

# EGLIF-CAR-T Cells Secreting PD-1 Blocking Antibodies Significantly Mediate the Elimination of Gastric Cancer

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**Objective:** To investigate the anti-tumor effects of programmed cell death protein 1 (PD-1) scFv-secreting EGFR-chimeric antigen receptor-modified (CAR)-T cells against gastric cancer.

**Methods:** Second-generation EGFR-CAR-T cells and fourth-generation PD-1 scFv-secreting EGFR-CAR-T cells were engineered. The anti-tumor activities of chimeric antigen receptor-modified (CAR)-T cells were analyzed in vitro by long-term co-culture with gastric cancer cells. The tumor scavenging capacity in vivo was evaluated in xenograft and PDX mouse models.

**Results:** EGFR-CAR-T cells secreting PD-1 scFv showed enhanced long-term tumor cell killing capacity in vitro. These cells also showed significant anti-tumor effect in the subcutaneous xenograft model of gastric cancer as well as in the PDX model, and autocrine PD-1 antibody secretion significantly increased tumor infiltration of the CAR-T cells.

**Conclusion:** EGFR-CAR-T cells secreting PD-1 scFv are highly effective against gastric cancer and offer new insights into anti-cancer immunotherapy.

**Keywords:** EGLIF-CAR-T cells, PD-1 blocking antibodies, elimination, gastric cancer

## Introduction

Gastric cancer is the third most common cause of cancer-related deaths worldwide, with approximately one million new cases diagnosed each year.<sup>1,2</sup> Despite significant advances in surgical and chemotherapeutic approaches, the five-year survival rate for patients with advanced gastric cancer is a dismal 5%–20%, while the median overall survival (OS) is only 10 months. Therefore, there is an urgent need for new strategies to extend patient survival and improve prognosis.<sup>3</sup> Chimeric antigen receptor-modified (CAR)-T cells offer an alternative immunotherapeutic strategy. The CAR consists of an extracellular single-chain variable fragment (scFv domain) that targets tumor cells, a short transmembrane domain, and an intracellular co-stimulatory domain comprising of tandemly assembled T cell signaling moieties.<sup>4,5</sup> Preclinical studies have shown that Her-targeted CAR-T cells can retard gastric cancer progression.<sup>6,7</sup>

EGFR is a transmembrane glycoprotein of the ERBB receptor tyrosine kinase family that is overexpressed in many human cancers due to amplification and/or mutation of the EGFR gene, and is closely related to tumor recurrence, angiogenesis and metastasis.<sup>8,9</sup> The extracellular domain of EGFR, which is overexpressed

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on the surface of tumor cells, is an ideal tumor-specific and immunogenic antigen. In fact, the monoclonal antibody against EGFR had shown encouraging results against lung cancer, and head and neck cancer.<sup>8,10,11</sup> Therefore, EGFR is a potential target for adoptive cellular immunotherapy as well.

Although CAR-T cell therapy has been effective against hematological tumors, it has not achieved the same success in solid tumors.<sup>12</sup> This is partially due to the immunosuppressive tumor microenvironment (TME) which blocks CAR-T cell activity by triggering inhibitory signals through the PD-1/PD-L1 pathway.<sup>13,14</sup> PD-1 is an inhibitory receptor that is not expressed on the resting T cells in cancer patients but increases significantly in the functionally depleted T cells.<sup>15</sup> Its ligand PD-L1 is expressed on the surface of antigen presenting cells (APCs) and tumor cells, and is significantly up-regulated upon stimulation by local inflammatory cytokines such as type I and type II interferons. The interaction of PD-L1 and PD-1 leads to the apoptosis and functional failure of T cells.<sup>16</sup> Therefore, blocking this immune checkpoint can partially restore T cell function and improve tumor cell clearance.<sup>17</sup> The anti-PD-1 monoclonal antibodies pembrolizumab and nivolumab were approved by the Food and Drug Administration (FDA) in 2014, and have achieved satisfactory results in melanoma, non-small cell lung cancer (NSCLC), renal cell cancer (RCC) and other malignant tumors.<sup>18–20</sup> However, most patients do not respond optimally to the immune checkpoint blockade (ICB), and some are completely unresponsive. Therefore, a better understanding of the pathways and molecular mechanisms that block the PD-1/PD-L1 pathway is needed. Studies on animal models and several clinical trials have shown that ICB therapy affects the tumor infiltrating lymphocytes (TILs) and other immune cells in the tumor microenvironment.<sup>21,22</sup> Based on these findings, we hypothesized that CAR-T cells producing anti-PD-1 antibodies will be resistant to immunosuppression and show enhanced toxicity against solid tumors. To this end, we engineered fourth generation EGFR-CAR-T cells secreting PD-1 scFv for ICB therapy against EGFR-overexpressing gastric cancer cells, and analyzed its anti-tumor efficacy.

