were measured by an enzyme-linked immunosorbent assay.

Results: $VHH^{Nectin4}$-CAR lentivirus treatment increased the proportion of CD4+ T and memory T cells. $VHH^{Nectin4}$-CAR-T cells had increased specific killing ability compared to the control using VH-VL-based CAR-T cells, specifically recognized $Nectin4^+$ BLCA cells, secreted cytokines, and mediated cell apoptosis. Furthermore, $VHH^{Nectin4}$-CAR-T had no effect on $Nectin4^-$ U87-MG cell growth.

Conclusions: $VHH^{Nectin4}$-CAR-T cells were established with potent killing ability that specifically recognized $Nectin4^+$ BLCA cells *in vitro*.

Statement of Significance: Second-generation targeted Nectin4 VHH-CAR-T cells were established. $VHH^{Nectin4}$-CAR-T cells with strong killing ability specifically recognized $Nectin4^+$ BLCA cells *in vitro*.

Keywords

Bladder cancer, CAR-T, CGT, Nectin4, VHH.

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Introduction

Bladder cancer (BLCA) is the most common urinary tract malignancy and one of the most prevalent cancers worldwide [1]. Although conventional therapies, such as tumor resection, chemotherapy, and radiation, have been proven to be effective, conventional therapies have many limitations, including drug resistance, cancer recurrence, and severe adverse effects [2–4]. Standard therapy for non-muscle invasive bladder cancer, including carcinoma *in situ*, involves intravesical instillation of bacillus Calmette Guerin, which triggers a local immune reaction that ultimately promotes elimination of BLCA cells [5]. However, BCG treatment of NMIBC has been shown to be ineffective in approximately 40% of patients [6]. Therefore, new therapeutic approaches for the treatment of BLCA are urgently needed.

In recent years new cancer treatments, such as immunotherapy (cell and gene therapy), have emerged. Chimeric antigen receptor (CAR) T-cell therapy is one of the most promising immunotherapeutic approaches. Indeed, six CAR T therapies have been approved by the Food and Drug Administration (FDA) for hematologic malignancies [7, 8]. However, CAR immunotherapy for solid tumors lags significantly behind liquid tumors due to CAR-T cell manufacturing, inefficient CAR-T cell trafficking and infiltration into tumor sites, therapy-associated toxicity [9], and antigen escape [10].

Ciltacabtagene autoleucel was the first variable domain of heavy chain of heavy-chain antibody (VHH)-based CAR-T product approved by the FDA, suggesting that nanobody-based CAR-T products could be an effective means to treat solid tumors [11, 12].

Previous studies have shown that Nectin4 has a pivotal role in the occurrence, invasion, and metastasis of BLCA and is one of the crucial targets for diagnosis and treatment [13]. The Nectin4-specific nanobody was screened from a phage display library constructed as described in our previous study [14]. Then, Nectin4 nanobody was utilized to construct a second-generation recombinant lentiviral CAR plasmid. Subsequently, lentivirus was transferred to cells to obtain Nectin4 targeted VHH-CAR-T ($VHH^{Nectin4}$-CAR-T) cells. $VHH^{Nectin4}$-CAR-T exhibited excellent cytotoxicity and targeting at the cellular level, and specifically killed tumor cells, and released corresponding cytokines, indicating the potential of $VHH^{Nectin4}$-CAR-T for BLCA immunotherapy.

Materials and methods

Cell lines and culture conditions

HEK293T, RT4, SW780, 5637, U87-MG, HGC-27, MDA-MB-231, SW620, ES-2, SKOV-3, and HOS cells were obtained from the Chinese Academy of Cell Bank Sciences (Shanghai, China). The HEK293T cell line was cultured in Dulbecco's modified DMEM medium (BI, Göttingen, Germany). HGC-27, MB-231, and 5637 cells were maintained in RPMI-1640 medium (Biosera, Cholet, France). SW780 and SW620 were maintained in L-15 medium (Gibco, Grand Island, NY, USA). RT4, ES-2, and SKOV-3 cells were maintained in McCoy's 5A medium (Gibco). HOS and U87-MG cells were maintained in MEM medium (Gibco). All cell lines were supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Biosera) and cultured in an incubator at 37°C with 5% CO_2. The humanized camelids single-domain phage library displays synthesized single-domain antibodies based on camelid VHH frames constructed by our laboratory [14].

CAR construction

Humanized VHHs for anti-Nectin4 VHH-CAR and anti-Nectin4 scFv-CAR were screened from a phage display library constructed, as described in our previous study [14]. scFv derived from hNec4.11 (CN 112088167 A) was incorporated into basic CARs composed of CD8α hinges, CD28 transmembrane domains, and CD28 and CD3ζ signaling domains. The two full-length DNA sequences, which had undergone human codon optimization, were synthesized by Sangon Biotech (Shanghai, China) and subsequently inserted into the lentiviral CAR expression vector (pCDH-CMV-EF1-GFP) via two specific restriction enzyme sites.

Production of lentivirus and T cell transduction

Nectin4 CAR lentivirus vector (pCDH-CMV-EF1-VHH-CAR-GFP and pCDH-CMV-EF1-scFv-CAR-GFP) with package plasmids (PLP1, PLP2, and PLP-VSVG) transfected HEK293T cells using PEI transfection reagent (Polysciences, Warrington, PA, USA) at a ratio of 23.1:16.5:16.5:9.9. The virus-containing supernatants were collected 72 h later, concentrated, and stored at −80 °C.

