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Original Article

Development of GPC3 and EGFR-dual-targeting chimeric antigen receptor-T cells for adoptive T cell therapy

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Abstract: Adoptive transfer of T cells expressing specific anti-glypican-3 (GPC3) chimeric antigen receptors (CARs) has demonstrated therapeutic potential against hepatocellular carcinoma (HCC). However, normal tissues with low expression of neoplasm-associated antigens often show on-target, off-tumor toxicity. Previous studies have revealed that the development of HCC xenografts in mice could be inhibited effectively by GPC3-targeting CAR-T cells. However, these studies did not provide information regarding on-target, off-tumor toxicity. We hypothesized that on-target, off-tumor toxicity may decrease in dual-targeting CAR-T cells that co-express GPC3 with epidermal growth factor receptor (EGFR)-targeted CARs characterized by CD3ζ and 28BB expression. Our research confirmed that dual-targeting CAR-T (CARgpc3-egfr) cells exhibited similar proliferative ability and cytotoxicity to CARgpc3 T cells against GPC3+EGFR+ HCC *in vitro*. However, EGFR-targeting CAR-T (CARegfr) cells showed poor proliferation activity and cytotoxicity against GPC3+EGFR+ HCC cells, similar to mock CAR-T cells. CARgpc3 and CARgpc3-egfr T cells showed enhanced cytokine secretion compared to CARegfr and mock CAR-T cells *in vitro*. *In vivo*, tumor growth suppression was better for CARgpc3-egfr T cells than for CARgpc3 T cells in GPC3+EGFR+ HCC, while it was not observed for CARegfr or mock CAR-T cells. Taken together, our data indicated that dual-targeting CAR-T cells with two CARs against GPC3 and EGFR may maintain relatively effective anti-neoplasm functions in GPC3+EGFR+ HCC *in vitro* and *in vivo*, a strategy that may reduce off-tumor toxicity.

Keywords: Hepatocellular carcinoma, Glypican-3, chimeric antigen receptors, EGFR

Introduction

Hepatocellular carcinoma (HCC) is a hepatic carcinoma that is currently the second most common cause of neoplasm-related death [1, 2]. HCCs mainly initiate within a cirrhotic liver, which usually results from chronic infection by hepatitis B or hepatitis C virus. However, other factors, including fatty liver disease, excessive alcohol consumption, obesity, and smoking are associated with liver metabolism and play an important role in HCC pathogenesis [3].

Treatment of patients with HCC remains a big challenge, with very few effective FDA-approved drugs. Thus, an efficient curative therapy for HCC remains elusive. After decades of failure of traditional therapies, immune therapies have emerged as potentially effective treatments for patients with advanced carcinoma [4-6]. In 2013, carcinoma immunotherapy was named as the “breakthrough of the year”, mainly because of the marked clinical effects of T-cell-based therapies [7]. Chimeric antigen receptors (CARs), as a powerful application of this approach, were first developed by the fusion of the antibody antigen-binding domain with the transmembrane and cytoplasmic domains of CD3ζ [8]. T cells expressing CARs showed *in vitro* cytotoxicity against neoplastic cells.

However, *in vivo* investigations of these T cells revealed that their efficacy was not adequate. Therefore, to overcome these defects, one or two costimulatory domains were fused with the intracellular domain of first-generation CARs to enhance their efficacy [9].

Glypican-3 (GPC3) is a common marker used to identify HCC [10]. Various studies have demonstrated that GPC3 expression is mainly present in most hepatoblastomas and absent in some typical subtypes, including teratoid hepatoblastomas and a few hepatoblastomas with mesenchymal differentiation [11-13]. Furthermore, GPC3 is more frequently expressed in poorly and moderately differentiated HCC, but it is less expressed in well-differentiated HCC [14, 15], suggesting that GPC3 could be used as a marker to differentiate between various HCC stages. However, GPC3 is also expressed in normal tissues; therefore, it is possible that CAR-T cells targeting only GPC3 may demonstrate off-tumor effects *in vivo*.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) of the ErbB family with essential functions in epithelial cell physiology. EGFR is used as an effective therapeutic target in multiple types of carcinoma because it is frequently mutated and/or overexpression in multiple human carcinomas. Gefitinib is an FDA-approved tyrosine kinase inhibitor specific for EGFR and is widely used in carcinoma treatment. Several reports have indicated that high EGFR expression is frequent in human HCC, which leads to more aggressive neoplasm growth, but EGFR is low in normal epithelial tissues [16, 17]. Moreover, EGFR overexpression could serve as a negative prognostic marker in poorly differentiated HCCs and a positive prognostic factor in early recurrence and metastasis of HCCs [16, 18]. Although EGFR is a potent target for innovative treatment strategies for HCC, it is also overexpressed in normal epithelial cells, thus enhancing the risk of potential on-target, off-tumor toxicity.

A previous study has revealed that third-generation CAR-T cells can effectively inhibit GPC3-positive HCC xenografts by targeting GPC3 *in vivo* [19], indicating that they may be a promising strategy for HCC treatment. However, GPC3 expression in normal tissues is not completely eliminated, thus possibly generating unwanted toxicity in humans [20]. CAR-T cells could attack HER2-positive parenchyma, which has led to a patient's death by third-generation HER2-targeted CAR-T cells targeting metastatic colon carcinoma [21], indicating that reducing on-target, off-tumor toxicity is urgent.

GPC3 is overexpressed in HCC, while its expression is low in normal cells, and it is not expressed in liver cells. EGFR is overexpressed in HCC, while its expression is low in liver epithelial cells. Dual-targeting CAR-T cells are activated by GPC3 and EGFR co-expression, and these cells can effectively reduce on-target, off-tumor toxicity [22, 23]. To the best of our knowledge, this is the first study to report the use of dual-targeting GPC3/EGFR-CAR-T (CARgpc3-egfr T) cells as therapy for HCC. Our data demonstrated that third-generation CARgpc3-egfr T cells have an obvious advantage compared with CARgpc3 T cells and exert similar anti-neoplastic effects and toxicity to first-generation CAR-T cells *in vitro* and *in vivo*, thus paving the way for dual-targeting immunotherapeutics.