## Materials and Methods

### Cell Lines and Culture Conditions

Fresh blood samples were collected from six healthy volunteers, and peripheral blood mononuclear cells

(PBMCs) were isolated by gradient centrifugation using Lymphoprep<sup>TM</sup> (Axis-Shield, Norseland). The CD3+ T cells were enriched by magnetic separation (Miltenyi Biotec Inc, Auburn, CA, USA), and cultured in X-VIVO15 medium (Lonza, Switzerland) supplemented with 5% human AB serum (Valley Biomedical Inc, Winchester, VA, USA.), 10 mM N-acetyl L-cysteine (Sigma Aldrich, St. Louis, MO, USA) and 300 U/mL human IL-2 (PeproTech, Rocky Hill, CT, USA). The gastric cancer cell lines MKN28, MGC-803, MKN7 and MGC-27 were obtained from the American Type Culture Collection (ATCC). MKN28 and MKN7 cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA), and the MGC-803 and MGC-27 cells in Dulbecco's modified Eagle medium (DMEM) (Hyclone), both supplemented with 10% fetal calf serum, 2 mM L-glutamine (Gibco, Gaithersburg, MD, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Sangong Biotech, Shanghai, China).

### Patient Tissue Samples

32 tumor tissues and three normal gastric tissue specimens were obtained from 32 gastric cancer patients, of which 3 normal tissues originated from 3 of 32 tumor patients, processed as per standard protocols and subjected to hematoxylin and eosin staining. The collection of human tissue samples was approved by the Seventh Affiliated Hospital of Xinjiang Medical University and all patients and healthy volunteers provided informed consent.

### Lentiviral Transduction of T Cells

Primary CD3+ T cells were activated with human CD3/CD28 beads (Invitrogen, Carlsbad, CA, USA) at the ratio of 2:1 (magnetic beads: cells) in T cell culture medium. Forty-eight hours later, the activated cells were transduced with the lentivirus at a MOI of 8 with 7 µg/mL polybrene (Yeasen Biotech, China), centrifuged at 1200 g for 60 minutes, and incubated overnight at 37°C with 5% CO<sub>2</sub>. After 5 days, the T cells were harvested and the percentage of transduced CAR-T cells was detected by flow cytometry.

### Flow Cytometry

The harvested cells were centrifuged, washed thrice with FACS wash buffer (1×PBS containing 0.5% BSA and 0.03% sodium azide), and stained with anti-EGFR-PE (BD, San Jose, CA, USA), anti-Fab-FITC (eBioscience, CA, USA), anti-CD3-FITC (eBioscience, CA, USA), anti-CD4-PE (BD, CA, USA), anti-CD8-APC (eBioscience,

CA, USA) and anti-PD-1-PB450 (eBioscience, CA, USA) antibodies as appropriate. The intracellular IFN- $\gamma$  in T cells was also stained using anti-IFN- $\gamma$ -FITC antibody (BD, CA, USA). In addition, the peripheral blood of the tumor-bearing mice was stained with the CD3-PerCP/CD4-FITC/CD8-PE TruCOUNT kit (BD Bioscience), and the CD4+ and CD8+ T cells were quantified according to the manufacturer's instructions.

## Enzyme-Linked Immunosorbent Assay (ELISA)

The effector and target cells were mixed at the ratio of 3:1 in a U-bottomed 96-well plate, and co-cultured for 24 h. The cells were then centrifuged, and the amount of IL-2, IFN- $\gamma$  and TNF- $\alpha$  in the supernatants were analyzed using specific ELISA kits according to the manufacturer's instructions (MultiSciences, China).

## Co-Culture of T Cells and Tumor Cells

MGC-803 cells were seeded in 6-well tissue culture plates at the density of  $1 \times 10^6$  cells/well and cultured for 4 days in complete medium. The CAR-T cells were then added at the effector/target ratio of 1:40, and the co-culture was maintained for 28 days. The T cells were harvested every 7 days and quantified by flow cytometry (CD3 positive) and manual counting. Prior to the third stimulation, the CAR-T cells were stained with Cell Trace Dilution (Invitrogen) and then co-cultured for 96 hours at the effector/target ratio of 3:1. The intensity of the dye was measured by flow cytometry.

## Establishment of Xenograft Model and Living Imaging

Five to seven-weeks-old female NOD-SCID IL-2 receptor gamma null (NSG) mice were purchased from Shanghai Runnuo Biotechnology Co. Ltd. (China), and housed at the Experimental Animal Research Center of the Seventh Affiliated Hospital of Xinjiang Medical University. The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animal published by National Academic Research Council of America. All animal experiments were approved by the Animal Care and Use Committee of the Seventh Affiliated Hospital of Xinjiang Medical University. A xenograft model was established by subcutaneously injecting the mice with MGC-803-Luc cells mixed with Matrigel. Seven days after inoculation, the

mice were intravenously injected with PBS,  $1 \times 10^7$  EGFR BB-z or  $1 \times 10^7$  EGFR BB-z/E30 (n=4). Bioluminescence was measured using the Xenogen IVIS Spectrum System (Life Technologies, USA). The tumors were measured every 7 days, and the mice were euthanized when they appeared morbid. For the PDX model, once the tumors grew to 100 mm<sup>3</sup>, the mice were randomized into control or treatment groups (n=5) and the latter were injected with  $1 \times 10^7$  effector cells via their tail vein. The tumor volume was measured every 3 days. The mice were euthanized if they lost more than 20% of their initial weight or appeared morbid. On the 20th day after tumor cell inoculation, 200  $\mu$ L blood was collected and the number of CD8+ and CD4+ T cells were quantified as described.