Fresh blood was obtained from healthy donors with consent and approval of the Ethics Committee. Human peripheral blood mononuclear cells (PBMCs) were isolated over a density gradient using a Ficoll kit (GE, Boston, USA). Next, T cells were enriched with ActSep® CD3/CD28 separation & activation magnetic beads (T&L Biotechnology, Beijing, China) to activate cells for 48 h prior to lentiviral infection. Then, activated T cells were transduced by lentiviral particles at a multiplicity of infection = 20. The cells were continuously cultured for 7 days and the cells were collected for *in vitro* experiments. Untransduced T cells (NC-T) and transfected GFP lentivirus (Mock-T) were prepared as a negative control and scFv-CAR-T was prepared as a positive control. All T cells were derived from different healthy donors.

All experiments were conducted in accordance with the guidelines and regulations of the Research Ethics Committees of East China University of Science and Technology (Shanghai, China), as approved by the mentioned committees (REC No. 20181223).

Flow cytometry and antibodies

mAbs against Nectin4 antibody (R&D, Minneapolis, MN, USA) and FITC-rabbit anti-goat IgG (H+L) secondary antibody (ABclonal Biotechnology Co., Ltd, Wuhan, China) were used to detect Nectin4 protein expression on cancer cells by flow cytometry. APC-Cy7-CD3, APC-CD4, PE-CD8, brilliant violet 421-CD197 (CCR7), FITC-CD45RA, PE-CD223 (LAG-3), brilliant violet 421-CD366 (Tim-3), and APC-CD279 (PD-1) (Biolegend, San Diego, CA, USA), HA-Tag (C29F4) rabbit mAb (PE conjugate; CST, Boston, Danvers, USA) were used for CAR expression. The cells were rinsed twice with PBS, dyed, and incubated with mAb for 20 min at 4 °C in the dark. The cells were collected for analysis on a flow cytometer (NovoCyte D3000; ACEA, Shanghai, China) and the data were analyzed using NovaExpress software (Shanghai, China).

Cytotoxicity assays

Nectin4-positive BLCA cells (SW780 and RT4) and Nectin4-negative U87-MG cells were cultured at a density of 2×10^4 cells/well in 96-well E-plates (ACEA) at 37 °C in a 5% CO_2 incubator overnight, then NC-T cells, mock-T cells, scFv-CAR-T, and VHH-CAR-T cells were co-cultured with target cells at different effector-to-target (E:T) ratios (0.5:1 and 1:1). The viability of target cells was detected using the Real-Time Cell Analysis system [RTCA] (ACEA).

Analysis of cytokine secretion

Tumor cells were co-cultured with NC-T cells, mock-T cells, scFv-CAR-T cells, and VHH-CAR-T cells in 96-well plates for 24 h without exogenous cytokines. Then, the supernatants were collected and cytokines (IFN-γ and TNF-α) were measured using ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis and graph generation were performed using GraphPad Prism software (v.8.0; Boston, MA, USA). All data are expressed as the mean ± SD. All experiments were repeated at least 3 times. Student's t-test was used to compare the two groups. One- or two-way ANOVA was used for multi-group comparisons to determine statistically significant differences between the samples. A P value < 0.05 was significant and the significance level is shown in figures as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Results

Establishment of Nectin4 targeting CAR molecules

Nectin4 targeting CAR was constructed and anti-Nectin4 scFv/nanoantibody was used to bind antigen outside of the cell. The extracellular antigen binding domain was connected to the intra-cellular domain via a CD8α hinge and a CD28 transmembrane region. The intracellular domain consisted of a CD28 co-stimulatory region and a CD3ζ T-cell activation region. The CARs in the present study were second generation [CAR^{2G}] (**Figure 1A and B**). scFv-CAR and VHH-CAR DNA sequence with or without an HA tag were