Materials and methods

Cell lines

Huh-7, SK-HEP1, and 293 T (purchased from ATCC) cells were grown in complete DMEM (Invitrogen, Carlsbad, CA) with 10% FBS. Peripheral blood mononuclear cells (PBMCs) from human donors were obtained from the Shanghai Blood Center. CD4+ and CD8+ T cells isolated from PBMCs were incubated in RPMI 1640 (Invitrogen) containing 10% FBS, 100 μg/mL penicillin, and 100 U/mL streptomycin.

Generation of vectors and lentivirus

As previously described, one construct was produced from the GPC3-CD3ζ (GPC3-Z) fragment that contains the anti-GPC3 scFv domain connected to human CD8α hinge and the transmembrane and intracellular domains from CD3ζ [19]. The other construct derived from the transmembrane and intracellular domains of human CD28 and CD137 linked to the EGFR-28BB fragment containing the anti-EGFR H chain. Finally, the two constructs were fused with the enhanced green fluorescent protein (eGFP) (GPC3-Z) or mCherry (EGFR-28BB) and inserted to the pLVX vector.

Recombinant lentiviral vectors were obtained from a polyethylenimine linear fragment (MW

25,000) [24] and 30X concentrated with ultracentrifugation (Beckman Coulter) at 28,000 rpm, 4°C.

Isolation, transduction, and culture of primary T cells

RosetteSep kits (STEMCELL) were used to isolate CD4+ and CD8+ T cells from PBMCs obtained from healthy donors at the Shanghai Blood Center. Fresh CD4+ and CD8+ T cells were mixed in equal quantities and cultured in AIM-V® Medium CTS™ (GIBCO) containing 2% human AB serum (Huayueyang) and 300 U/mL recombinant human interleukin 2 (rhIL-2) (Huaxin). Next, we stimulated T cells with anti-CD3 and anti-CD28 fixed on tosyl-activated paramagnetic beads (Invitrogen) for 24 h. We transduced the lentivirus into 24-well plates packed with RetroNectin (TaKaRa) at a multiplicity of infection (MOI) of 15. Finally, we cultured cells at a density of 5×10^5 cells/mL with rhIL-2 (300 U/mL). Fourteen days post-transduction, functional assays were performed by adjusting engineered T cell populations to the same proportion of Gpc3-Z+ T cells with non-transformed T cells.

T cell proliferation

Neoplastic cells expressing antigens were co-cultured (1.0×10^6 cells) at 1:1 E/T ratio and treated with 50 Gy. Viable T cell number was estimated with trypan blue staining the next day, and cells were re-stimulated every week with radioactive neoplastic cells. No exogenous cytokines were used during the tests.

ELISA

Supernatants were treated with the medium, using an ELISA kit (Multi Sciences Biotechnology) to detect IFN-γ, TNF-α, IL-2, and IL-4. OD was measured at 450 nm.

Cytotoxicity of GPC3/EGFR CAR-T cells on GPC3+EGFR+ HCC in vitro

The redirected cytotoxicity was tested with CAR-T cells as effector cells and GPC3+EGFR+ HCC cells as target cells to explore whether GPC3/EGFR CAR-T cells can target specifically GPC3+EGFR+ HCC cells. Target cells were incubated with T cells at effector: target ratios of 1:3, 1:1, and 3:1, with different concentrations of the GPC3/EGFR CAR-T in 96-well plates for 18 h. Cytotoxicity was measured with the CytoTox 96 ®Non-Radioactive Cytotoxicity Assay kit. Three independent experiments with five replicates of each were performed.

Western blot analysis

Lysates from neoplasms were obtained and centrifuged for 15 min twice at 13,000 rpm at 4°C. Proteins were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes, cultured with primary antibodies at 4°C overnight, and then incubated with appropriate secondary antibodies for 2 h. Proteins were quantified with chemiluminescence.

GPC3/EGFR CAR-T cells hindering of neoplasmigenesis of subcutaneous GPC3+EGFR+ Huh-7 xenografts

Non-obese diabetic/severe with immunodeficiency disease (NOD/SCID) mice, 1.5 months old, were bred under specific pathogen-free conditions. Procedures were conducted according to the Shanghai Cancer Institute Experimental Animal Care Commission. Twenty-four hours before incubation, 200 mg/kg cyclophosphamide was administered intraperitoneally to remove the remaining immune cells. For the established orthotopic HCC model, mice were separated into four groups and inoculated with a mixture of 2×10^6 CAR-T cells (each group n=6, Mock, CARgpc3, CARegfr, and CARTgpc3-egfr T cells) and 2×10^6 GPC3+ EGFR+ Huh-7 cells with luciferase overexpression in the liver on day 0. On the 14^{th} and 21^{st} days, mice received intravenous injections of 5×10^6 modified T cells (each group: Mock, CARgpc3, CARegfr, and CARTgpc3-egfr T cells). Mice were observed every week following T-cell administration to explore the role of CAR-T cells in neoplasm growth and survival.

Immunohistochemical (IHC) analysis

IHC staining was used to investigate T cell infiltration in neoplasms. First, fresh neoplasm tissues from mice were collected and immediately fixed in 4% formalin for 24-72 h. Paraffin-embedded neoplasm blocks were then sectioned at a thickness of 4 m. Neoplasm sections were dried at 60°C for 1 h, deparaffinized and rehydrated with xylene and gradient alcohol concentration, and treated in citrate buffer

(pH 6.0) for 10 min. Endogenous peroxidase was quenched in quenching solution (3% hydrogen peroxide) for 10 min, following which blocking solution (1% bovine serum albumin) was added for 10 min. Anti-CD3ζ and anti-CD8 antibodies (Thermo Scientific) were added and incubated at 4°C, followed by peroxidase-conjugated secondary antibodies for 1 h and DAB solution for 5 min (ChemMate™ DAKO EnVision™ Detection Kit, Peroxidase/DAB, DAKO). Sections were counterstained with hematoxylin and cover-mounted.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Student's *t* test or one-way ANOVA with Bonferroni's post-test was applied to test the differences. $P < 0.05$ indicated statistical significance.

