

Original Article

Bispecific tumor-penetrating protein anti-EGFR-iRGD efficiently enhances the infiltration of lymphocytes in gastric cancer

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Abstract: Efficient trafficking of lymphocytes to the tumor microenvironment is crucial for the success of an effective antitumor immunotherapy. A major challenge to achieve effective adoptive immunotherapy is poor tumor penetration and inefficient migration of T cells to the tumor site. Several approaches to facilitate the trafficking of lymphocytes to the tumor microenvironment have been suggested to overcome these obstacles. Here, we address this issue with a focus on the tumor-penetrating peptide iRGD, which can specifically increase the permeability of the tumor vasculature and tumor tissue, enhancing drug penetration. We previously constructed a bispecific tumor-penetrating protein, anti-EGFR-iRGD, which consists of the variable region of the heavy chain of anti-EGFR antibody and a tumor-penetrating peptide iRGD, and verified its ability to improve the penetration of antitumor drugs. Herein, we introduce a novel method of co-administering T cells and anti-EGFR-iRGD to enhance the trafficking, penetration and antitumoral activity of T cells. Our results provide new insights for effectively enhancing T-cell infiltration in tumors and demonstrate a preclinical translational approach for the use of anti-EGFR-iRGD as a therapeutic modifier of cancer immunotherapy to improve clinical outcomes.

Keywords: T-cell infiltration, recombinant protein, anti-EGFR single domain antibody, iRGD, gastric cancer

Introduction

Gastric cancer (GC) is the fourth most prevalent cancer and the second leading cause of cancer-related deaths throughout the world [1]. The early signs of GC are not apparent; symptoms often appear late, and tumors are prone to metastasis with a poor prognosis. Although significant progress has been made in recent years, surgery, chemotherapy, radiation and targeted therapies only prolong the overall survival of GC patients by a few months. Thus, it is important to develop alternative treatment methods. Adoptive cell therapy (ACT) as a promising therapeutic approach has recently resulted in dramatic clinical responses to different cancer types including GC [2, 3].

The presence of tumor-infiltrating lymphocytes (TIL) has been reported to correlate well with

positive clinical outcomes in many types of cancer [4, 5]. One of the most important rate-limiting steps in ACT is the inefficient migration of T cells to tumors. In addition, solid tumors are generally characterized by increased interstitial fluid pressure and dense tumor stroma, leading to the poor homing of tumor-specific T cells to the tumor site. To overcome the poor penetration of T cells into tumor sites, several new strategies have been tested in mouse models and clinical settings. For example, chimeric-antigen receptor (CAR) T cells bearing CCR2, a functional chemokine receptor, can overcome the inadequate tumor localization that limits conventional CAR targeting strategies [6]. Achieving a sufficient number of tumor-infiltrating T cells is critical to the success of eliciting antitumor immune responses. Thus, exploring methods to improve T-cell infiltration into tu-

mors holds significant value for the development of future cancer immunotherapy.

Here, we focus on the peptide iRGD containing the RGD sequence, which has been demonstrated to improve tumor-targeting and cell penetration abilities [7, 8]. Epidermal growth factor receptors (EGFRs) are overexpressed in a wide variety of human cancers and cancer cell types, including GC cells, and expressed in 27.4% of 511 GC tissues. Furthermore, abnormal EGFR expression levels in GC influence tumor growth and survival in malignant states [9, 10]. Our group previously constructed a bispecific highly permeable recombinant protein named anti-EGFR-iRGD based on anti-EGFR single-domain antibody and the tumor-penetrating peptide iRGD. The protein can target both the EGFR extracellular domain and integrin $\alpha_v\beta_3/\beta_5$, which are highly expressed in tumor tissues [11]. Moreover, the anti-EGFR-iRGD fusion protein has been shown to improve the penetration of drugs (from 0.6 kDa to 100 nm) into the deep zone of GC 3D multicellular spheroids, enhancing the efficacy of the co-delivery of drugs [12].

Based on these findings, we hypothesized that the co-administration of T cells with anti-EGFR-iRGD could alter the tumor immune microenvironment, increasing the vascular extravasation and effective trafficking of lymphocytes into deep tumor regions. However, there is currently no clear evidence of the benefit of T cells co-administered with anti-EGFR-iRGD. The present study aimed to determine whether the combination of ACT and the highly permeable protein anti-EGFR-iRGD could enhance the efficacy of cancer immunotherapy.

Materials and methods

Ethics statement

All experimental methods were carried out in accordance with approved manuals. The blood collection procedure was developed in accordance with the guidelines verified and approved by the Ethics Committee of Drum Tower Hospital. All donors signed an informed consent for samples to be used for scientific research.

Reagents and cell lines

Recombinant protein anti-EGFR-iRGD had a verified history and was prepared as reported

previously [8]. Human gastric adenocarcinoma cell lines BGC-823, MGC-803 and MKN45 were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology. BGC-823 cells, MGC-803 cells and MKN45 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C and 5% CO₂ (Invitrogen, Grand Island, NY, USA). All animal experiments were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Cell viability

The in vitro cytotoxicity of recombinant protein anti-EGFR-iRGD was evaluated by MTT assays in both lymphocytes and tumor cells. After cells were seeded into a 96-well plate at a density of 5000 cells per well, anti-EGFR at indicated concentrations was added to each well. One row of a 96-well plate served as a control into which only 100 μ l culture medium was added. Cells were incubated for 6 h, 12 h, 24 h or 48 h, and 200 μ L MTT (0.5 mg/mL) was added to each well. After incubation for another 4 h, MTT was replaced by 100 μ l dimethylsulfoxide under constant shaking at 100 rpm in the dark. The absorbance at 490 nm was measured by using a Varioskan flash multimode plate reader (Thermo, NH, USA). Cell viability was calculated according to the absorbance values.

CFSE-based cytotoxicity assay

The lymphocytic antitumoral activity was assessed by lytic activities using carboxy fluorescein succinimidyl ester (CFSE)/propidium iodide (PI) labeling cytotoxicity assays to measure the cytotoxic reactivity at the end of cell expansion. After 6 h, PI was added, and the relative fraction of double-positive cells of the CFSE-labeled tumor cell population was analyzed by fluorescence-activated cell sorting (FACS). Target tumor cells were labeled with 5 mM CFSE (Invitrogen) at 10⁷/ml for 15 min at 37°C in PBS. Labeling was ended by adding a 10-fold volume of sterile PBS and extensively washing twice with PBS before seeding into 24-well plates and resuspending in RPMI-1640 complete medium. T cells were prepared in fresh medium and cultured with 1×10⁵ target cells for 6 h at different effector cell: target cell (E:T) ratios (40:1, 20:1, 10:1). PI (Sigma) was added to determine the ratio of tumor cell death. The toxicity of anti-EGFR-iRGD to lym-

phocytes was tested using the same method. Anti-EGFR-iRGD at the indicated concentrations was cultured with 1×10^7 lymphocytes for 6 h, 12 h, 24 h or 48 h, and finally, PI was added to determine the percentage of dead cells. Samples were analyzed by flow cytometry.