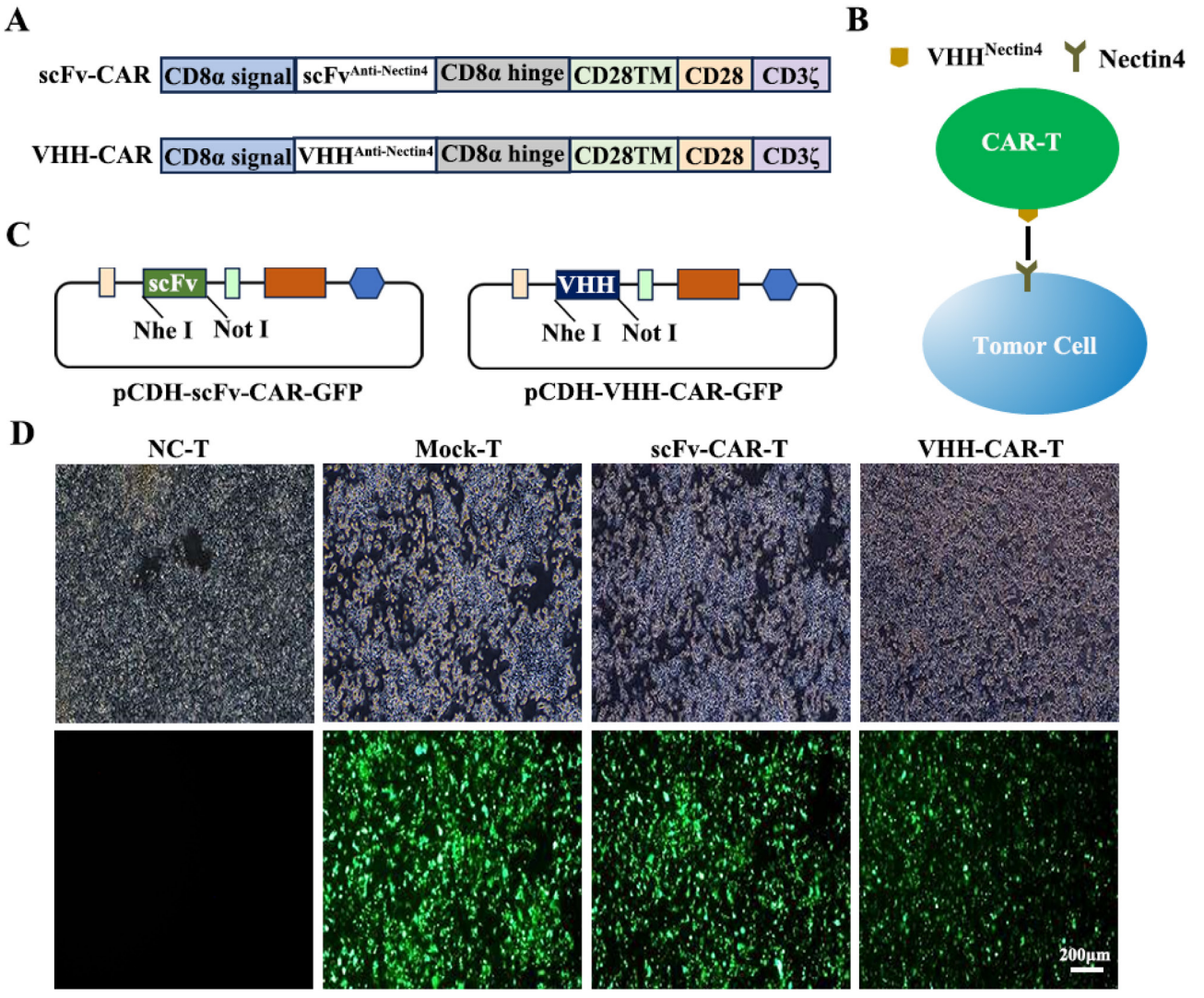

Figure 1 Establishment and expression of the Nectin4 targeting CAR molecules. (A) Schematic diagram of CARs, including the CD8 signal peptide (SP), scFv/VHH, CD8α hinge, CD28TM, CD28, and CD3ζ domain. (B) Diagram of a lentiviral vector encoding CARs. (C) Schematic diagram of pCDH-VHH-CAR-GFP and pCDH-scFv-CAR-GFP. (D) GFP expression was observed by fluorescence microscope in HEK-293T cells transfected with NC, mock, scFv-CAR, and VHH-CARs after 72 h. Bar = 200 μm.