Results

Establishment of CAR-EGFR-CD28BB (CARegfr) and CAR-GPC3-z (CARgpc3) T cells

We constructed the first-generation CAR (GPC3-cd3ζ, thereafter GPC3-z), carrying the scFv of GPC3, gc33, named as CARgpc3, and CCR (CARegfr) containing the T cell costimulatory signaling molecules CD28 and 4-1BB (thereafter, EGFR-CD28BB) fused with EGFR (including egfrv III) scFv hu7b3. Co-expression of GFP, m-cherry, CAR, and CCR was accomplished by the ribosome hopping sequence F2a, and these fragments were inserted into the pLVX lentivirus vector (**Figure 1A**).

CAR-T cells were generated after T cells were transduced with the encoding CAR genes, and mock T cells expressed only eGFP. The transduction efficiency of mock, CAR-EGFR, CAR-GPC3, and CAR-GPC3/EGFR cell were 83.2%, 55.4%, 65.6%, and 52.0%, respectively, according to flow cytometry (**Figure 1B**). To further confirm the infection efficiency of CARs in T lymphocytes, we performed western blotting to investigate CD28BB and CD3ζ protein expression. As expected, T lymphocytes infected with the CAR-GPC3/EGFR lentivirus expressed both EGFR-CD28BB and GPC3-CD3ζ, whereas T lymphocytes infected with CARegfr or CARgpc3 only expressed EGFR-CD28BB or GPC3-CD3ζ, respectively (**Figure 1C**).

GPC3+EGFR+ Huh-7 and GPC3+EGFR+ SK-HEP1 HCC establishment

To compare the *in vitro* cytotoxic activities of the dual-targeting and single-targeting CAR-T cells, Huh-7 and SK-HEP1 cells were infected with lentivirus expressing GPC3 and EGFR; therefore GPC3+EGFR+ Huh-7, and GPC3+EGFR+ SK-HEP1 cell lines were established. Western blot analysis suggested that GPC3 and EGFR-infected Huh-7 and SK-HEP1 cells had higher expression of GPC3 and EGFR than non-infected cells (**Figure 2A** and **2B**).

Central memory characteristics of CARgpc egfr T cells

Adoptive transfer of central memory T cells (TCM) can reduce the number of experimental cells used and improve the complete remission rate of the disease. Therefore, more specialized T cell subsets, such as TCM and TSCM, can effectively improve survival and provide better therapeutic effects *in vivo*.

Clinical trials have also been performed to test the potential of T cell subsets from the peripheral blood. Next, we tested the phenotype of CARgpc3-egfr T cells. Expression of four differentiation markers, CD28, CD62L, CD45RO, and CD45RA, was examined by flow cytometry 14 days after T cell infection. Based on the four differentiation markers used, the majority of CAR-T cells showed a central memory T cell phenotype (**Figure 3**).

Dual-targeting T cells showed robust proliferative capacity a gainst GPC3+EGFR+ HCC cells in vitro

CD4+ and CD8+ T lymphocytes were activated by αCD3/αCD28 magnetic beads. Then, they were infected with lentivirus, expanded, and treated with 300 μg/mL IL-2 the next day. After recording the number of cells in each group every day, we found that CAR-positive T-lymphocytes and untreated T-lymphocytes grew at a similar rate - 100-200-fold in approximately 14 days (**Figure 4A**).

In order to explore whether neoplastic cells expressing EGFR and GPC3 can stimulate CAR-T cell-specific expansion, we designed two-step expansion experiments. Huh-7-GPC3+/EGFR+ and SK-HEP1-GPC3+/EGFR+ neoplastic