## Immunofluorescence Assay

The infiltration of human CAR-T cells in the tumors was assessed by immunostaining cryosections of tumor tissues with anti-CD3, anti-EGFR, and anti-EGFR antibodies (1:150, Thermo Scientific) as per the manufacturer's instructions.

## Statistical Analysis

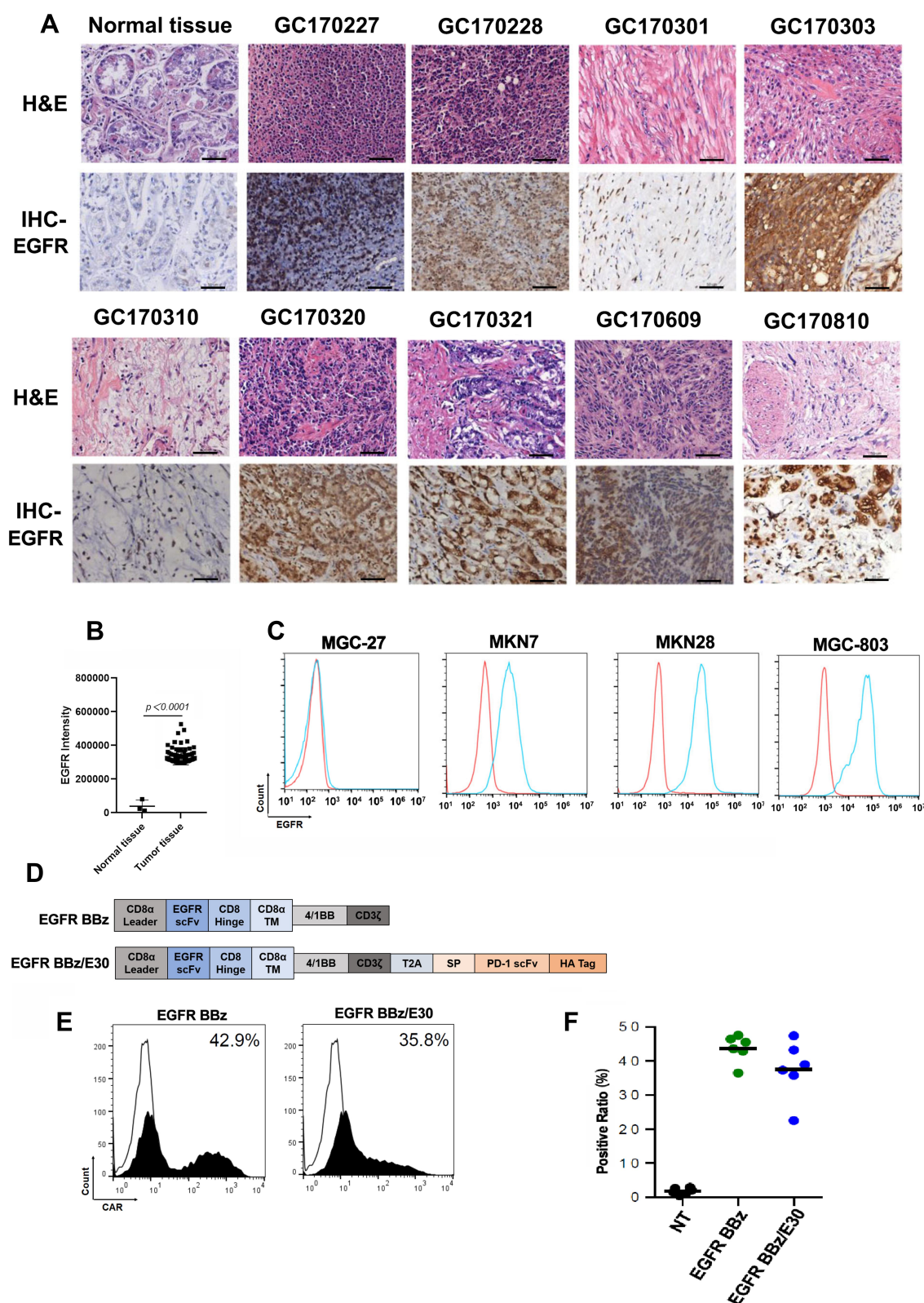
Statistical analysis was performed using GraphPad Prism 6.0. The groups were compared by Student's *t*-test and *p* < 0.05 was considered statistically significant.

## Results

### Generation of EGFR-Targeting CAR-T Cells

EGFR was highly expressed in 75% of the human gastric cancer tissues (24/32) compared to 3 of the normal tissues (Figure 1A and B). Consistent with this, EGFR was also significantly upregulated in the MKN28, MGC-803 and MKN7 gastric cancer cell lines, while MGC-27 was EGFR-negative (Figure 1C). Subsequently, a second-generation CAR sequence targeting EGFR (EGFR BB-z) was constructed, followed by EGFR BB-z/E30 which incorporated the PD-1 scFv sequence as well (clone 332.8H3).<sup>23</sup> The CD3 $\zeta$  and 4-1BB sequences were included in each construct as the T cell activation and costimulatory signals respectively (Figure 1D). The transduced cells were probed using the anti-Fab antibody in order to detect the EGFR BB-z and EGFR BB-z/E30 sequences (Figure 1E), and around 40% CAR-T cells were stably transduced (Figure 1F).