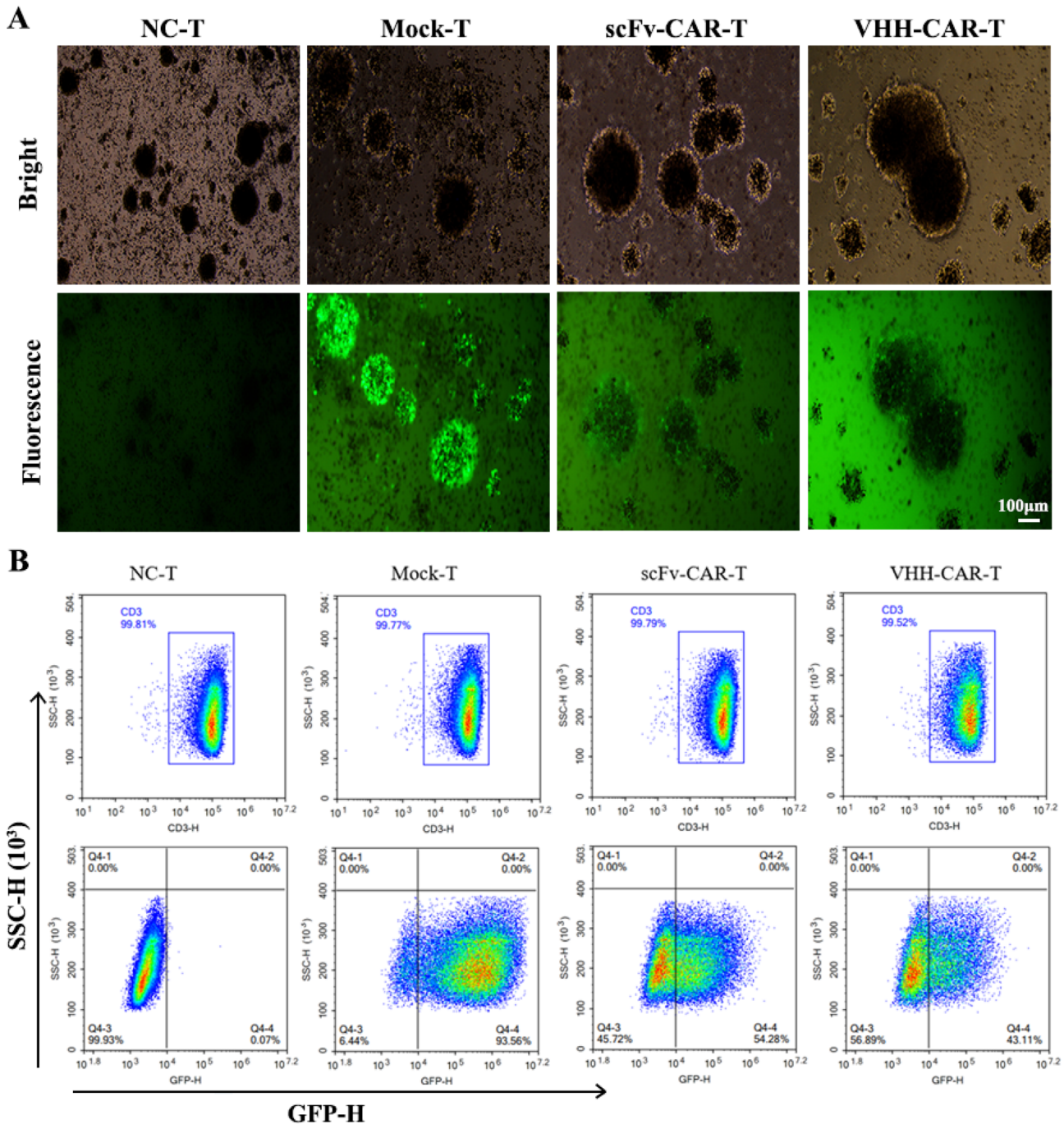

Figure 2 **Generation of Nectin4 CAR-T cells.** (A) The fluorescence images of NC-T, mock-T, scFv-CAR-T, and VHH-CAR-T cells transfected by lentiviral vectors after 48 h. (B) The positive rate of GFP in the T cells detected by FCM. Bar = 100 μm.

synthesized and inserted into the lentiviral vector (pCDH-CMV-EF1α-GFP; **Figure S4**, **Figure 1C**). The single-chain variable fragment (scFv)-CAR and VHH-CAR sizes were 1478 and 1148 bp, respectively (**Figure S1**). The fluorescence pattern and lentivirus titer were determined (**Table S1**) to verify whether the recombinant lentiviral vector plasmid was successfully transfected and the corresponding lentiviral particles were released. The fluorescence pattern revealed positive CAR expression in HEK-293T cells (**Figure 1D**), indicating that the recombinant lentiviral vector plasmid was successfully transfected and the corresponding lentiviral particles were released.

Preparation and evaluation of CAR-T cells

Mock, scFv-CAR, and $VHH^{Nectin4}$-CAR lentiviruses successfully infected T cells to determine whether the prepared CAR was expressed in T cells. As shown in **Figure 2A**, green fluorescence (GFP) was observed in the lentivirus-infected T cells after 48 h as expected, except in the NC-T cells, suggesting that the three lentiviruses were successfully transfected into T cells. The percentage of $CD3^+$ T cell (**Figure 2B**) was detected by flow cytometry. The purity of $CD3^+$ T cells was > 99%, indicating that the T cells were