Flow cytometric analysis

The expression of cell surface molecules was determined by flow cytometry using multicolor standard methodology. FACS Aria (BD Bioscience) was used for fluorescence expression analysis. Cells were stained with the following mouse anti-human antibody labeled by fluorescence for 30 min in 4°C in the dark: CD3-PerCP-Cy5.5 (OKT-3, eBioscience) or CD3-FITC (HIT3a, BD Bioscience), CD8C-PE (HIT8a, BD Bioscience), CD62L-FITC (DREG-56, eBioscience), CD27-PE (O323, eBioscience), CD28-PE (CD28.2, eBioscience), CD45RO-PE (UCHL1, BD Bioscience), and CD69-PE (FN50, BD Bioscience). For multiple cytokine detection, a BD Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Kit was used.

Induction of lymphocytes and determination of their cytotoxic activity

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Conray density gradient centrifugation and were collected using blood cell separators (Baxter, Deerfield, IL, USA). To generate lymphocytes, PBMCs were transferred to 6-well cell plates and incubated at 37°C in 5% CO₂. They were then cultured at 2×10^6 cells/ml in RPMI 1640 medium-10% heat-inactivated FBS and penicillin/streptomycin, supplemented with 1000 U/ml IFN-γ on day 1 and 50 ng/ml OKT-3 (eBioscience, USA) and 300 U/ml of human recombinant IL-2 (eBioscience, USA) on day 2. Half of the cell culture medium was replaced by fresh complete medium containing IL-2 (100~300 U/ml), IL-7 (10 ng/ml), and IL-15 (10 ng/ml) every 2~3 d. Phenotypic analysis was performed weekly, and cytotoxic activity was assessed at the end of 2 weeks of culture. Before infusion, the viability of lymphocytes was tested by dye-exclusion with no less than 95% of viable cells.

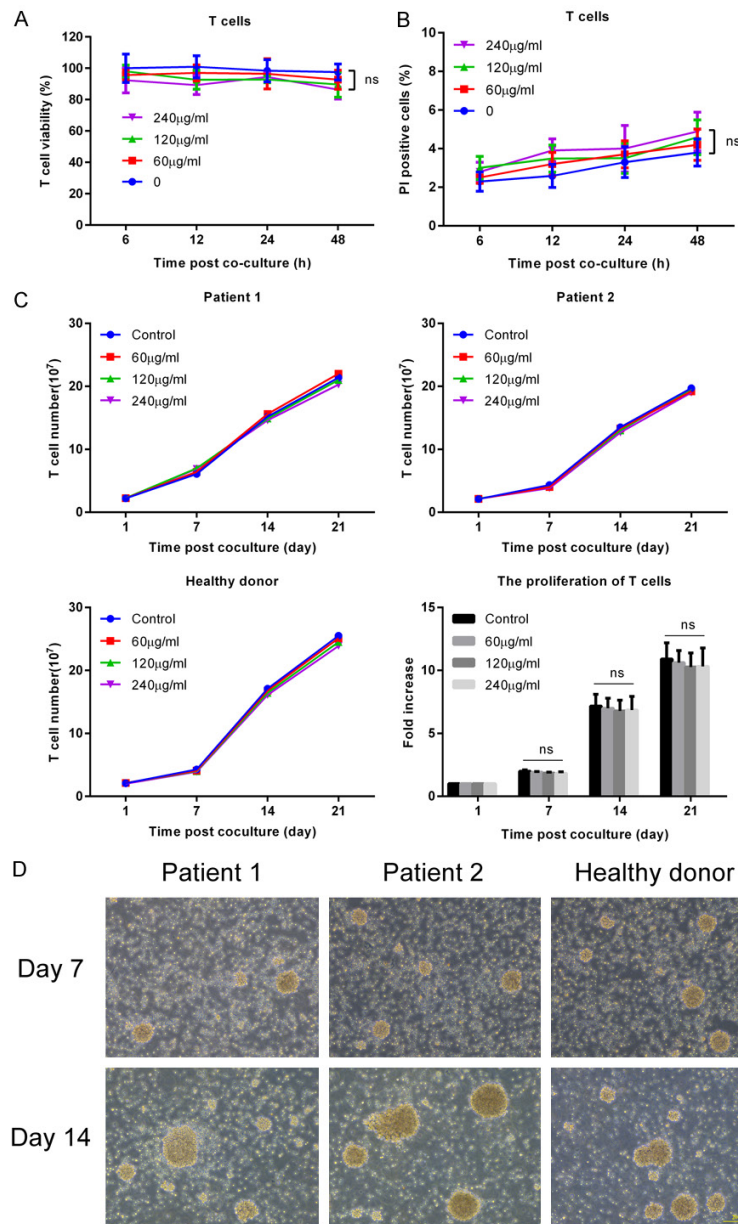
In vivo antitumor effect

Athymic nude BALB/c mice (5-6 weeks, female, 18-22 g) were subcutaneously inoculated with six million BGC-823, MGC-803, and MKN-45

GC cells into the lower right axilla and randomly divided into 4 groups (n=6 mice per group). On days 15, 21 and 27 after tumor cell implantation (when tumors were at least 200 mm³), lymphocytes in combination with or without anti-EGFR-iRGD (1×10^7 lymphocytes, 50 mg/kg anti-EGFR-iRGD) were injected into mice via the tail vein. Saline was used as a control. Thereafter, 40,000 U human recombinant IL-2 was intraperitoneally administered once a day for three consecutive days after adoptive transfer. During the study, tumor volume was recorded every 3 d. Tumor size was measured across 2 diameters using a Vernier caliper, and the tumor volume was calculated as $(L \times W^2)/2$, where L is the longest diameter, and W is the widest diameter of the tumor. At the end of the experiment, the mice were sacrificed, and the main organs (liver, lung, spleen, heart and kidney) were dissected. Hematoxylin-eosin staining was performed to assess systemic toxicity after 15 days of treatment (n=3 mice per group). The slices obtained were examined by optical microscopy.