**Figure 1** Generation of EGFR-targeting effector T cells. **(A)** Representative images of HE and immuno-stained normal gastric tissues and tumor tissues. **(B)** Quantitative analysis of immunohistochemical pictures. **(C)** Flow cytometry plots showing EGFR expression levels in different gastric cancer cell lines. **(D)** Schematic representation of EGFR BB-z and EGFR BB-z/E30 sequences. **(E)** Flow cytometry plots of T cells showing transfection of EGFR BB-z and EGFR BB-z/E30. **(F)** Percentage of T cells stably expressing EGFR BB-z and EGFR BB-z/E30 (n=6).

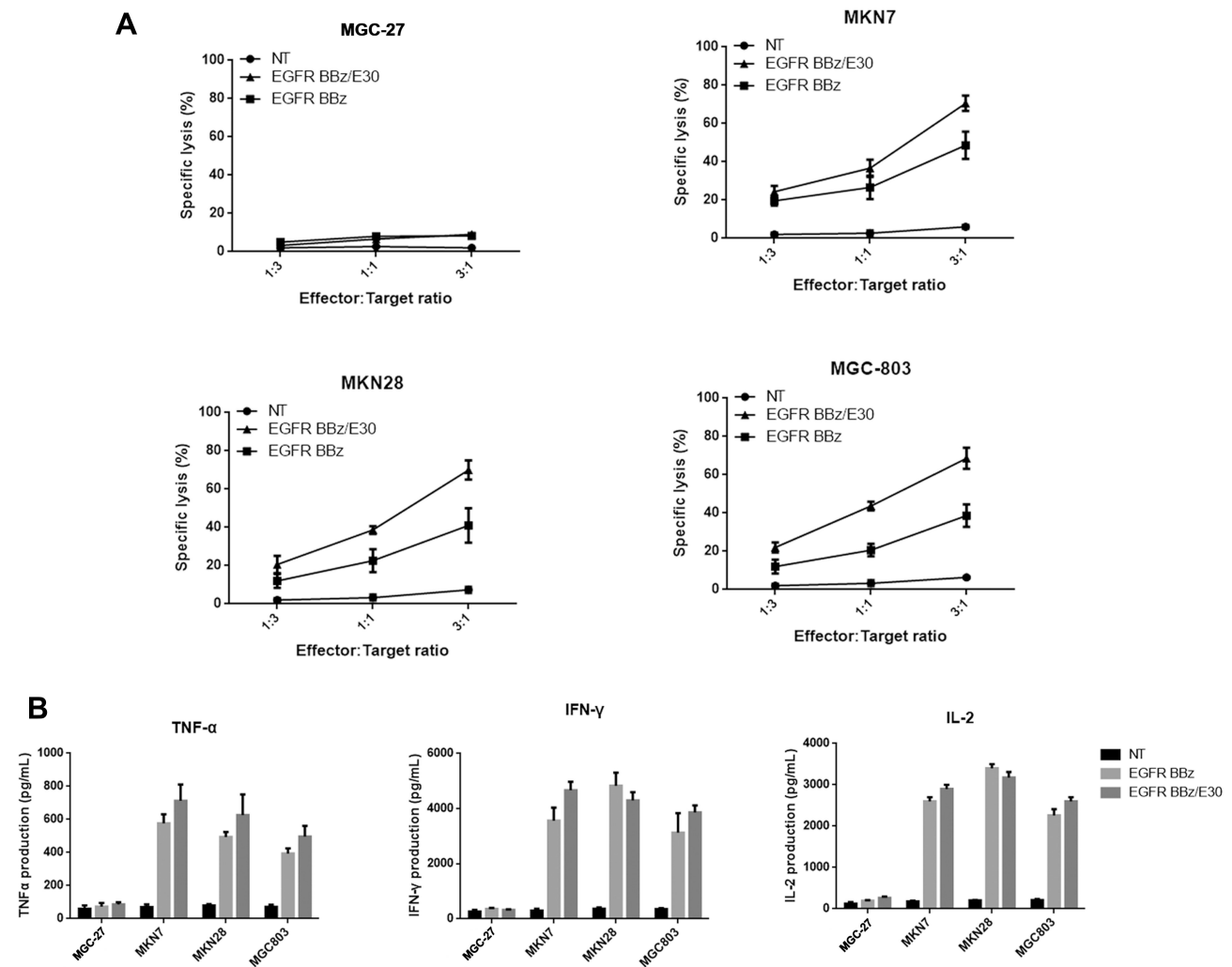
## EGFR-CAR-T Cells Show Antigen-Specific Activation in vitro