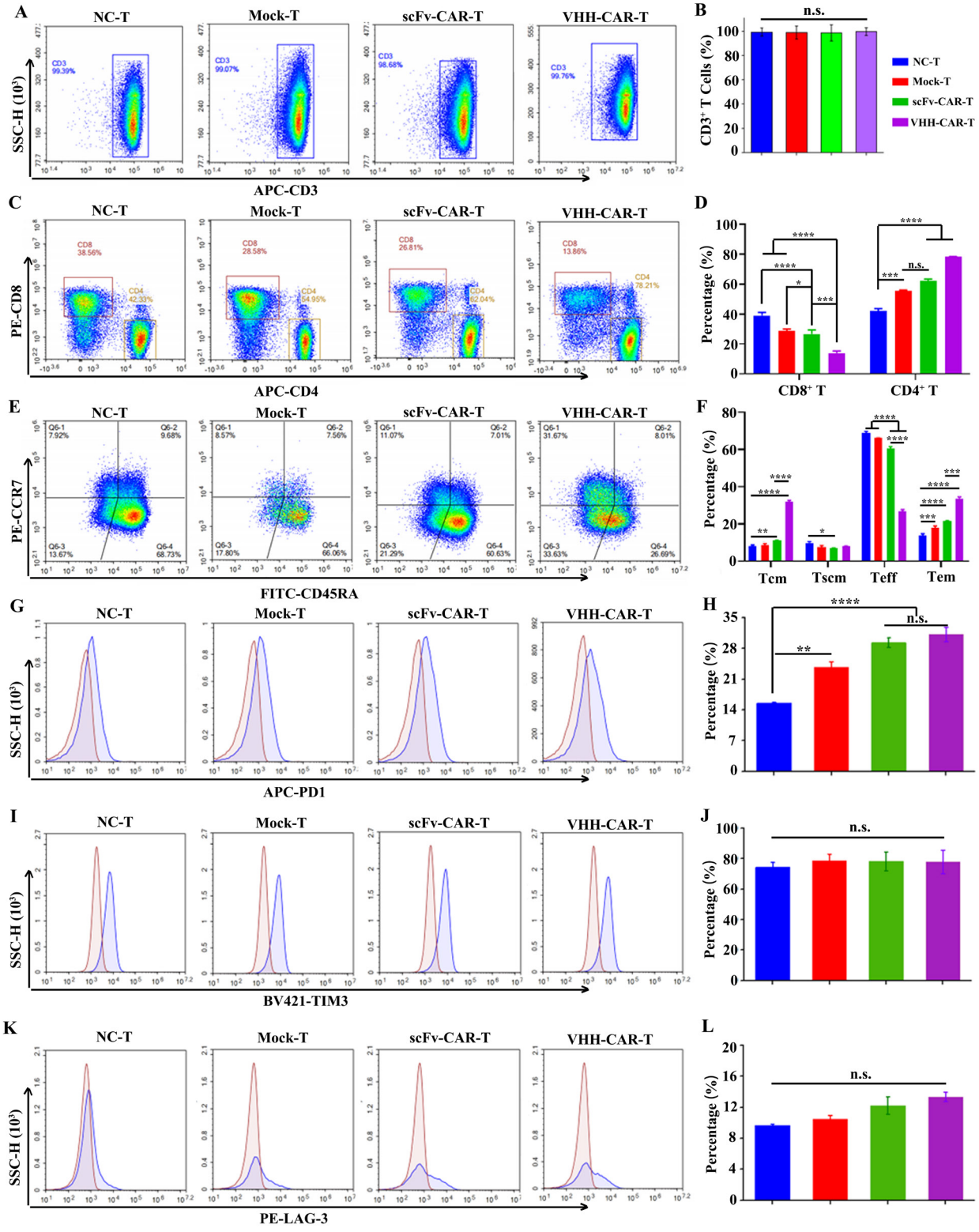

Figure 3 **Cell phenotype of VHH-CAR-T cell.** The ratios of CD3⁺ (A-B), CD4⁺/CD8⁺ (C-D), and CD45RA⁺/CCR7⁺ (E-F) in CAR-T and NC-T cells were detected by flow cytometry. PD1 (G-H), TIM3 (I-J), and LAG3 (K-L) expression in CAR-T and NC-T cells was detected by flow cytometry. All data are shown as the mean ± SD (n = 3) and analyzed by Student's t-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001; n.s., not significant.

specifically proliferating in large numbers. Next, flow cytometry (FCM) showed that the GFP-positive rates in mock-T, scFv-CAR-T, and VHH^{Nectin4}-CAR-T cells were 93.56%, 54.28%, and 43.11%, respectively (**Figure 2B**), which indicated that scFv/VHH affects GFP expression. In addition, the HA rates in VHH^{Nectin4}-CAR (HA tag)-T cells were also detected using flow cytometry and the results indicated that the percentages of GFP and HA-positive cells in

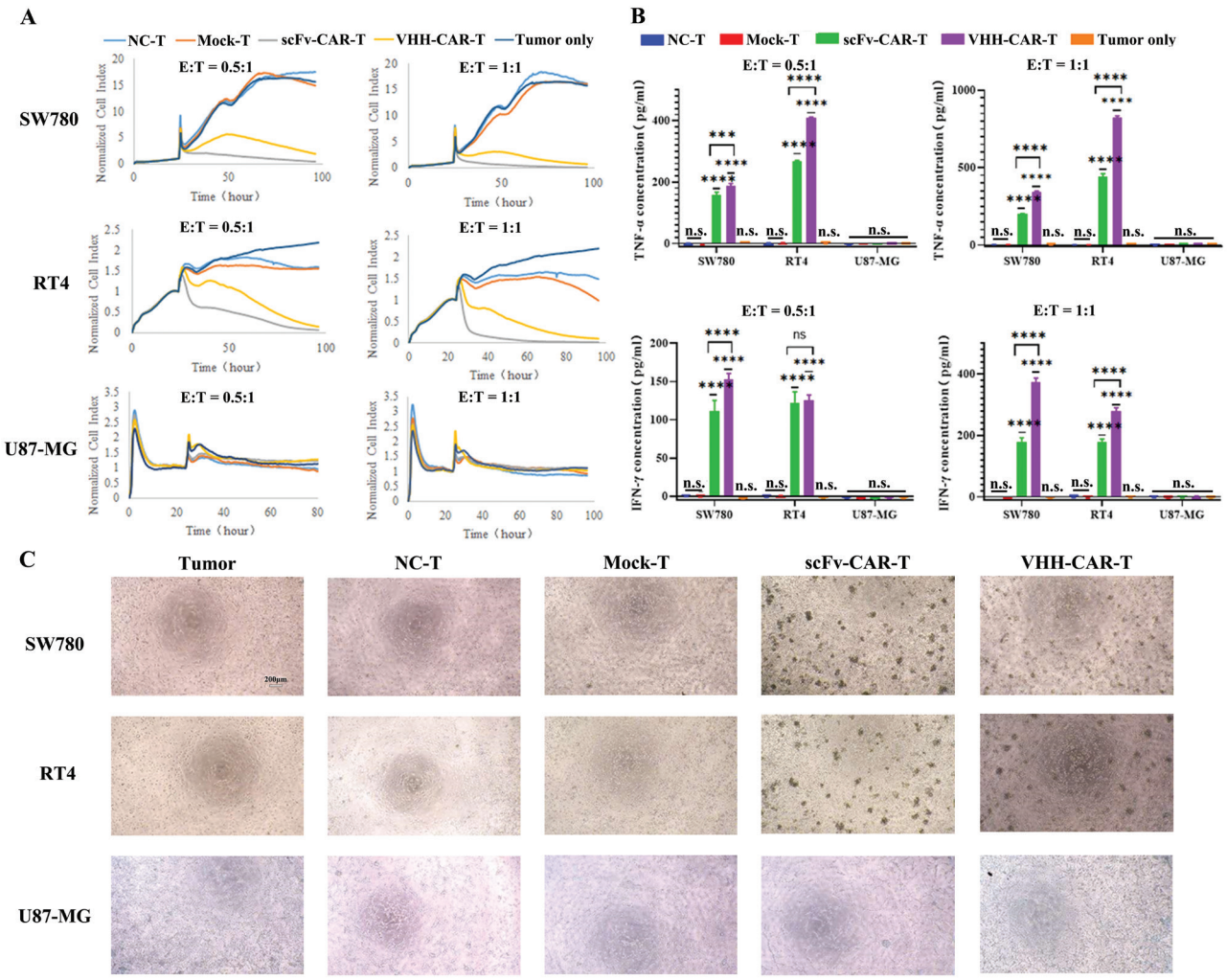

Figure 4 **Evaluation of the killing ability of VHH-CAR-T cells *in vitro*.** (A) The cytotoxic effect of VHH-CAR-T cells on tumor cells detected using the RTCA system. RT4, SW780, and U87-MG cells were co-cultured with different CAR-T cells for 24 h at different E:T ratios. The cell killing assay was detected using the RTCA system. (B) TNF-α and IFN-γ expression was detected by ELISA analysis. (C) The morphology of different T cells co-cultured with cancer cell lines (SW780, RT4, and U87-MG). All data are shown as the mean ± SD (n = 3) and analyzed using Student's t-test. ***$P < 0.001$ and ****$P < 0.0001$; n.s., not significant.