In vivo distribution and tumor penetration

Female BALB/c mice (20±2 g) bearing BGC-823 and MKN-45 or MGC 803 tumors were used for the *in vivo* tumor penetration study. Briefly, six million cancer cells suspended in 200 μL PBS were subcutaneously injected into the lower right axilla of female BALB/c mice (20±2 g). Once the volume of tumors reached 200 mm³, lymphocytes in combination with or without anti-EGFR-iRGD (1×10^7 lymphocytes, 50 mg/kg anti-EGFR-iRGD) were injected into the mice via the tail vein. Mice were sacrificed at 24 h after treatment to obtain the tumors. The obtained tumors were observed using an *in vivo* imaging system (Near-infrared Quick View 3000, Bio-Real, Austria). The fluorescence intensity of tumors was analyzed by semi-quantitative analysis of the *ex vivo* fluorescent images. For tumor the penetration study, the obtained tumors were fixed in 4% paraformaldehyde to prepare frozen sections (10 μm thickness). Lymphocytes were stained with CFSE before injection. The tumor slices were stained with mouse anti-CD31 antibody (SANTA, USA), Cy3-labeled rabbit anti-mouse secondary antibody (SANTA, USA) and DAPI (SANTA, USA). The prepared tumor sections were observed by confocal microscopy (LSM710, Carl Zeiss, Germany).



iRGD on leukomonocyte proliferation. *In vitro* cytotoxicity tests using MTT assays of anti-EGFR-iRGD at different densities against lymphocytes were conducted. The differences in anti-proliferation activity between the control group and the co-culture group at 6 h, 12 h, 24 h and 48 h were not significant (all $P>0.05$). Even at the highest anti-EGFR-iRGD concentration of 240 $\mu\text{g/ml}$, no obvious decrease in cell viability was observed (**Figure 1A**). Likewise, no significant apoptotic responses of lymphocytes were observed under treatment with anti-EGFR-iRGD by flow cytometry at 6 h, 12 h, 24 h and 48 h, which is consistent with the result of the MTT assay (**Figure 1B**). These results suggest that anti-EGFR-iRGD even at the highest concentration induced little cytotoxicity to T cells.

To verify the effects of anti-EGFR-iRGD on lymphocytic growth, when co-culturing with different concentrations of anti-EGFR-iRGD from 60 $\mu\text{g/ml}$ to 240 $\mu\text{g/ml}$, we assessed the capacity of lymphocytes to proliferate *in vitro* by stimulation with IL-2, IL-7 and IL-15. We did not observe significant effects of recombinant protein on the proliferation of lymphocytes over the prolonged 21 days of the culture period, during which cell numbers ranged from 2×10^7 cells on day 7 to 2×10^8 on day 21 (**Figure 1C**). On days 1, 7, 14, and 21, there were no differences in the proliferation of lymphocytes when co-cultured with the different concentrations of anti-EGFR-iRGD (all $P>0.05$). The lymphocytic clones were observed and grew larger during the following days, indicating that there was no difference in the proliferation or activation of lymphocytes (**Figure 1D**). These results demonstrate that the ability of lymphocytes is not affected by anti-EGFR-iRGD.

Lymphocytic characterization upon co-culture with anti-EGFR-iRGD

To investigate whether anti-EGFR-iRGD may affect the differentiation of PBMCs, we harvested lymphocytes co-cultured with or without 240 $\mu\text{g/ml}$ anti-EGFR-iRGD on day 21 and measured the expression of CD8, CD4, and CD56 and the expression of activation markers including CD28, CD27, and CD69 on CD3⁺ cells. At the end of incubation, we found that lymphocytes in the co-cultured group displayed a similar phenotype to those of the untreated group.

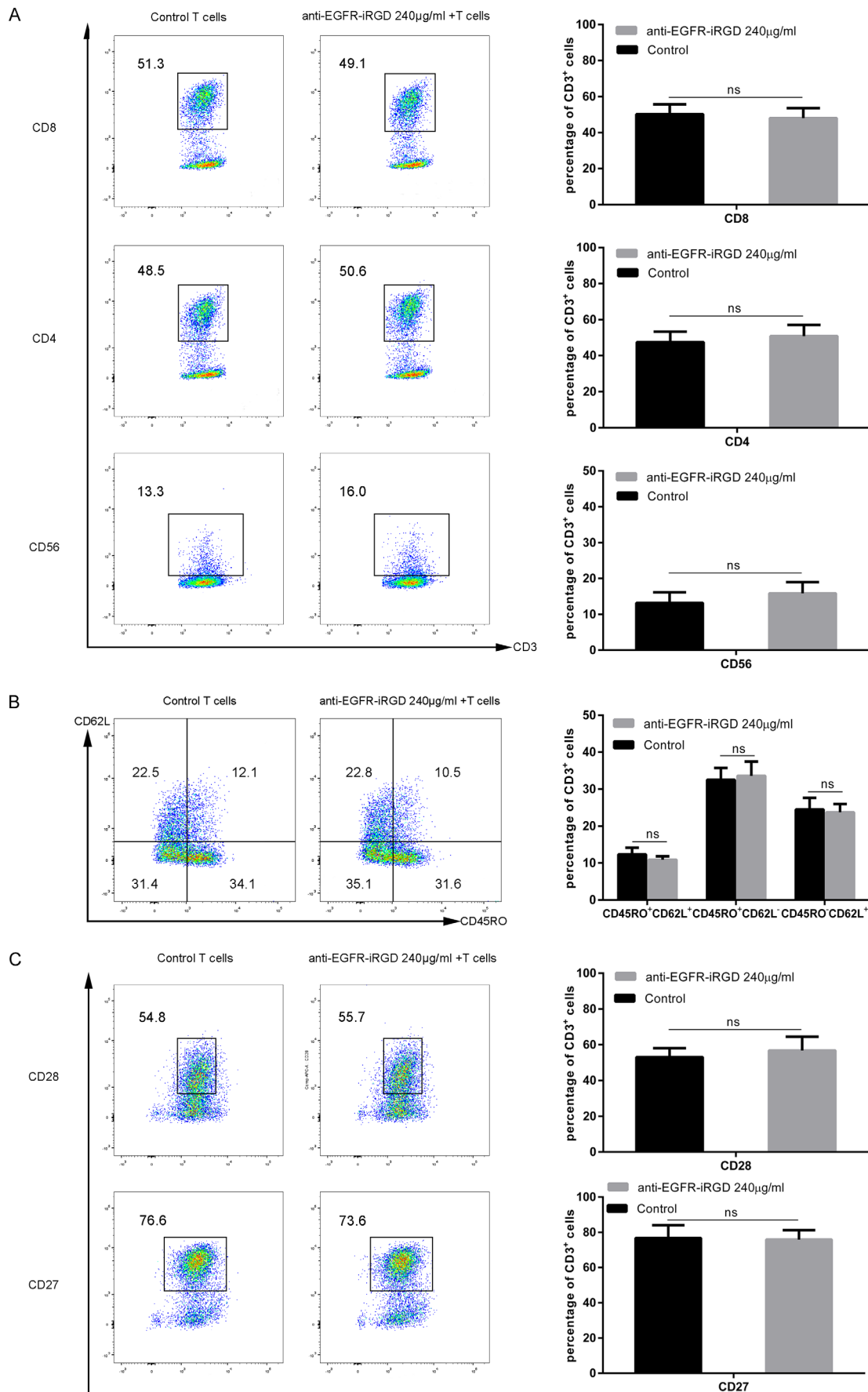
As shown in **Figure 2A**, lymphocytes co-cultured with anti-EGFR-iRGD exhibited no differences in the percentage of CD8⁺ T cells ($46.2\pm 5.49\%$ in control cells vs $43.05\pm 5.61\%$ in co-cultured cells; $P=0.5253$, $n=5$) or CD4⁺ T cells ($47.5\pm 5.85\%$ in control cells vs $50.9\pm 6.2\%$ in co-cultured cells; $P=0.5278$, $n=5$). Although a decrease in CD3⁺CD56⁺ cells among cocultured cells ($15.85\pm 3.14\%$ vs $13.2\pm 2.94\%$; $P=0.3026$, $n=5$) was observed, the difference was not statistically significant. CD62L and CD45RA expression in CD3⁺ cells after 3 weeks of culture were also measured to define the differentiation stage of lymphocytes. No differences in memory markers including central memory CD45RO⁺CD62L⁺ T cells ($12.33\pm 1.83\%$ vs $10.9\pm 0.9\%$ $P=0.2909$ $n=5$), effector memory CD45RO⁺CD62L⁺ T cells ($32.53\pm 3.20\%$ vs $33.60\pm 3.83\%$ $P=0.7304$ $n=5$) and naïve T cells ($24.50\pm 3.15\%$ vs $23.73\pm 2.25\%$ $P=0.7491$ $n=5$) were detected (**Figure 2B**).