The different gastric cancer cell lines were incubated with the EGFR-CAR-T cells at the effector/target cell ratios of 3:1, 1:1, and 1:3. As indicated by the secreted LDH levels, both EGFR BB-z and EGFR BB-z/E30 cells showed potent dose-dependent cytotoxicity against the EGFR<sup>+</sup> MKN28, MGC-803 and MKN7 cells, while the EGFR<sup>-</sup> MGC-27 cells were unaffected (Figure 2A). In addition, the CAR-T cells secreted significantly higher levels of IL-2, IFN- $\gamma$  and TNF- $\alpha$  compared to the un-transfected T cells in the presence of the EGFR<sup>+</sup> tumor cells, while the EGFR<sup>-</sup> MGC-27 cells did not increase the basal secretion levels of these cytokines (Figure 2B). Furthermore, there was no significant difference in the cytotoxicity and cytokine secretion levels of the EGFR BB-z and EGFR

BB-z/E30 CAR-T cells during the co-culture. Taken together, the EGFR BB-z and EGFR BB-z/E30-CAR-T cells were selectively toxic against the EGFR<sup>+</sup> tumor cells, and secreted high levels of cytokines upon antigen stimulation.

## PD-1 scFv Secretion Maintains the Functional Potency of EGFR-CAR-T Cells After Long-Term Stimulation in vitro

To determine the effect of the secreted PD-1 scFv on EGFR-CAR-T cell activity after long-term antigen stimulation, the effector cells were co-cultured with MGC-803 for 28 days. The total number of EGFR BB-z/E30 CAR-T cells were significantly higher compared to the EGFR BB-z cells after long-term stimulation (Figure 3A), which was also confirmed by the higher proliferation rates in the former (Figure 3B).

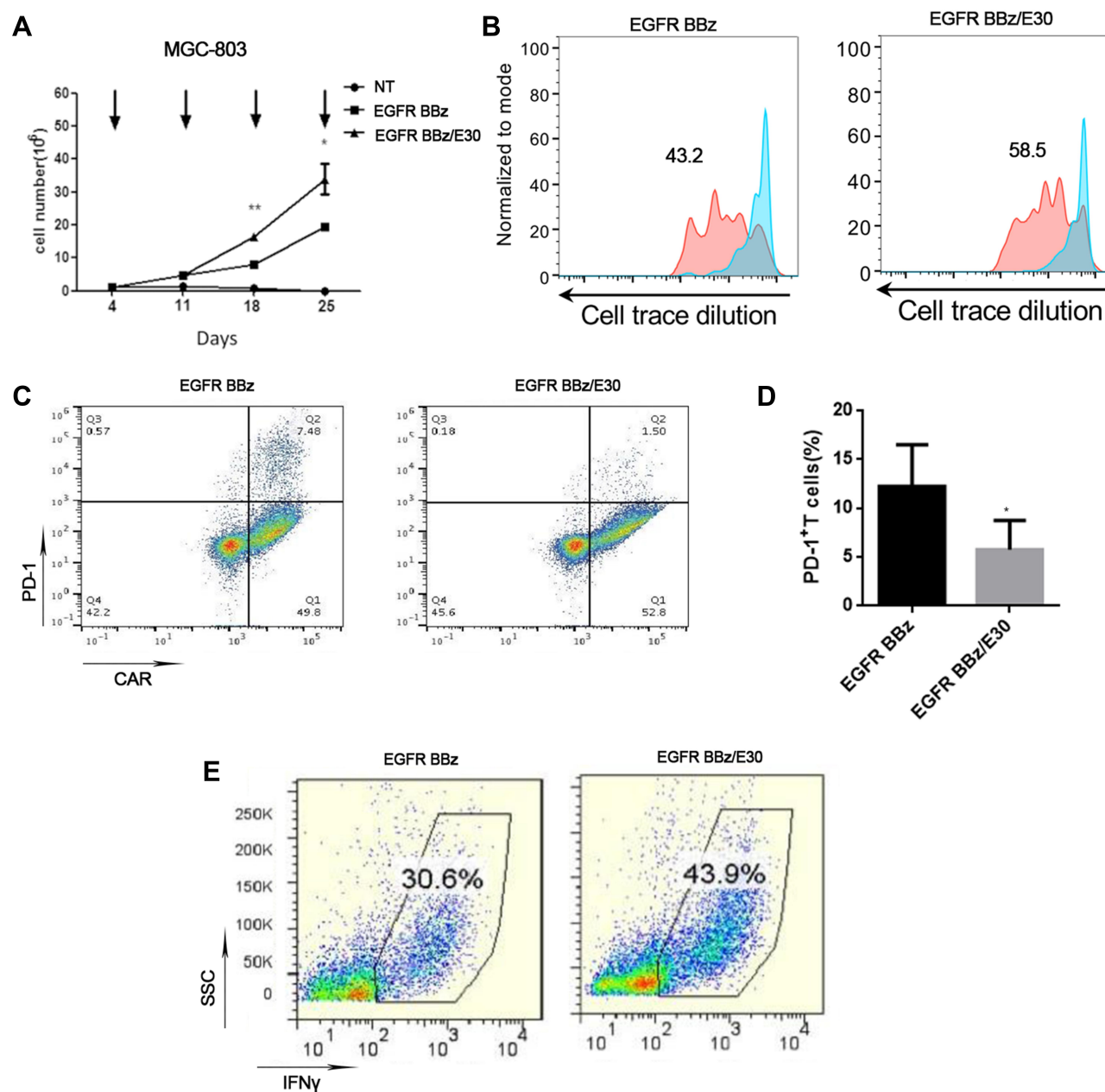


**Figure 2** EGFR-CAR-T cells release cytokines and kill tumor cells in vitro upon antigenic stimulation. **(A)** Secreted LDH levels following co-incubation of effector cells and target cells at the ratios of 3:1, 1:1 and 1:3 (n = 3). **(B)** Secreted levels of IFN- $\gamma$ , IL-2 and TNF- $\alpha$  from effector cells cultured with target cells at the ratio of 3:1 (n = 3).