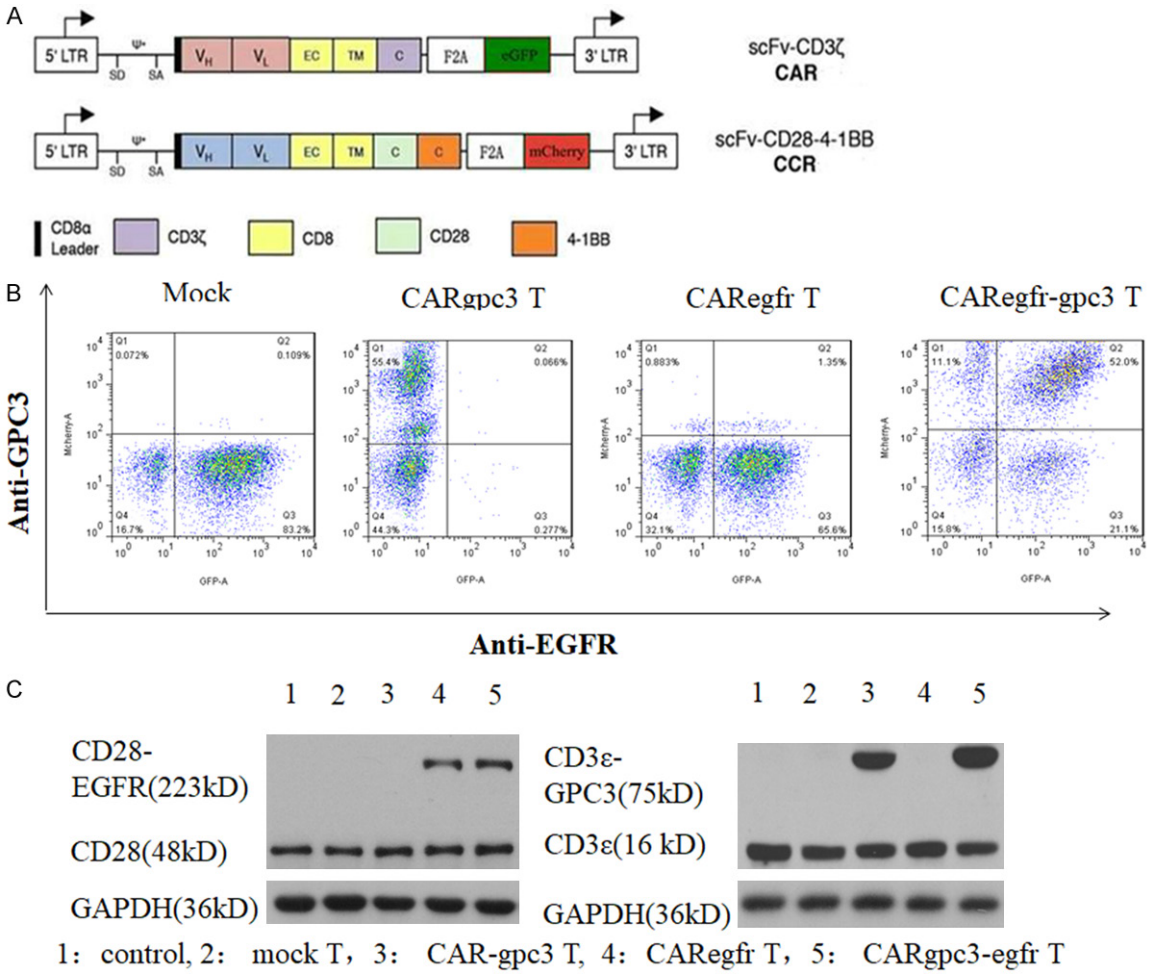

Figure 1. Establishment of EGFR-CD28-BB and GPC-3-zeta T cells. A. The construct of CAR, CCR and dual target CARs. GPC3-cd3ζ, GPC3-z for short, carrying the scFv of GPC3, gc33, named as CARgpc3; CCR (CAR-egfr) containing T cell costimulatory signal CD28, 4-1BB (EGFR-CD28BB for short) carrying EGFR (including egfrv III) scFv hu7b3. B. The CARs were expressed by engineered T cells upon letiviral infection. The EGFR-CD28-BB and GPC-3-zeta CARs were detected by staining for GPC3 and EGFR antibodies. Cells were tested by flow cytometry. T cells without CAR (Mock) served as control. C. Western blot analysis of EGFR-CD28-BB and GPC3-Z expression in T cells after transduction. Anti-human CD28 and CD3-zeta antibodies were used to detect endogenous and chimeric EGFR and GPC3 CARs protein levels.

cells were incubated with mock, CARgpc3, CAREgfr, and CARgpc3-egfr T lymphocytes, following which we counted the number of T cells. Our results revealed that CARgpc3 and CARgpc3-egfr T-lymphocytes co-incubated with GPC3+EGFR+ HCC cells expanded better than T-lymphocytes with single EGFR positivity when co-stimulatory signals were activated (**Figure 4B-E**). Four groups of genetically modified T-lymphocytes showed obvious expansion with artificial antigen-presenting cells ak562-64/86 and 100 ng/ml OKT3 stimulation. However, there was no obvious expansion for mock and CAREgfr cells after incubation with neoplastic cells.

Dual-targeting T cells exert cytotoxicity against GPC3+EGFR+ HCC cells in vitro

Next, we investigated the cytotoxicity of CAR-T cells against GPC3+EGFR+ HCC cells. Our results revealed that when the ratio of effector: target was 3:1, CARgpc3-egfr T cells achieved a better killing effect on neoplastic cells, with a killing rate of 70.34 ± 5.49% and 71.2 ± 6.43% for Huh-7 and SK-HEP1 cells, respectively, while the killing rate of CARgpc3 T lymphocytes was 33.16 ± 5.69% and 28.55 ± 4.53%, respectively (**Figure 5A**, **5B**). In addition, the effect of GPC3 and EGFR expression on CAR-T cell cytotoxicity was examined. Our results

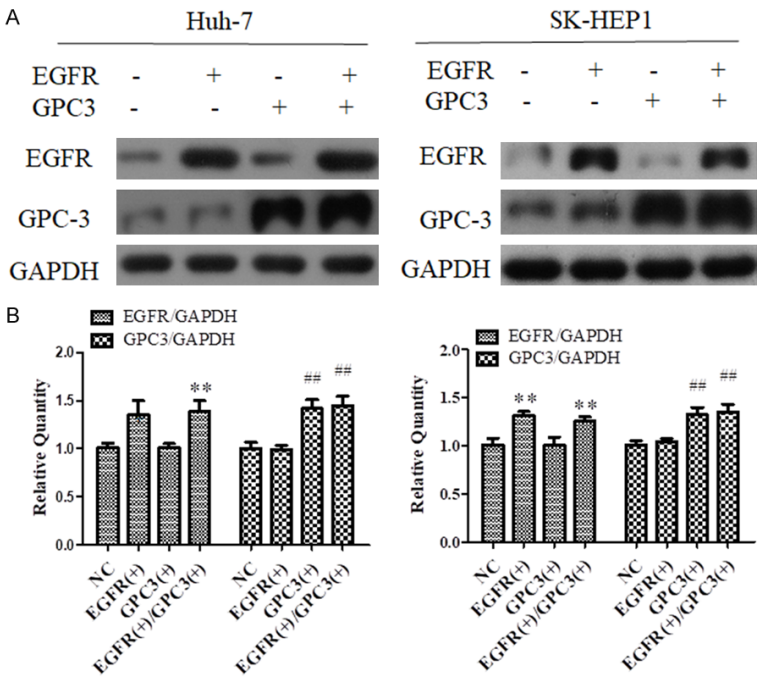

Figure 2. Establishment of GPC3+EGFR+ HCC cell models. (A) Western blot analysis of GPC3 and EGFR in Huh-7 cells after transduction. Lysates of untransduced, GPC3-transduced, EGFR-transduced and GPC3 and EGFR double transduced Huh-7 cells were separated by SDS-PAGE and detected EGFR and GPC3 protein levels. (B) Intensity of EGFR and GPC3 bands, relative to intensity of GAPDH bands, from three independent tests conducted as in (A) Values are represented as means ± s.e.m. of three independent tests conducted in triplicate. Each result was compared with its negative control (NC), $*P < 0.05$, $**P < 0.01$, $\#\#P < 0.01$ Student's *t*-test.