It is noteworthy that we did not find any significant changes in the expression of activation markers CD28 and CD27 on CD3⁺ T cells between the co-cultured cells and control cells ($P>0.05$). While CD69 expression on CD3⁺ T cells only showed minor upregulation by stimulation of anti-EGFR-iRGD (**Figure 2C**). In addition, multiple cytokine assays were conducted to determine the poly-function of T cells. In this assay, IFN- γ , TNF- α and IL-2, which are defined as important for lymphocytic cytotoxicity were similar between the untreated group and the co-cultured group. After incubation with anti-EGFR-iRGD, we found that secretion of the immunosuppressive cytokine IL-10 was slightly downregulated and production of IL-6, which plays a role in the inflammation of lymphocytes, was slightly upregulated in the co-culture group compared with the control group, although the differences between the two groups were not significant (**Figure 2D**). These results indicated that the constitution of the two groups and the activation status and function of lymphocytes are relatively stable when co-cultured with anti-EGFR-iRGD.

Enhancement of the antitumoral effect on gastric cancer cell lines by the combined use of T cells and anti-EGFR-iRGD

In the previous study, we proved that the binding ability of recombinant protein anti-EGFR-iRGD to GC cells mainly depends on the EGFR

T-cell infiltration in gastric cancer by anti-EGFR-iRGD protein



T-cell infiltration in gastric cancer by anti-EGFR-iRGD protein

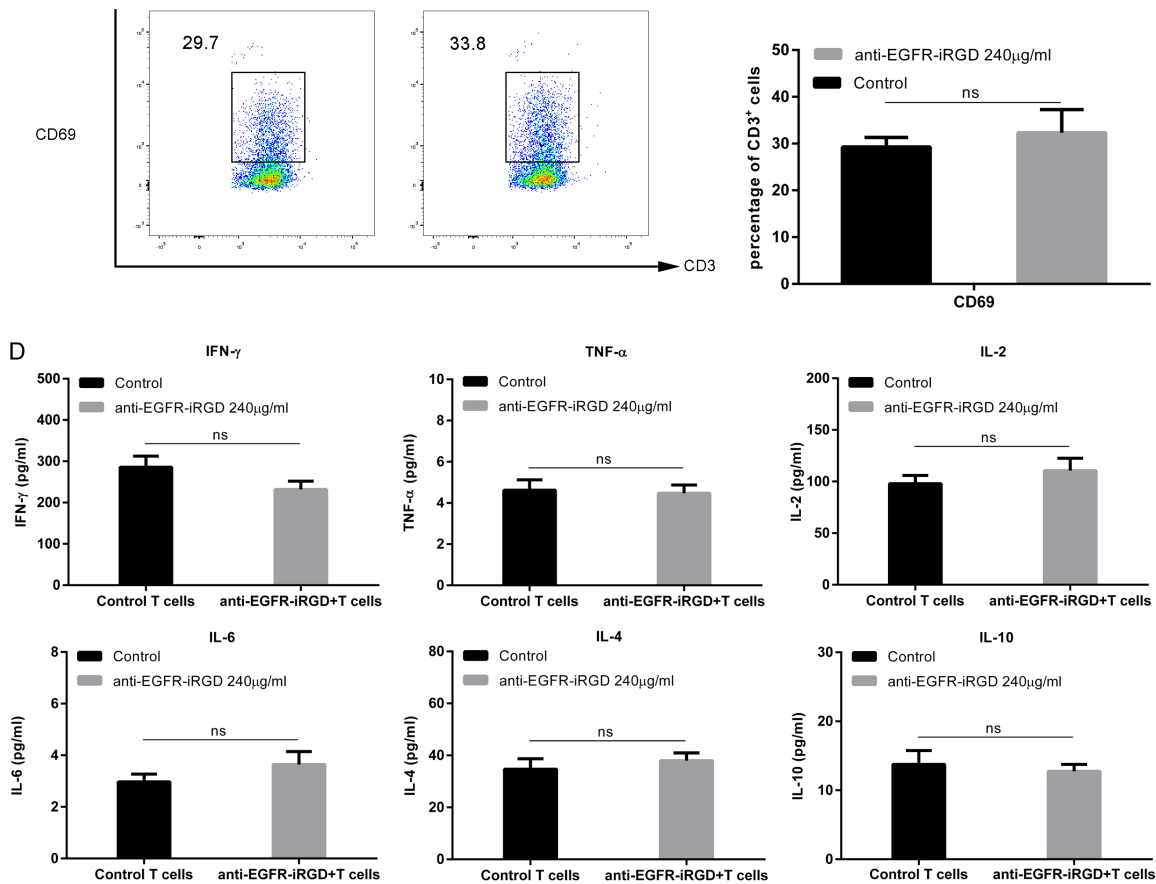


Figure 2. The phenotype of *in vitro* co-cultured lymphocytes. Lymphocytes were harvested on day 21 to evaluate the phenotype change after incubation with anti-EGFR-iRGD at a concentration of 240 μg/ml. A. The expression of CD3⁺CD8⁺, CD3⁺CD4⁺ and CD3⁺CD56⁺ on control leukomonocytes and co-cultured leukomonocytes. B. CD45RO⁺CD62L⁺, CD45RO⁺CD62L⁻ and CD45RO⁻CD62L⁺ were analyzed by gating on CD3⁺ cells. C. Cell surface expression of the activation markers CD28, CD27 and CD69 was measured on CD3⁺ cells. D. Cell culture supernatant was harvested and used for Th1/Th2 cytokine detection by FACS. Graphs show quantification of FACS data. Data are presented as the mean ± SEM of 3 independent experiments. ns: not significant.