VHH^{Nectin4}-CAR (HA tag) T cells were comparable, suggesting that GFP+ cells effectively represent CAR+ cells (**Figure S4**).

CAR affects T-cell phenotype

Different T cell typing is significant for CAR-T cell immunotherapy and can significantly influence the effect of immunotherapy [15, 16]. Cell surface marker expression of CAR-T (CD3, CD4, CD8, CD45RA, and CCR7) was detected by FCM to explore the effect of CAR on T cell subtypes. The proportion of CD4$^+$ cells in mock-T, scFv-CAR-T, and VHH^{Nectin4}-CAR-T cells was increased and the percentage of CD8+ cells was significantly decreased compared to NC-T cells (**Figure 3A-D**), indicating that CD4+ and CD8+ T cell immunophenotyping changed under the influence of lentivirus transfection. Compared to mock-T cells, the proportion of CD8+ significantly decreased and the proportion of CD4+ significantly increased in scFv-CAR-T and VHH^{Nectin4}-CAR-T cells, suggesting that the recombinant CAR lentivirus had a greater impact on CD4+ and CD8+ T cell immunophenotyping. The proportion of CD8+ T cells decreased and the proportion of CD4+ cells increased significantly in the VHH^{Nectin4}-CAR-T group compared to scFv-CAR-T.

T cells can be divided into four subsets based on the surface expression of CD45RA and CCR7 [17] during T cell differentiation. T_{eff} cells can proliferate and differentiate after stimulation by an antigen. Contact with host cells and activation of lysosomal enzymes in target cells cause lysis and death of host cells [18]. Therefore, flow cytometry was used to detect immunophenotyping on the surface of T cells. The proportion of T_{eff} cells based on VHH^{Nectin4}-CAR-T cell typing was significantly reduced compared to scFv-CAR-T cells and the total number of CD4+ T cells and remaining memory T cells were significantly increased (**Figure 3E-F**). A previous study showed that CD4+ T cells are essential for formation of cytolytic CD8+ T cells against tumors [19]. The higher proportion of CD4+ T cells in VHH^{Nectin4}-CAR-T cells suggested that there might be significant differences in the anti-tumor mechanisms and functions between VHH^{Nectin4}-CAR-T and scFv-CAR-T, warranting further investigation.