Furthermore, the PD-1 expression levels were significantly lower on EGFR BB-z/E30 cells compared to EGFR BB-z cells during the long-term stimulation (Figure 3C and D), while the proportion of IFN $\gamma$ -secreting cells was significantly higher in the former (Figure 3E). In conclusion, secretion of PD-1 scFv maintains the proliferative capacity and anti-tumor function of EGFR-CAR-T cells following long-term antigenic stimulation, which can translate to better therapeutic effects.

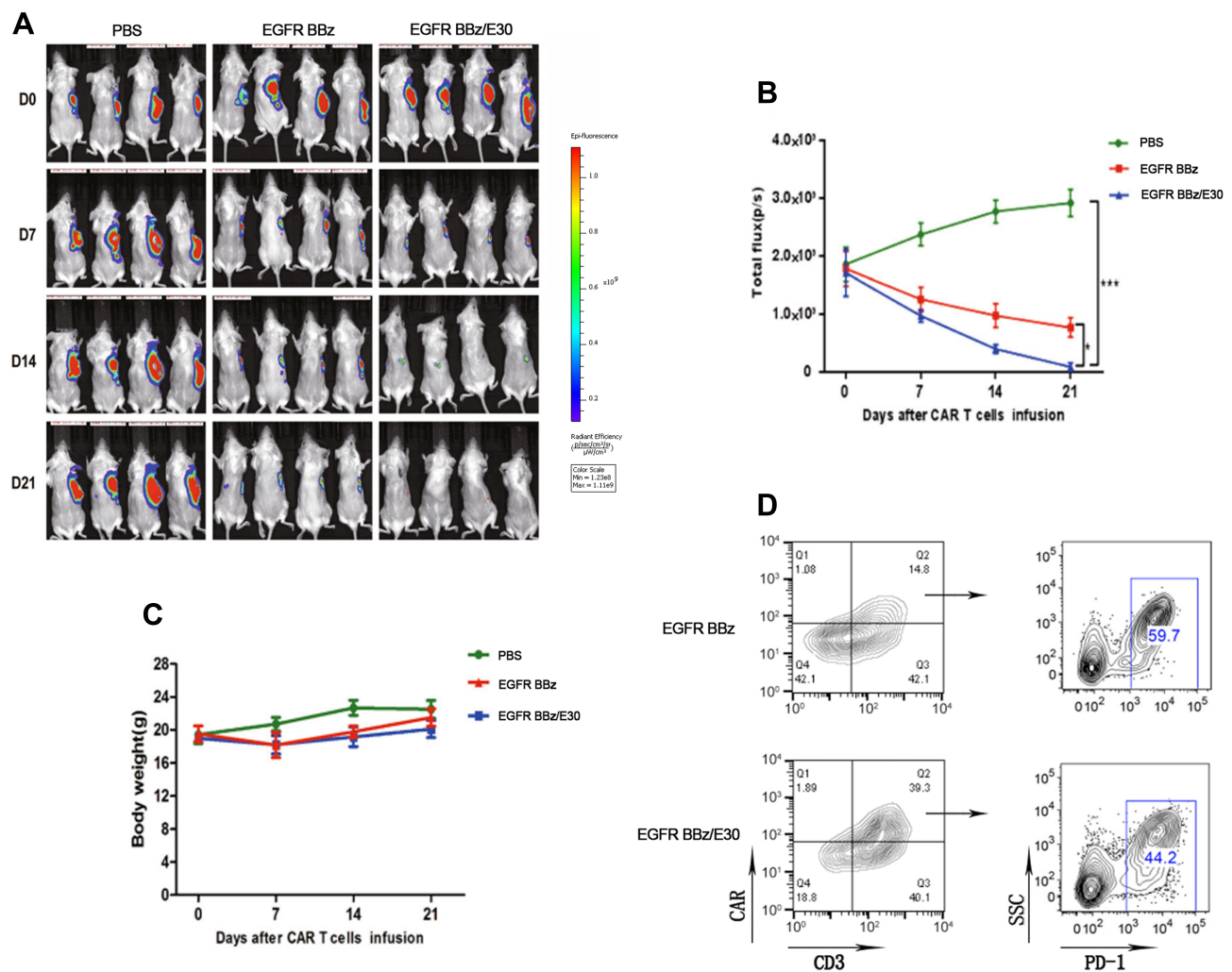
## PD-1 scFv Enhanced the Anti-Tumor Effect of EGFR-CAR-T Cells in a Heterologous Tumor Model