expression level of cells. Therefore, we investigated the total EGFR expression levels in the GC cell lines. The cellular expression levels of EGFR decreased in the following order: BGC-823>MKN-45>MGC-803 (Figure 3A and 3B). Meanwhile, recombinant protein anti-EGFR-iRGD exhibited basal antitumoral activities against the BGC-823, MKN-45 and MGC-803 cell lines (Figure 3C). Anti-EGFR-iRGD induced slightly stronger inhibition of proliferation in the BGC-823 cell line than in the MKN-45 ($P=0.0258$) and MGC-803 cell lines ($P=0.013$) at the highest concentration of 240 μg/ml. For the MGC-803 cell line (low EGFR expression), no obvious anti-proliferative activity was observed.

As demonstrated above, anti-EGFR-iRGD does not impact the proliferation, phenotype or function of T cells when incubated together. We sub-

sequently sought to determine whether the combined utilization of T cells and anti-EGFR-iRGD could enhance the antitumoral effect of T cells *in vitro*. As shown in Figure 3, T cells exhibited lytic capability on GC cells in a dose-dependent manner. Notably, the cytotoxicity of the combination group was superior to the cytotoxicity of T cells alone. Furthermore, we found that the cytotoxicity of the combination group on tumor cells was significantly enhanced at the ratio of 40:1 (E:T) compared with ratios of 20:1 or 10:1 in all three GC cell lines. The percentages of apoptosis of BGC-823, MKN-45 and MGC-803 cells were $54.47\pm3.28\%$, $42.90\pm3.30\%$ and $27.07\pm1.90\%$, respectively, upon treatment with T cells alone, while those treated in the combination group were significantly increased to $65.5\pm5.76\%$, $51.2\pm3.28\%$ and $32.5\pm2.15\%$ (all $P<0.05$) at a ratio of 40:1 (Figure 3D). These results suggest that

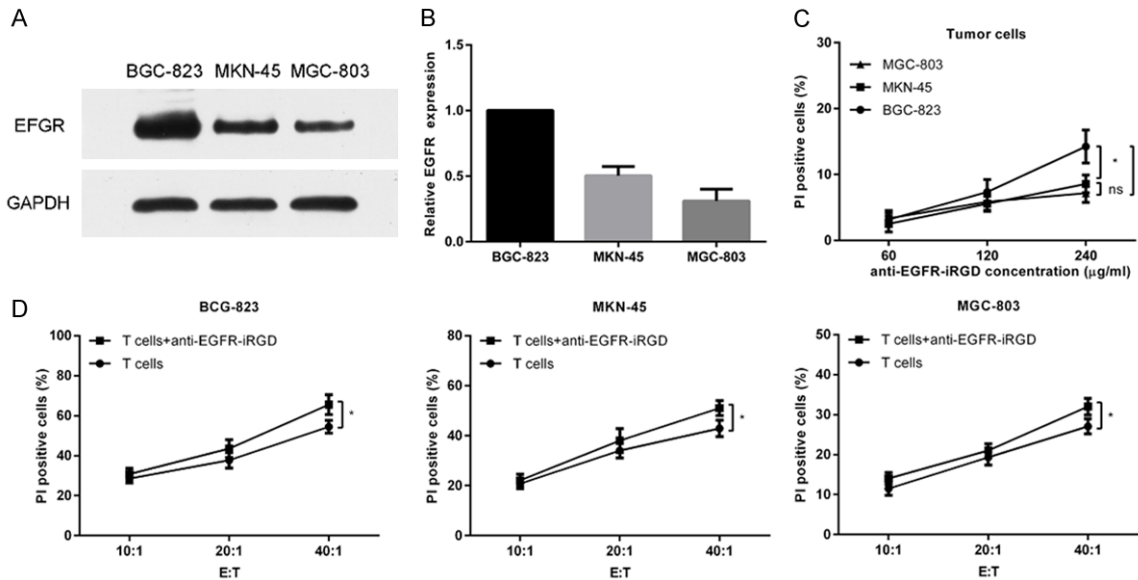


Figure 3. Investigation of the total EGFR expression levels in MGC-803, BGC-823 and MKN45 cell lines by western blot. Cancer cell apoptosis after treatment with T-cell monotherapy or T cells combined with recombinant protein anti-EGFR-iRGD was investigated by FACS: A. Western blot analysis of whole cell line lysates for EGFR. B. GAPDH and the ratio of the gray value of the destination strip and the gray value of the inner parameter. C. Single treatments of anti-EGFR-iRGD at concentrations of 60 μg/ml, 120 μg/ml, and 240 μg/ml were co-cultured with CFSE-labeled gastric cells BGC-823, MKN-45 and MGC-803 at ratios (E:T) of 10:1, 20:1, and 40:1. After 6 h, PI was added, and the cells were analyzed by flow cytometry. D. A single treatment of T cells or T cells combined with anti-EGFR-iRGD at concentrations of 60 μg/ml, 120 μg/ml, and 240 μg/ml were co-cultured with CFSE-labeled gastric cells BGC-823, MKN-45 and MGC-803 at ratios (E:T) of 10:1, 20:1, and 40:1, respectively. After 6 h, PI was added, and the cells were analyzed by flow cytometry. The above experiments were repeated 3 times with similar results. ns: not significant.

the combined use of T cells and anti-EGFR-iRGD had advantageous antitumoral effects *in vitro* on tumor cell lines compared with T cell monotherapy.

Inhibitory effect on tumor growth in BGC-823 tumor models

As the co-administration of T cells and anti-EGFR-iRGD induced an increase in cell-mediated cytotoxicity *in vitro*, we assumed that T cells combined with anti-EGFR-iRGD would also induce enhanced cytotoxicity *in vivo*. Thus, we established BGC-823 tumor models to evaluate their antitumoral effects. Human T cells were activated *ex vivo* and then transferred into tumor-bearing mice with anti-EGFR-iRGD or saline on days 15, 21, and 27 after tumor cell implantation (Figure 4A).