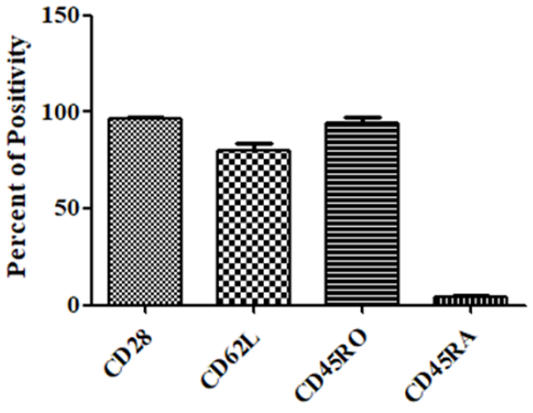

Figure 3. Expression of central memory phenotype in CARgpc3-egfr T. The immunophenotype was determined by flow cytometry, four differentiation markers CD28, CD62L, CD45RO and CD45RA, were detected and tested.

revealed that there was no obvious difference between CARgpc3-egfr T cells on Huh-7 or huh-7-GPC3+/EGFR+ and SK-HEP1 or SK-HEP1-GPC3+/EGFR+ cells (**Figure 5C**, **5D**). Expression of neoplasm cell antigen reached a certain level, and the expression level did not have a great impact on the killing effect of CAR-T cells. CARgpc3 and CATgpc3-egfr T-lymphocytes revealed targeted and specific killing effects on GPC3+EGFR+ HCC cell lines *in vitro*; however, neoplastic cells were not killed with mock and CARegfr T cells. In this study, CARgpc3-egfr T cells are theoretically equivalent to third-generation CAR-T cells, while CARgpc3 T lymphocytes are equivalent to first-generation CAR-T cells; third-generation CAR-T cells should have a better killing effect on neoplasm cells than first-generation CAR-T cells, in theory.

Enhanced cytokine production of dual-targeting T cells

Cytokines are important markers of lymphocyte activation. To confirm that GPC3+EGFR+ neoplasm cells can effectively activate CAR-T cells, we co-incubated CAR-T cells with Huh-7-GPC3+/EGFR+, or SK-HEP1-GPC3+/EGFR+ at a ratio of 1:1, and we examined the level of cytokine production.

Our results revealed that CARgpc3 and CARgpc3-egfr T-lymphocytes promoted higher secretion of IFN-γ, TNF-α, IL-2, IL-4, and IL-10 in GPC3+EGFR+ Huh7 and GPC3+EGFR+ SK-HEP1 cell lines than mock and CARegfr T cells (**Figure 6**). Therefore, EGFR and GPC3 could stimulate secretion of T-lymphocyte factors in CARgpc3 and CARgpc3-egfr T cells. In addition, CARgpc3-egfr T cells could be more robustly activated and play a more critical role in killing neoplasm cells due to the presence of co-stimulatory signals, compared to CARgpc3 cells. As reported, the effect including CTL response, cytokine release, proliferation, and prevention of death could be enhanced by the combination of co-stimulatory signals [25]. Thus, cytokine production is thought to be synergistically enhanced by combining CD28, CD3ζ, and co-stimulatory signals in dual-targeting T cells.

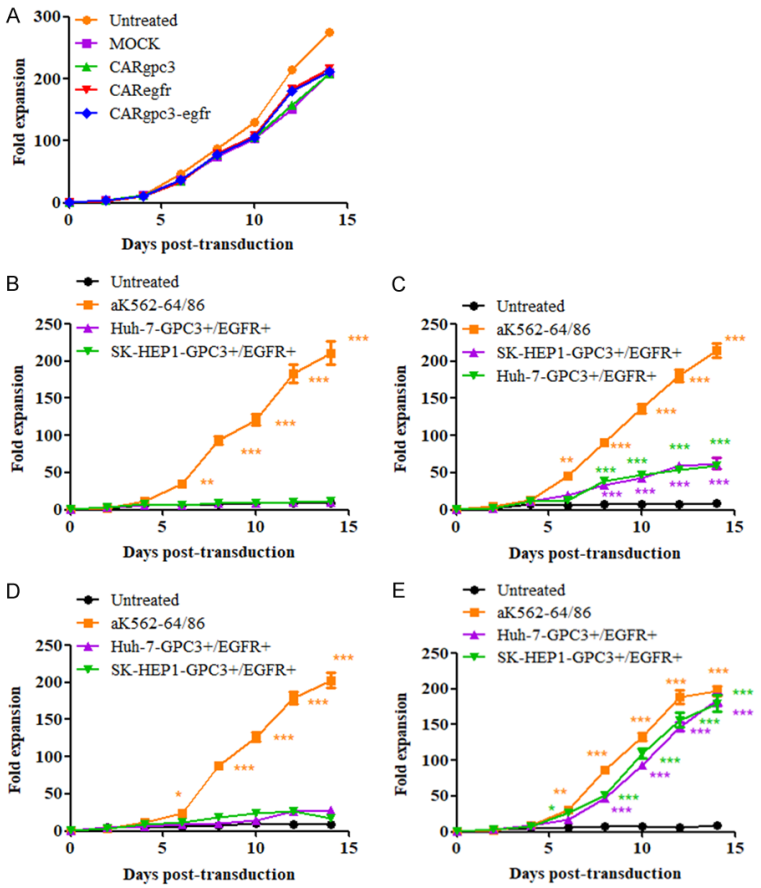

Figure 4. Dual-targeted CART cells exert evident proliferation capacity *in vitro*. (A) 1 × 10^6 mock, CARgpc3, CARegfr and CARgpc3-egfr T cells were activated by αCD3/αCD28 magnetic beads. The viable T cell was counted the next day. (B-E) 1 × 10^6 mock (B), CARgpc3 (C), CARegfr and CARgpc3-egfr T cells (D) were co-cultivated with irradiated mock, GPC3+EGFR+ Huh-7 and SK-HEP1 cell lines with freshly irradiated neoplasm cells stimulated every week. The viable T cell were counted the next day.