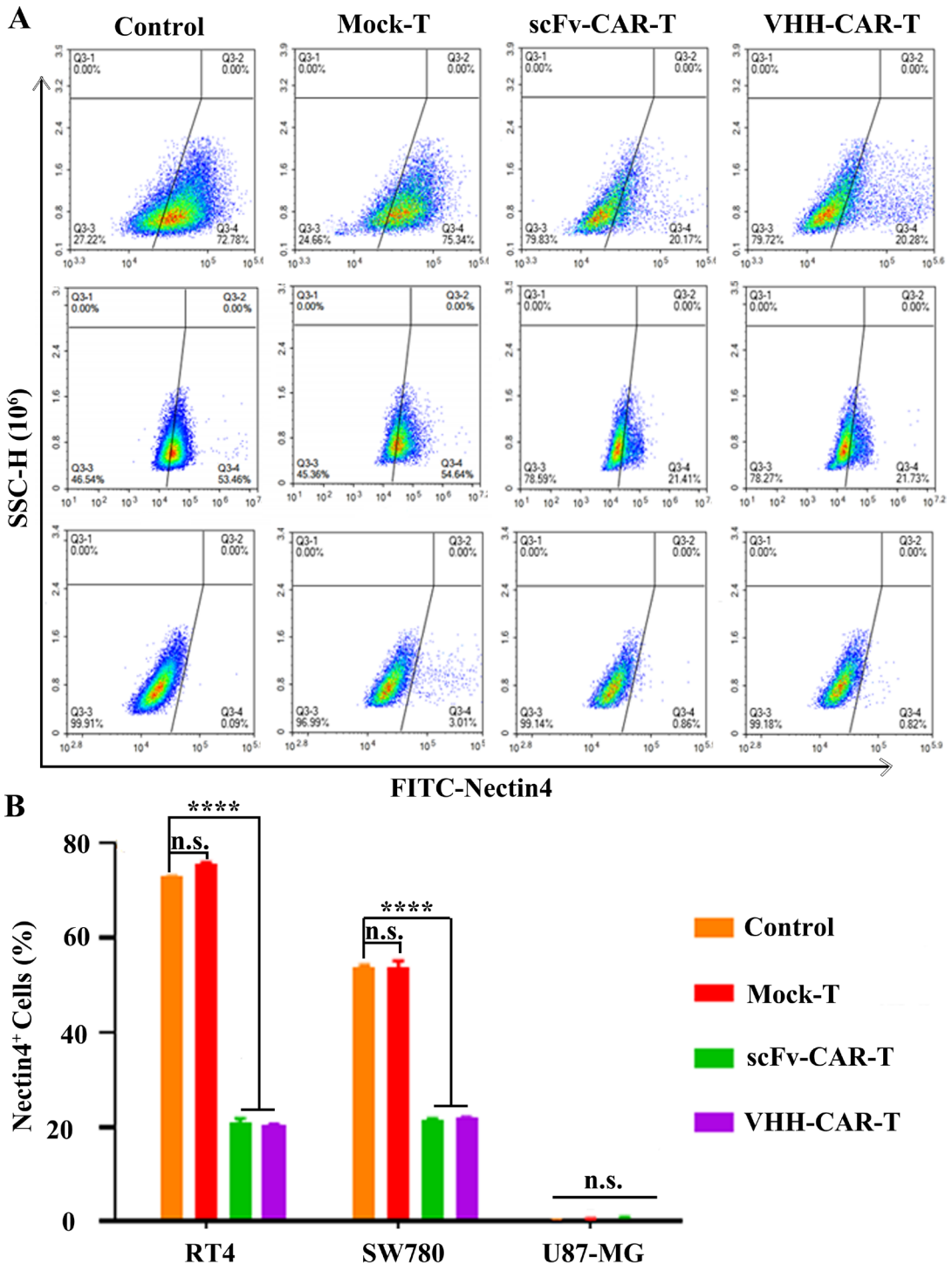

Figure 5 **Nectin4 expression on tumor cells surface was detected by FCM.** (A) Nectin4 expression on the surface of cancer cells treated with Mock-T, scFv-CAR-T, and VHH-CAR-T cells was detected by FCM. (B) Quantitative analysis of (A). All data are shown as the mean ± SD (n = 3) and analyzed using Student's t-test. ****P < 0.0001; n.s., not significant.

VHH ^{Nectin4}-CAR affects immune checkpoint expression

Emerging research suggests that T-cell activation and associated immune responses are influenced by immune checkpoints [20, 21]. Checkpoint-related protein expression on the surface of T cells (PD-1, TIM-3, and LAG-3) was detected by FCM to determine the effect of CAR on T cell immune checkpoints. PD-1 protein expression on the surface of lentivirus-transfected mock-T, scFv-CAR-T, and VHH^{Nectin4}-CAR-T was increased compared to NC-T (**Figure 3G-H**), suggesting that PD-1 expression on the surface of T cells was affected by VHH^{Nectin4}-CAR lentivirus. However, there was no significant differences in the levels of TIM-3 (**Figure 3I-J**) and LAG-3 expression (**Figure 3K-L**). These results indicate that the level of PD-1 expression on the surface of T cells was affected by scFv-CAR and VHH^{Nectin4}-CAR lentivirus.

VHH^{Nectin4} CAR-T cells display targeted toxicity to BLCA cells

RT4 and SW780 cells were co-cultured with Nectin4 CAR-T cells at different E:T Ratio to determine the presence of targeted toxicity of Nectin4 CAR-T to Nectin4$^+$ tumor cells (**Figure S2**). The cell index was detected using the RTCA system, which showed that the VHH ^{Nectin4}-CAR-T and scFv-CAR-T cell index decreased significantly in RT4 and SW780 cells (**Figure 4A**). Similarly, the release of cytokines (TNF-α and IFN-γ) was significantly increased in SW780 and RT4 cells treated with VHH ^{Nectin4}-CAR-T. In addition, cytokines (TNF-α and IFN-γ) released by the VHH ^{Nectin4}-CAR-T cell group was significantly higher than the scFv-CAR-T cell group (**Figure 4B**). Notably, there was no significant difference in the Nectin4$^-$ cell line (U87-MG) and VHH ^{Nectin4}-CAR-T and scFv-CAR-T group cell index and cytokine release, indicating that the VHH ^{Nectin4}-CAR-T and scFv-CAR-T cells were targeted and unresponsive to Nectin4$^-$ cells. The aggregation of T cells to the site of tumor cells signifies the killing ability of T cells [22]. The effects of different T cells on tumor cells were directly observed under a microscope and the results showed that there were obvious T cell clusters in the scFv-CAR-T and VHH-CAR-T groups (**Figure 4C**). These findings indicate that VHH ^{Nectin4}-CAR-T may exhibit strong killing activity against Nectin4**+** cancer cells by releasing related cytokines.

VHH ^{Nectin4}-CAR-T reduced Nectin4 expression on the surface of cancer cells

Surface expression of Nectin4 in SW780, RT4, and U87-MG cell lines was examined by flow cytometry after co-incubation with different T cells (**Figure 5**, **Figure S5**) to determine the changes in surface Nectin4 expression after co-culturing target cells with different T cells. Nectin4 expression on the surface of SW780 and RT4 cells decreased significantly compared to control and mock-T groups after co-culture with scFv-CAR-T and VHH^{Nectin4}-CAR-T cells for 5 h (**Figure 5A and B**), while there was no significant difference in the Nectin4-negative cell line (U87-MG). The experimental results demonstrated that VHH^{Nectin4}-CAR-T and scFv-CAR-T target Nectin4$^+$ cells. Furthermore, the surface antigen of cancer cells was shown to decrease gradually, which may be a mechanism underlying immune escape of cancer cells.

Discussion

There are currently >1000 CAR-T therapies undergoing clinical trials with the majority targeting relapsed/refractory hematologic malignancies. More and more studies involving solid tumors focus on finding tumor-associated antigens but only a few clinical trials have shown satisfactory results due to severe side effects and toxicity [23]. Li et al. reported that the fourth-generation Nectin4-7.19 CAR-T cells exhibit greater proliferation, migration, and cytotoxicity than second-generation Nectin4 CAR-T cells, in which Nectin4-7.19 CAR-T consist of a scFv derived from an antibody against human Nectin4 [24]. VHHs have also been used as the antigen-recognition domains of CAR-T cells due to the small size, high stability, and ability to recognize hidden antigen epitopes. Safarzadeh Kozani et al. developed a VHH-based CD19-redirected CAR-T cells capable of mediating robust anti-tumor effects [25]. Herein second-generation Nectin4 CAR-T cells are described, in which VHH^{Nectin4} CAR-T consisted of single-domain antibodies (VHHs), which were shown to possess potent proliferation, and cytotoxicity and significant anti-tumor effects *in vitro*.