As shown in Figure 4B, the BGC-823 tumors grew rapidly in mice treated with saline or anti-EGFR-iRGD alone. Following a single treatment of anti-EGFR-iRGD, we did not observe significant tumor regression compared with the

saline group. Both the saline and anti-EGFR-iRGD groups displayed poor antitumoral efficiency, as the average tumor volumes of the saline and anti-EGFR-iRGD-only groups increased by approximately 9-fold and 8-fold, respectively, by day 30. However, the ACT delivered 3 times over the course of 30 days induced significant inhibition of tumor growth. In the ACT-treated group, the tumor volume in the mice increased by approximately 5.3-fold by day 30, representing approximately a 24.3% reduction in tumor volume compared with the saline group. The ACT group had a significantly better inhibitory effect against tumor growth than the saline and anti-EGFR-iRGD groups. Although ACT alone could inhibit tumor growth, there was a significantly greater retardation in tumor growth for the combination treatment with anti-EGFR-iRGD. The differences in tumor volumes between the co-administration group with either saline ($P < 0.01$) or anti-EGFR-iRGD ($P < 0.05$) were both statistically significant. In the combination-treated mice, the tumor volume increased by 5.5-fold by day 30, representing a

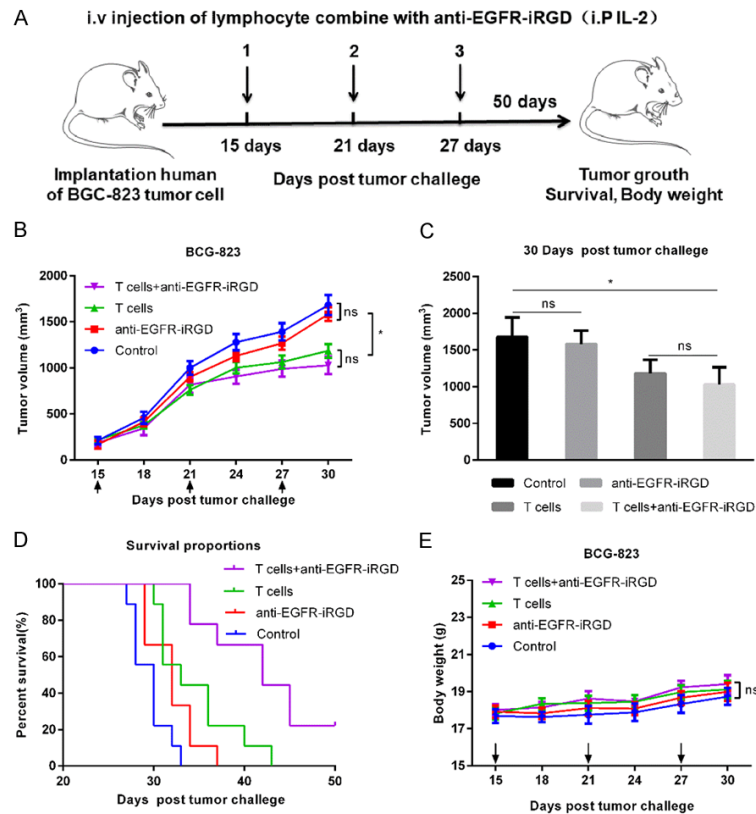


Figure 4. Mice bearing subcutaneous BGC-823 were treated by intraperitoneal injections of saline, anti-EGFR-iRGD or ACT combined with or without anti-EGFR-iRGD every seven days: (A) schema for adoptive cell transfer therapy combined with anti-EGFR-iRGD. The tumor volume growth curves (B) and tumor volume (C) of the treated mice at 30 d after tumor inoculation. The survival curve (D) and body weight change (E) of BGC-823 tumor-bearing mice that received the indicated treatments. Experimental groups contained at least five mice for the antitumor efficacy experiment. Data are presented as the mean $\pm$ SEM (standard error of the mean). One-way ANOVA was used for the analysis of tumor volume and body weight. ns: not significant.

reduction in tumor volume by approximately 28.7% compared with the saline control group (Figure 4C). Strikingly, the combination group resulted in further inhibition of tumor growth that exceeded the inhibition observed for either treatment alone. However, the difference between the combination group and monotherapy ACT group was nonsignificant ($P>0.05$), although there was still a trend that the combined use of ACT and anti-EGFR-iRGD could yield better outcomes.

In the survival study, we observed that ACT improved the overall survival of tumor-bearing mice, and better results were achieved when ACT was co-administered with anti-EGFR-iRGD. The median survival of female BALB/c mice bearing BGC-823 tumors in the co-administra-

tion group (42 days) was significantly longer than the survival of mice treated with saline (30 days), anti-EGFR-iRGD (32 days), and T cells (33 days). Two mice that were co-administered ACT and anti-EGFR-iRGD survived more than 50 d after tumor inoculation (Figure 4D).

None of the mice treated with saline or anti-EGFR-iRGD exhibited any body weight loss (Figure 4E). Although the mean body weight of the anti-EGFR-iRGD group showed a slight decrease during the treatment, the differences in body weight between the group treated with anti-EGFR-iRGD and the other groups were not significant ($P>0.05$).

In vivo distribution and tumor penetration study

To explain the reason behind the prolonged survival of the group that received the combination of ACT and anti-EGFR-iRGD, we hypothesized that anti-EGFR-iRGD could further improve the tumor homing and penetration ability of ACT by facilitating the migration of lymphocytes into tumors. To evaluate whether the delayed tumor growth and the extended survival of tumor-bearing mice were a result of the increased trafficking of cytotoxic T cells, a tumor penetration study of the BGC-823 GC mouse model was performed. As shown in Figure 5A, the accumulation of T cells in the tumor tissue of the co-administration group was much greater than that of the monotherapy group. The observation indicated that the combination of ACT with anti-EGFR-iRGD increased the lymphocyte distribution in tumor parenchyma.

Furthermore, T cells were entrapped in the tumor blood vessels or mainly observed around the tumor blood vessels with little extravasation when administered alone. However, the lymphocyte fluorescence in the combination