Dual-targeting T cells inhibited neoplasm proliferation in vivo

To further explore the antineoplastic roles of dual-targeting CAR-T cells, T lymphocytes expressing mock, CARgpc3, CARegfr, and CARgpc3-egfr mixed with GPC3+EGFR+ Huh-7 cells (Luciferease-overexpression) were inoculated orthotopically into the liver of NOD/SCID mice at 1:1 ratio. Twenty-four hours before inoculation, 200 mg/kg cyclophosphamide was administered intraperitoneally to remove the remaining immune cells. After two weeks of treatment, CARgpc3-egfr T lymphocytes inhibited the growth of GPC3+EGFR+ Huh-7 (LUC) transplanted neoplasm, which was obviously different from saline, mock, CARgpc3, and CARegfr groups (***$P < 0.001$). The inhibition rate of the CARgpc3 group was also better than that in the saline, mock, and CARegfr T groups (***$P < 0.001$). In the CARegfr T cell group, only costimulatory signals were activated, but T cells could not be fully activated; therefore, the inhibition rate of the CARegfr T cell group was lower than that of the CARgpc3 T cell group and slightly higher than that of the saline and mock groups (**Figure 7A**, **7B**).

Even more importantly, each mouse treated with GPC3 and EGFR dual-targeting T cells survived for more than 67 days, while the median survival duration of the mock, CARegfr, and CARgpc3 T cells treatment was 32, 35, and 50 days, respectively (**Figure 7C**).

Previous studies have shown that T cells *in vivo* are closely correlated with neoplasm regression. In this study, after two weeks of adoptive infusion of CAR-T lymphocytes, survival of GFP+ CAR-T cells was examined by estimating the average cell concentration (cells/μL) of T cells in the peripheral blood of mice. The number of CARgpc3-egfr T cells in the experimental group was higher than that in the other three groups (***$P < 0.001$), and the number of CARgpc3 T cells was higher than that in mock and CARegfr T cells (***$P < 0.001$). This study revealed that CARgpc3-egfr T lymphocytes could survive better than single-targeting T cells in mice (**Figure 7D**).

IHC results suggested that dual-targeting CAR-T cells had the highest infiltration rate, compared with other CAR-T cells (**Figure 7E**, **7F**). Thus, neoplasm antigen recognition drove survival of infused T cells *in vivo*. It has been reported that the CD137 signaling domain could enhance Bcl-XL expression and promote the survival of T cells. We also found an increase in Bcl-XL in dual-targeting T cells caused by GPC3 and

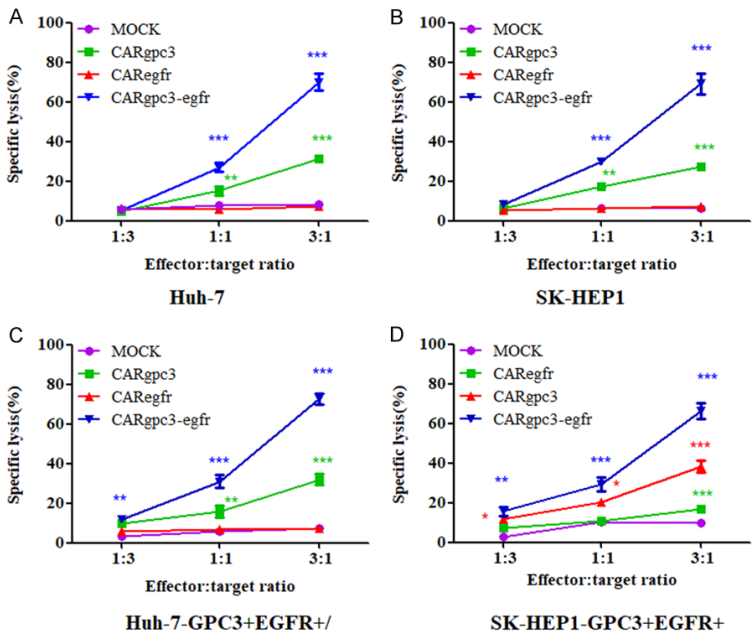

Figure 5. Dual-targeted T cells exert cytotoxicity against GPC3+EGFR+ HCC cells *in vitro*. The cytotoxicity of CAR T cells against GPC3+EGFR+ HCC cells at the ratio of effect: target 1:3, 1:1, and 3:1 for HCC cell lines Huh-7 (A), SK-HEP1 (B), Huh-7- GPC3+/EGFR+ (C) and SK-HEP1-GPC3+/EGFR+ (D), respectively.

EGFR, suggesting that the increase in CAR-T cells may be caused by upregulation of Bcl-XL expression (**Figure 7G**).

Discussion

CAR-T cells targeting varying levels of traditional TAA have been commonly used in experimental models and humans [26]. Although most TAAs can have serious on-target, off-neoplasm side effects, or even fatal toxicity, TAA is not completely limited to neoplasm tissue [27]. Therefore, it is conceivable that on-target, off-neoplasm toxicity could be reduced by dual-targeting CAR-T cells. For dual-targeting CAR-T cells, the first target of the stimulatory signal was enhanced in the neoplasm tissue, but not in the normal tissue, while the second target of the co-stimulatory signal was a tissue-specific protein with a high level of expression in the neoplasm tissue. GPC3 is found in liver carcinoma but is absent in normal liver tissues [20, 28]. EGFR is a type of overexpressed cell surface molecule in many kinds of neoplasms, with a small amount of expression in normal tissues. Therefore, EGFR serves as a co-stimulatory signal to ensure optimal activation of double-targeting T cells in HCC. In HCC, GPC3 and EGFR are highly expressed at the same time. Therefore, GPC3 and EGFR are ideal target combinations for double-targeting CAR-T cells.

In this study, we designed two-step expansion experiments to investigate whether neoplasm cells expressing EGFR and GPC3 can stimulate CAR-T cell-specific expansion. Huh-7-GPC3+/EGFR+ and SK-HEP1-GPC3+/EGFR+ neoplasm cells were incubated with mock, CARgpc3, CARegfr, and CAR-gpc3-egfr T lymphocytes. Our results revealed that the dual-targeting CARgpc3-egfr T lymphocytes had a higher expansion rate than single CARgpc3 T lymphocytes, due to activating EGFR co-stimulatory signals. Four groups of genetically modified T lymphocytes and artificial antigen-presenting cells ak562-64/86 were stimulated with 100 ng/mL OKT3, suggesting obvious expansion. However, there was no obvious expansion in mock and CAREgfr T lymphocytes after co-incubation with neoplasm cells. GPC3+EGFR+ HCC cells were specifically killed by CARgpc3 and CARgpc3-egfr T lymphocytes, but not by mock and CAREgfr T cells. In this study, CARgpc3-egfr T cells are theoretically equivalent to third-generation CAR-T cells, whereas CARgpc3 T cells are equivalent to first-generation CARs, which should theoretically have a better killing effect on neoplasm cells.