Recent reports have revealed the correlation between variations in the function of T cell subpopulations and the efficacy of CAR-T cell immunotherapy [26]. T_{SCM}, expressing a CD45RA+ CCR7+ T population, possesses higher effectiveness and persistence against tumors than T_{CM} [27]. Both CD8+ and CD4+ T subsets exhibit synergistic anti-tumor CAR-T activities because CD4+ cells are conducive to developing CD8+ memory functions [28]. The expression of CAR in the CD4+ T subset was markedly increased, whereas the CD8+ subset significantly decreased. The proportion of T_{em} subpopulation of VHH-CAR-T was higher than NC-T cells but the VHH^{Nectin4}-CAR-T T_{eff} subpopulation decreased compared to NC-T and scFv-CAR-T, which may be related to the weak killing ability of VHH^{Nectin4}-CAR-T. Studies have shown that increased memory T-cell (Tem) phenotypic markers may delay CAR T-cell exhaustion [29]. Interestingly, the increased VHH^{Nectin4} CAR-T T_{em} subpopulation may delay CAR T-cell exhaustion, thereby improving CAR T-cell persistence.

T cells rapidly expand to reach the appropriate number relative to the tumor load after being transported to the tumor and encountering homologous antigens [30, 31]. Immune checkpoints can lead to T cell exhaustion and suppression of immune function [32]. For example, PD-1 inhibits T cell activation after binding to PD-L1 on the tumor [33] and TIM-3 and LAG-3 regulate T cell depletion [34]. TIM-3 and LAG-3 assist tumor cells to inhibit the activity of immune cells and act as a tumor immune checkpoint. The present study showed that there was no significant difference in LAG-3 and TIM-3 expression among the four types of T cells but PD-1 expression in the three types of T cells infected with virus was significantly increased. PD-1 expression was significantly increased in VHH^{Nectin4}-CAR-T cells compared to scFv-CAR-T, which could lead to a greater likelihood of VHH^{Nectin4}-CAR-T cell failure, which was liin agreement with the results of other CAR-T studies [35].

Enfortumab vedotin (EV) is an antibody-drug conjugate targeting Nectin4 and approved for treatment-refractory metastatic urothelial cancer. Despite the unprecedented success of CAR-T cell therapy in hematologic malignancies, CAR-T cell therapy has not had similar results in solid tumors. Success in this area is hampered by several factors, including the lack of desirable tumor antigens, poor transport of T cells to the tumor, and a hostile tumor microenvironment [36]. In the current study, Nectin4 targeted CAR-T (VHH^{Nectin4}-CAR-T) cells was established as a therapeutic target in the clinic setting based on the safety and efficacy

of Nectin4 and capability and security was confirmed *in vitro*. Nanoantibodies (VHH), which are the smallest antigen-binding fragments reported to date, were used instead of traditional single-chain antibodies (scFv). Nanoantibodies have been utilized in CAR-T therapy as the extracellular antigen-binding structural domain of CAR-T. Nanoantibodies have the advantages of small molecular weight, easy expression, strong penetration, good stability, low immunogenicity, and homology with humans [37, 38], demonstrating excellent therapeutic efficacy.

Conclusion

Construction of second-generation VHH^{Nectin4}-CAR-T cells was demonstrated in the current study that target Nectin4, which is abnormally expressed on BLCA cell surface but not on normal cell surfaces. Specifically, VHH^{Nectin4}-CAR lentivirus treatment increased the proportion of CD4+ T and memory T cells. *In vitro* experiments demonstrated that VHH^{Nectin4}-CAR-T cells with strong specific killing ability can specifically recognize Nectin4^{+} bladder cancer cells, secrete cytokines, and kill cancer cells. Fortunately, VHH^{Nectin4}-CAR-T had no effect on the growth of U87-MG cells due to the lack of Nectin4 expression on the surface of U87-MG cells. Taken together, the data herein will hopefully support the potential of VHH^{Nectin4}-CAR-T as a targeted drug for the treatment of BLCA. Moreover, VHH ^{Nectin4}-CAR-T cells must be further investigated in preclinical xenograft models of Nectin4+ BLCA before advancing into clinical trials.

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Contributions

XYM, XLT, and WYZ participated in the research design and guidance. JPN and SZ performed the experiments, analyzed the data, and wrote the manuscript. CNX, GDL, and ZWX assisted with the mouse experiments and modified the manuscript. All authors have read the manuscript and approved the content.

Availability of data and materials

All datasets generated and analyzed during this study are included in the published article. Additional data are available from the corresponding author upon reasonable request.

Ethics declarations

Ethics approval and consent to participate

Fresh blood was obtained from healthy donors with their consent after approval by the Ethics Committee.

Consent for publication

Not applicable

Animal Research Statement

Not applicable

Conflict of Interest Statement

The authors declare no competing interests. Guodi Liu is an employee of Shanghai Yihao Biological Technology Co., Ltd.

Supplementary materials

Supplementary Material can be downloaded from https://bio-integration.org/wp-content/uploads/2025/04/bioi20250041_Supplemental.pdf.

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