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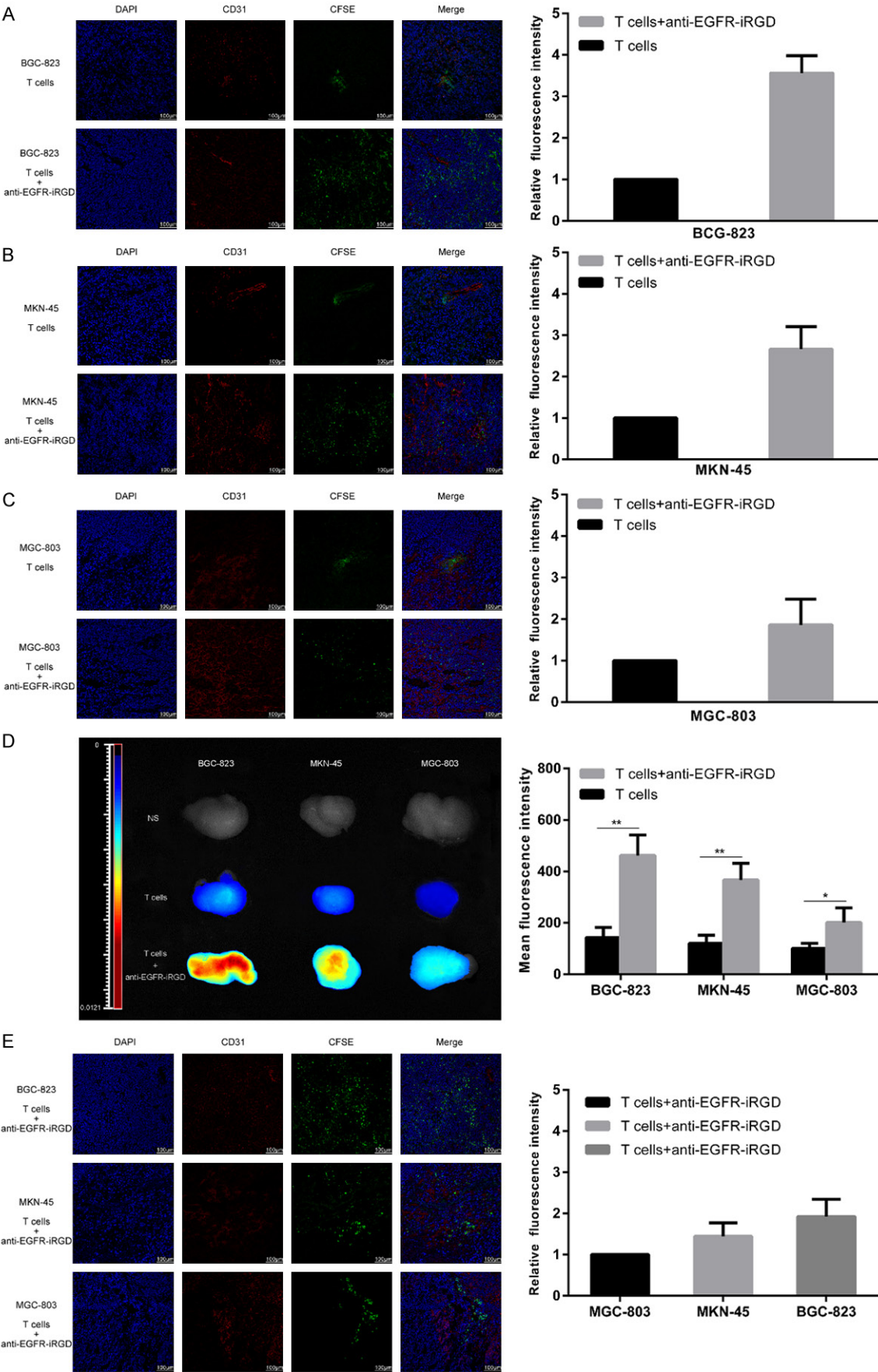


Figure 5. Tumor distribution of lymphocytes from three different gastric cancer mouse models is presented: T cells (stained with CFSE) combined with anti-EGFR-iRGD (1×10^7 lymphocytes, 50 mg/kg anti-EGFR-iRGD) were injected into the tail vein. Tumor sections were stained with anti-CD31 (red) for blood vessels and DAPI (blue) for nuclei. The green fluorescence represents lymphocytic nuclei. Bars represent 100 μ m. Distribution of lymphocytes in BGC-823 (A), MKN-45 (B) and MGC-803 (C) tumors from mice 24 h after intravenous administration of lymphocytes combined with or without anti-EGFR-iRGD. (D) *Ex vivo* imaging of three gastric tumors at 24 h post injection with lymphocytes stained with DiR combined with or without anti-EGFR-iRGD. (E) T-cell distributions in three kinds of tumors are shown at 24 h after intravenous co-administration of lymphocytes and anti-EGFR-iRGD.

group was distributed significantly further from the tumor vessels and covered a much wider range. The *in vivo* quantitative analysis of cellular infiltration revealed a trend that anti-EGFR-iRGD could significantly increase the lymphocyte fluorescence signal in tumor cells compared with lymphocyte monotherapy (**Figure 5A**), which was consistent with the lymphocyte distribution in tumors.

In the other two human GC mouse models, ACT combined with or without anti-EGFR-iRGD also displayed similar pattern of fluorescence signals. All the combination group displayed the strongest fluorescence signal in MKN-45 and MGC-803 GC mice model (**Figure 5B** and **5C**). In addition, lymphocyte fluorescence of the co-administered groups was found at the central regions of the tumor parenchyma far from the tumor vessels, which was similar to that in the BGC-823 mouse model (**Figure 5A**). Thus, the anti-EGFR-iRGD treatment could significantly enhance the infiltration of activated T cells in the three GC models. Furthermore, we evaluated the differences in lymphocyte accumulation in three different tumor models. Near-infrared scanning was used to observe the developed tumor, as shown in **Figure 5D**, lymphocytes combined with anti-EGFR-iRGD significantly enhanced the lymphocytic distribution signal in the tumors compared with lymphocyte monotherapy-treated groups. In addition, the co-administration group in the BGC-823 tumor mouse model displayed the strongest tumor distribution fluorescence signal intensity and the best accumulation among all the co-administration groups of the three tumor models, and these results are consistent with the *in vivo* distribution observed by laser scanning confocal microscopy (LSCM) (**Figure 5E**).

Discussion

In this study, we demonstrated for the first time that dual-target tumor penetration protein, anti-EGFR-iRGD, can improve the effect of lymphocytic infiltration at the site of tumor tissue

and enhance the antitumoral activity both *in vitro* and *in vivo*. In addition, our data revealed that the synthetic recombinant protein, anti-EGFR-iRGD, has no effect on the vitality, proliferation, phenotype and function of lymphocytes. These results imply that the co-administration of ACT and anti-EGFR-iRGD can elevate lymphocytic localization to tumors and improve antitumoral immune responses. This strategy may ultimately enable personalization of cancer therapies based on the combined use of ACT with anti-EGFR-iRGD.

In the past few years, immunotherapy has emerged as a relatively safe and effective alternative for the treatment of cancers. However, many factors result in lymphocytic exhaustion in the tumor site, such as the expression of inhibitory receptors CTLA-4 and PD-1 and the presence of immunosuppressive regulatory T cells and immunosuppressive cytokines within the tumor microenvironment [13, 14]. New methods utilizing cytokines, CTLA-4 and PD-1/PD-L1 blockers, and ACT have been developed and approved to treat solid carcinomas and have exhibited dramatic results in some cancers [15, 16].

The number and efficiency of adoptively transferred lymphocytes migrating to the tumor microenvironment and the persistence of these cells have been found to positively correlate with clinical responses and outcomes in patients [17, 18]. Thus, a sufficient amount of high-avidity leukomonocytes migrating to inner solid tumors is an important goal of cancer immunotherapy. Strategies that enhance the function of lymphocytes are critical for immunotherapy, but efficient lymphocyte trafficking to tumors remains a major challenge for inducing adequate therapeutic effects. Of the large number of adoptively transferred lymphocytes required for effective treatment, only a small fraction of the total *ex vivo* expanded cells eventually reaches the tumor tissue. Most cells mainly stay around blood vessels owing to the high interstitial fluid pressure in tumors and the

binding-site barrier [19, 20]. Therefore, these lymphocytes are not effectively exposed to cancer cells, especially cancer cells located in deep tumor regions.