Cytokines are important markers of lymphocyte activation. EGFR and GPC3 stimulated secretion of T-lymphocyte factors. The difference between CARgpc3 and CARgpc3-egfr T cells revealed that CARgpc3-egfr T cells could be better activated and kill neoplasm cells because of the presence of co-stimulatory signals. In addition, it has been suggested that cytokine release syndrome may appear in clinical treatment.

Less differentiated T cell subsets are closely related to longer survival time and better therapeutic effects and clinical responses. *In vivo* studies revealed that adoptive transfer of TCM

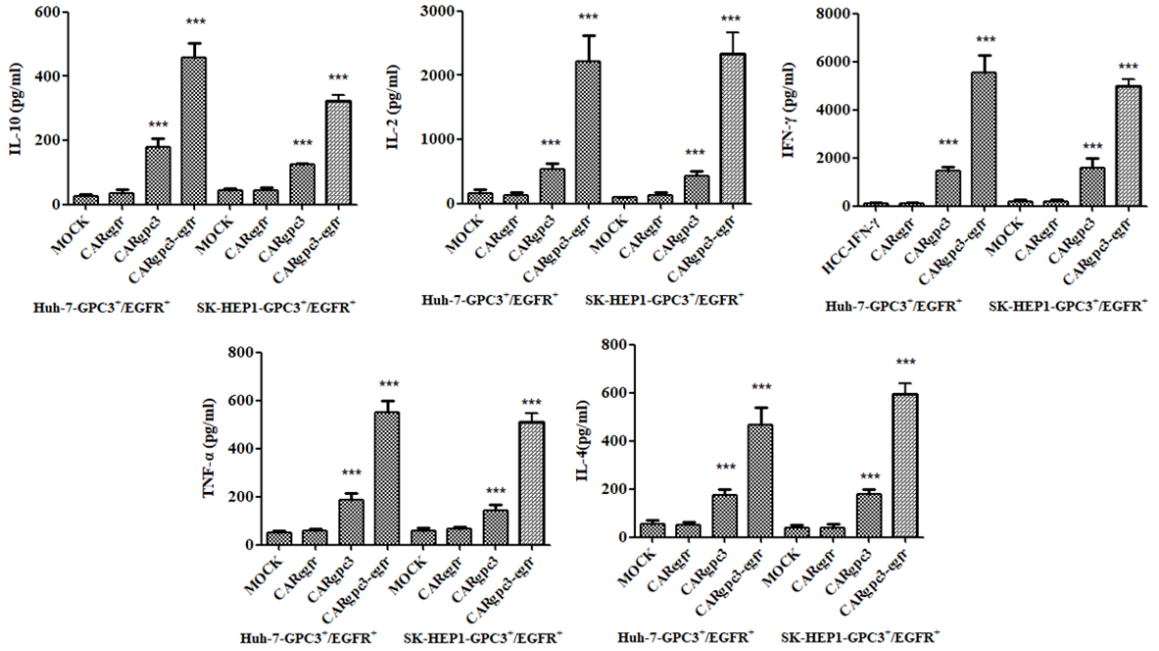

Figure 6. Enhanced cytokines production of dual-targeted T cells. 1 × 10^6 engineered T cells were co-cultivated with equal quantity of indicated engineered Huh-7 and SK-HEP1 cells for 24 h. IFN-γ, TNF-α, IL-2, IL-4 and IL-10 in the supernatants were detected by ELISA.

cells could reduce the number of experimental cells used and improve the complete remission rate of the disease. Therefore, more specialized T cell subsets, such as TCM and TSCM, can effectively improve survival and provide better therapeutic effects *in vivo*. Clinical trials have also been performed to test the potential of T cell subsets from the peripheral blood. We tested four differentiation markers of CARgpc3-egfr T cells, namely, CD28, CD62L, CD45RO, and CD45RA. Fourteen days after T cell transfection, flow cytometry analysis was conducted to explore the surface immunophenotype. The results indicated that most CAR-T cells showed a central memory T cell phenotype.

In order to further confirm that CARgpc3-egfr T lymphocytes can specifically target and kill EGFR and GPC3-positive neoplasm cells *in vivo*, we established a NOD/SCID xenograft model with GPC3+EGFR+ Huh-7 (LUC) neoplasm cells. Our results revealed that CARgpc3-egfr T lymphocytes inhibited the growth of GPC3+EGFR+ Huh-7 transplanted neoplasm volume, and the inhibition rate of the dual-targeting CAR-T cell group was the highest, which was equal to that of third-generation CAR-T cells; additionally, the CARgpc3 T cell group performed better than the mock and CARegfr T cell groups (**P < 0.001). CARegfr T cells included only co-stimulatory signals; hence, T cells could not be fully activated. There was no obvious difference in inhibition rate between CARegfr and mock T cells (**Figure 6A**, **6B**).

Compared with GPC3-28bbz [19] in Huh-7 xenografts, dual-targeting CAR-T cells have poor antineoplastic ability. We speculate that the dual-targeting CAR-T cell structure is not simply to separate the two signal domains; some unknown factors may also affect the ultimate therapeutic effect. A recent report indicated that the structure of scFvs may affect downstream signals and their final antineoplastic activity [29]. In this study, we used the antibody domain of EGFR [30] as the antigen-binding region for the first time, although it has been proven that it is a common single-chain antibody that transduces costimulatory signals by binding to the original. However, according to previous reports, antibody domains tend to aggregate [31]. Therefore, it is not clear whether cross-linking of related receptors and early senescence of T cells would result from the antibody domain of EGFR.