The anti-tumor activity of EGFR BB-z and EGFR BB-z/E30-CAR-T cells was analyzed in NOD/SCID mice bearing subcutaneous MGC-803-luc xenograft tumors, and tumor growth inhibition was tracked through in vivo fluorescence imaging. As shown in Figure 4A, both EGFR BB-z and EGFR BB-z/



**Figure 3** PD-1 antibody production increases the proliferative capacity and viability of T cells after long-term stimulation in vitro. **(A)** Effector-target cell co-culture for 28 days with the arrow indicating the time point of adding fresh tumor cells. Number of viable T cells counted on a weekly basis ( $n = 3$ ,  $*p < 0.05$ ,  $**p < 0.01$ ). **(B)** Cell Trace Dilution staining indicating the long-term proliferation of T cells in the co-culture system. **(C)** Flow cytometry plots showing PD-1 expression levels on the surface of EGFR BB-z and EGFR BB-z/E30-CAR-T cells after 28 days of co-culture. **(D)** Bar graph showing quantification of PD-1 levels ( $n = 3$ ,  $*p < 0.05$ ). **(E)** Flow cytometry plots showing the expression of IFN- $\gamma$  in EGFR BB-z and EGFR BB-z/E30-CAR-T cells.





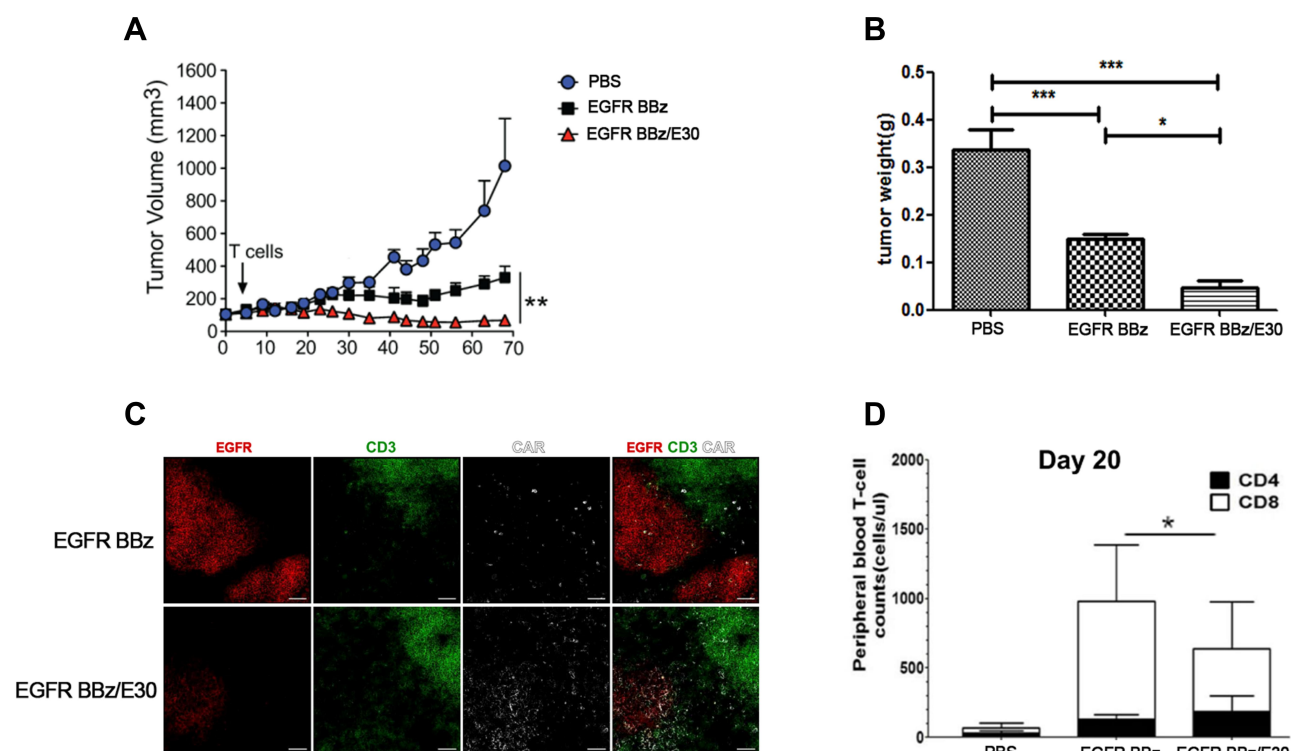
**Figure 4** EGFR-CAR T cells inhibit tumor growth in a xenograft model. **(A)** Representative fluorescence images showing the MGC-803-Luc allogeneic tumor cells after treatment. **(B)** Fluorescence intensity changes in the tumor-bearing mice after treatment ( $n=4$ ,  $*p < 0.05$ ,  $***p < 0.001$ ). **(C)** Changes in bodyweight of tumor-bearing mice. **(D)** Flow cytometry plots showing presence of EGFR BB-z and EGFR BB-z/E30-CAR T cells in tumor samples, and the surface PD-1 expression levels. Each set of data represents three independent experiments.