The trafficking of leukomonocytes involves complex interactions between leukomonocytes and endothelial cells (ECs), chemokine receptors, selectins, and integrins [21]. It has been reported that increased α v integrin expression on effector T cells is largely dispensable for extravascular trafficking of leukocytes and that β 1 and β 3 integrin play important roles in the function of cytotoxic T lymphocytes (CTLs), both in sensing changes in the extracellular environment and in target cell recognition [22, 23]. Studies have reported that the tumor-penetrating peptide iRGD can bind integrin α v, which is overexpressed on the vascular endothelium in tumors, and then the proteolytically cleaved peptide can activate neuropilin-1 (NRP-1) to increase tumor vascular and tissue permeability, thus improving tumor penetration by antitumor drugs of various compositions, including small molecules, nanoparticles and antibodies. We successfully constructed a recombinant protein named anti-EGFR-iRGD, a dual-target and highly permeable protein that can extensively spread throughout both multicellular spheroids and the tumor mass. Therefore, we hypothesized that the interaction of anti-EGFR-iRGD with integrins and NRP-1 could also lead lymphocytes to extravasate from tumor vessels and penetrate tumor tissue.

To further confirm this hypothesis *in vivo*, we used the established BGC-823 tumor model, which allows us to monitor the trafficking of transferred lymphocytes. From the results of the distribution study *in vivo*, we found that lymphocytic trafficking to tumor sites was improved following co-administration of anti-EGFR-iRGD, the co-administration group in the BGC-823 mice model exhibited a higher increase in lymphocyte accumulation in the tumor site than the co-administration groups of the MKN-45 and MGC-803 mouse models. Thus, adoptively transferred T cells exhibited significantly more intense penetration into the high-EGFR-expressing tumor with the combination of anti-EGFR-iRGD. These findings support our hypothesis that anti-EGFR-iRGD enhances lymphocyte migration and penetration to tumors. However, the mechanisms underlying this promotion of T cell infiltration into tumors following anti-EGFR-iRGD treatment have not yet been elucidated.

We hypothesized that a possible mechanism is that more anti-EGFR-iRGD can bind to cells that highly express EGFR, which later helps lymphocytes migrate and kill tumors. The enhancement may be associated with the ability of anti-EGFR-iRGD to improve the permeability of tumor tissue, although this needs to be further studied.

Recent therapies such as chemotherapy, radiotherapy or anti-angiogenic therapy have been shown to enhance lymphocytic penetration by altering the local tumor microenvironment. The combination of chemotherapy and vaccines induced increased lymphocyte infiltration into cutaneous melanoma lesions which largely enhance immune responses. In addition, T cells in the tumor tissue of mice were enriched and infiltrated into the tumor parenchyma when treated by radiation [24]. However, high doses of chemotherapeutics are immunosuppressive, and radiotherapy also has side effects on the human body and may damage leucocyte populations, impeding the widespread use of these therapeutic strategies [25-29]. However, of all the methods established to-date, this is the first report of the combination of ACT with an iRGD-fused protein anti-EGFR-iRGD, which has been proven effective and safe. We observed promoted cytotoxicity of T cells on tumor cell lines and enhanced tumor infiltration of lymphocytes, while no altered functions of T cells. Importantly, mice in the co-administration group displayed the highest tumor inhibition rate and the longest overall survival among all the treated groups. The difference in T cell distribution *in vivo* correlated with different outcomes, which suggested that the retardation of tumor growth and prolonged survival were likely to be immune mediated.

Interestingly, we observed that the mice treated with anti-EGFR-iRGD and lymphocytes did not show a marked reduction in tumor volume but had a longer survival time than mice in the other groups. The potential mechanisms underlying this phenomenon are unclear, however, possible reasons for the insignificant difference in tumor volume among groups could be as follows. It is not only the number of tumor-infiltrating T cells but also the antigen-specificity of T cells that determines the antitumor effect. We propose that no significant tumor regression was observed because most of the T cells were not tumor antigen specific, and thus, only a small percentage of effective

immunologic cells were directly involved in the antitumoral response. In addition, due to the short half-life of anti-EGFR-iRGD whose molecular weight is 18 kDa, it is difficult for anti-EGFR-iRGD to exert a continual, long-lasting aid to ACT. In the future, we will increase the T cell immune efficacy and the durability of anti-EGFR-iRGD.

Furthermore, in the context of immunotherapy, the conventional response evaluation criteria were all based on changes in tumor burden, such as WHO or RECIST. These criteria may not adequately assess the activity of immunotherapeutic agents due to stable disease (SD), and even partial response (PD) does not necessarily indicate therapeutic failure. A case study showed that an ipilimumab-treated patient who appeared to have PD after two cycles of treatment, underwent resection, and histological analyses confirmed that the increase in lesion volume was likely related to T cell infiltration rather than tumor cell proliferation [30]. Although we did not find a difference in tumor volume, our results suggest that anti-EGFR-iRGD combined with lymphocytes can significantly improve overall survival (OS), as the improved OS of the co-administration group was correlated with the function of anti-EGFR-iRGD in improving the internalization of lymphocytes into the tumors. Nevertheless, OS as a gold standard to reflect therapeutic outcome is a reliable tool to evaluate the efficiency of immunotherapy.

Notably, no significant abnormal damage and no side effects were observed in the mice that received multiple injections of T cells with anti-EGFR-iRGD. Taken together, the studies show that anti-EGFR-iRGD may be an effective and safe adjunct to ACT considering the pressing need to find agents that can enhance the tumor-infiltrating capacity of antitumoral T cells.

In summary, our study assessed an alternate strategy to increase the effectiveness of ACT by promoting lymphocyte homing and penetration to the tumor site, and we developed a safe and rational method for GC treatment. We showed that anti-EGFR-iRGD could enhance the antitumoral efficacy of ACT both *in vitro* and *in vivo* and displayed low toxicity to lymphocytes. When co-administered with anti-EGFR-iRGD, the tumor accumulation and penetration of lymphocytes were greatly elevated. Moreover, more effective penetration and higher affinity

of T cells into high-EGFR-expressing tumors were observed. Finally, this recombinant protein with bispecific targets offers potential synergy with other small-molecule antitumoral agents for promoting the efficacy of immunotherapy. Our strategy exclusively targets the delivery of immune cells to tumor tissue and provides a possible solution for the optimization of therapeutic options in cancer immunotherapy.

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Disclosure of conflict of interest

None.

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