In summary, dual-targeting CAR-T (CARgpc3-egfr) cells exhibited better proliferation ability

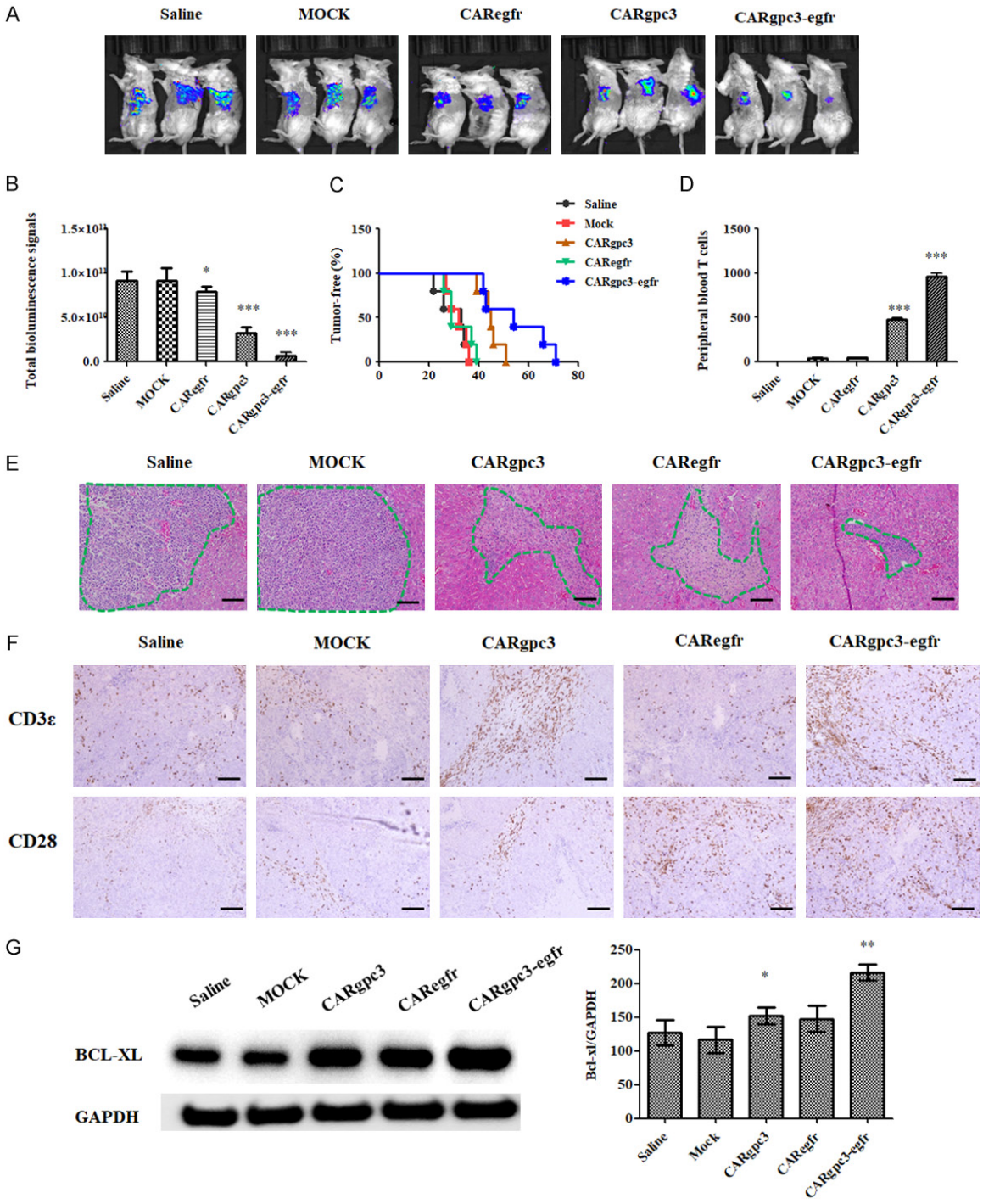

Figure 7. Enhanced neoplasm suppression capacity of dual-targeted T cells *in vivo*. A. NOD/SCID mice bearing GPC3+EGFR+ Huh-7 (Luc) neoplasm were intravenous injected with 5 × 10^6 genetically-modified T cells on the 14th and 21st day, respectively. Neoplasm growth was tested by total bioluminescence signals of image weekly. Compared with control groups, the neoplasm growth treated with EGFR-CD28-BB and GPC-3-zeta T cells was potently suppressed. B. The quantification of bioluminescence signal. C. The overall survival of mice bearing GPC3+EGFR+ Huh-7 (Luc) neoplasm and treated with indicated T cells or saline. D. Survival of CAR T cells in peripheral blood. E. Histology of the neoplasms from mice transplanted with GPC3+EGFR+ Huh-7 (Luc) cells and treated with indicated T cells or saline. Mice were sacrificed in week 7, and paraffin sections of neoplasm were stained by hematoxylin and eosin by immunohistology. Microscope magnification: 40×, the black straight lines stand for 100 μm. F. Dual-targeted CAR T cells could be located in GPC3+ and EGFR+ SK-HEP1 neoplasms. neoplasms were obtained from mice bearing GPC3+EGFR+ Huh-7 (Luc) subcutaneous xenografts treated with indicated T cells or saline. IHC stain-

ing of human CD28 and CD-3-zeta was used for detect T cells infiltration of neoplasms (brown), the black straight lines stand for 100 μm. G. Western blot analysis of anti-apoptotic protein BCL-XL expression in indicated CAR T cells. Lysates of saline CAR T, mock, GPC-3-zeta-transduced, EGFR-CD28-BB-transduced and EGFR-CD28-BB and GPC-3-zeta double transduced T cells were separated by SDS-PAGE to detect BCL-XL expression. The quantitative analysis of band density. *P* values were tested by log-rank test analysis; n.s., not statistically evident, $*P < 0.05$, $**P < 0.01$, $*P < 0.001$. Results indicate three independent experiments.

and cytotoxicity than GPC3-targeting CAR-T (CARgpc3) cells against GPC3+EGFR+ HCC cells *in vitro* and *in vivo*, which may contribute to anti-HCC treatment with lower off-neoplasm toxicities.

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Disclosure of conflict of interest

None.

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