E30-CAR-T cells inhibited tumor growth, although the latter showed greater efficacy (Figure 4B). In addition, more EGFR BB-z/E30-CAR-T cells infiltrated the tumor tissues compared to the EGFR BB-z cells, and the surface expression of PD-1 was significantly lower on the infiltrating EGFR BB-z/E30 cells (Figure 4D). In terms of biocompatibility, no significant changes were observed in the bodyweight of the mice throughout the experiment (Figure 4C). Taken together, EGFR BB-z/E30-CAR-T cells are highly potent against gastric tumors without any obvious side effects.

### PD-1 scFv Enhanced the Anti-Tumor Activity of EGFR-CAR-T Cells in a PDX Model

We next established a gastric cancer PDX model, and injected the tumor-bearing mice with PBS, EGFR BB-z

or EGFR BB-z/E30-CAR-T cells. While both the engineered CAR T cells inhibited tumor growth, the EGFR BB-z/E30 cells showed greater tumor clearance and a prolonged inhibitory effect compared to the EGFR BB-z cells (Figure 5A). Consistent with this, the tumors were significantly smaller in the EGFR BB-z/E30 versus the EGFR BB-z-CAR-T cells-treated mice (Figure 5B). The EGFR BB-z/E30-CAR T cells also infiltrated to a greater extent in the tumors, which corresponded to significantly lower fewer EGFR<sup>+</sup> tumor cells compared to that in the EGFR BB-z group (Figure 5C). However, 20 days after reintroduction of effector cells, the number of circulating EGFR BB-z/E30 cells was less than that of EGFR BB-z cells (Figure 5D). This could be due to the inherent differences between both cell types in terms of proliferative capacity. In conclusion, EGFR BB-z/E30-CAR T cells



**Figure 5** EGFR-CAR T cells inhibit tumor growth in a PDX model. **(A)** Time-dependent change in tumor volume in the differentially treated groups ( $n=5$ ,  $**p < 0.01$ ). **(B)** Tumor weight in the different groups at the end of the treatment regimen ( $n=5$ ,  $*p < 0.05$ ,  $***p < 0.001$ ). **(C)** Representative immunofluorescence images showing infiltration of EGFR BB-z and EGFR BB-z/E30-CAR T cells in tumor tissues. Each set of data represents three independent experiments. **(D)** Number of circulating CD4 + and CD8 + T cells on day 20 of treatment ( $n = 5$ ,  $*p < 0.05$ ).

effectively inhibited the growth of PDX gastric tumors by targeting the EGFR+ cells.

## Discussion

CAR-T cell therapy has achieved excellent clinical outcomes in hematological malignancies and is currently being developed for the treatment of solid tumors as well.<sup>12</sup> Since gastric tumor tissues overexpress multiple tumor-specific antigens, including EGFR, Her2, MSLN etc., CAR-T cells are a highly promising option for treating this malignancy. However, CAR-T cells are often ineffective against solid tumors due to the immunosuppressive microenvironment.<sup>24–26</sup> Immune checkpoint blockers, especially antibodies targeting PD-1 and PD-L1, can overcome the inhibition of CAR-T cells and significantly increase their efficacy against solid tumors.<sup>27</sup> Although the combination of anti-PD-1 antibody and CAR-T cells has shown encouraging results in some clinical trials, systemic administration of the antibody is associated with adverse effects.<sup>28</sup> In addition, the high cost of the combination therapy limits its widespread use. An alternative strategy is to engineer CAR-T cells that release PD-1 antibody in an autocrine

manner. To this end, we constructed a second-generation EGFR BB-z-CAR sequence targeting the EGFR-overexpressing gastric cancer cells, and then incorporated the PD-1 scFv sequence resulting in fourth-generation EGFR BB-z/E30-CAR T cells that secrete the variable fragments of anti-PD-1 antibody. While both CAR-T cells displayed cytotoxic effects against the gastric cancer cells in an antigen-dependent manner, autocrine PD-1 antibody secretion significantly augmented the tumor cell killing activity of the EGFR BB-z-CAR-T cells after long-term stimulation. In the xenograft tumor model as well, the EGFR BB-z/E30 cells achieved better clearance of the EGFR+ tumor cells compared to the EGFR BB-z cells. It is worth noting that when evaluating the antitumor activity of EGFR BB-z/E30 in the PDX model, EGFR BB-z and EGFR BB-z/E30 can still be detected in peripheral blood on the 20th day. The number of EGFR BB-z/E30 is lower than that of EGFR BB-z, but the tumor infiltration of EGFR BB-z/E30 is much higher than that of EGFR BB-z. It shows that after CAR-T cell therapy, only T cells that fully infiltrate the tumor tissue can play a good anti-tumor effect.



## Conclusion

These PD-1 scFv-secreting CAR-T cells provide a novel immunotherapeutic strategy against EGFR+ gastric cancer, and can be adapted to target other tumor-specific antigens like MSLN and Her2 as well.<sup>29</sup> However, since CAR-T cells targeting a single antigen may have off-target effects, we will consider adopting the dual-receptor CAR-T cell strategy.<sup>30,31</sup> In addition, we will also improve the efficacy of transfecting large fragment genes encoding both dual-receptor CAR and PD-1 antibody by electroporation, so as to increase its clinical potential.

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## Disclosure

The authors declare that they have no conflict of interests for this work.

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