

## Cellular Immunotherapy for Carcinoma Using Genetically Modified EGFR-Specific T Lymphocytes<sup>1,2</sup>

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### Abstract

Epidermal growth factor receptor (EGFR) is overexpressed in a variety of human malignancies, including pancreatic cancer, breast cancer, colon cancer, and non–small cell lung cancer. Overexpression of EGFR is a predictive marker of therapeutic response and several lines of evidence suggest that EGFR is an excellent target for tumor therapy. However, the effective antitumor capacity of EGFR-specific T cells against EGFR-overexpressing tumor cells has not been fully elucidated. In our previous study, we identified an anti-EGFR single-chain variable fragment (scFv) with specific and high affinity after screening by ribosome display. In this study, the anticancer potential of anti-EGFR scFv was investigated on the basis of cell-targeted therapy. A chimeric antigen receptor (CAR) targeting EGFR was constructed and expressed on the cell membrane of T lymphocytes. These CAR-modified T cells demonstrated antitumor efficacy both *in vitro* and *in vivo*. In addition, the safety evaluation showed that CAR-modified lymphocytes have no or very minimal acute systemic toxicity. Taken together, our study provided the experimental basis for clinical application of genetically engineered lymphocytes; moreover, we also evaluate a new and interesting cell therapy protocol.

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### Introduction

The epidermal growth factor receptor (EGFR) is overexpressed on the surface of the cell membranes in a wide variety of solid tumors, particularly in non–small cell lung cancer (NSCLC). Overexpression of EGFR has marked effects on various aspects of carcinogenesis, like cell growth and invasion, angiogenesis, and metastasis. The potential value of EGFR as a target for the diagnosis and therapy of human tumors has been recognized for several years [1–4]. A number of targeting agents directed at tumors that express high levels of EGFR have been approved for clinical use of treating NSCLC, and antibody therapy plays an important role in these agents [5,6]. Monoclonal antibodies (mAbs), such as cetuximab, have shown promising results in providing a survival advantage for advanced-stage NSCLC patients [7–10]. However, due to EGFR mutations and the poor penetration of antibodies into the tumor tissue, these agents are not able to kill a sufficient number of malignant cells and cause tumor regression

Abbreviations: EGFR, epidermal growth factor receptor; ACT, adoptive cell therapy; CAR, chimeric antigen receptor

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[11,12]. It is necessary to direct the antibody with a cytotoxic agent to the target to enhance the efficacy of its antitumor activity.

Adoptive cell therapy (ACT) is a new cancer therapy strategy that is usually approached through infusion of antitumor immune effector cells to obtain or enhance antitumor responses in patients. Dr Rosenberg initiated the prelude of ACT in 1985 when he treated melanoma with lymphocyte-activated killer cells for the first time [13]. Many tumor-specific antigen *T cell receptor* (*TCR*) genes, such as the melanoma antigen tumor-specific *TCR* gene, have been isolated and identified along with the rapid development of genetic engineering technology in recently years [14–18]. Furthermore, chimeric antigen receptor (CAR) technology has combined both the specificity of mAbs and the efficient cytotoxic of T lymphocytes. The biggest advantage of CAR is that it is not subject to major histocompatibility complex (MHC) restriction, as natural TCR is. Nonspecific lymphocytes can be endowed with the ability to specifically recognize tumor antigens and to release cytokines by *CAR* gene transfection [19,20]. Using this approach in the clinical treatment of a variety of tumors, such as lymphoma [21,22], neuroblastoma [23], and metastatic renal cell carcinoma [24], has yielded encouraging results.

In our previous study, after screening by ribosome display, we generated an anti-EGFR single-chain variable fragment (scFv) with high antigen specificity and affinity [25]. An rGel-based EGFR-specific immunotoxin was constructed, expressed, and purified; this immunotoxin resulted in significant antigen specificity and antitumor effect both *in vitro* and *in vivo*. Here, we aimed to further investigate the antitumor potential of the anti-EGFR scFv using the cell-targeted therapy strategy, with a view to establishing whether the CAR-modified T lymphocytes have potential for application in the treatment of EGFR-positive carcinoma. To this end, the CAR was constructed to consist of the human interleukin-2 (IL-2; hIL-2) signal peptide, anti-EGFR scFv, hemagglutinin (HA) tag, IgG2 Fc, and the transmembrane region and part of the cytoplasmic region of the human CD28 signaling chain fused to the cytoplasmic region of CD3 $\zeta$  chain. Then, the *CAR* gene was cloned into the *NheI* and *BglII* sites of a CMV promoter-based nonviral vector, pmaxCloning. After verification of the expression of the CAR on the cell membrane of T lymphocytes, the antitumor effects of CAR-modified T cells were evaluated both *in vitro* and *in vivo*. The systemic acute toxicity of this strategy was also evaluated.

## Materials and Methods

### Cell Lines and T Cells

Human epithelial carcinoma cell line A431, human NSCLC cell line A549, human Burkitt's lymphoma cell line Raji, and human ovarian cancer cell line A2780 were obtained from American Type Culture Collection (Manassas, VA). A549, A2780, and Raji cell lines were cultured in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated FBS. The A431 cell line was maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS.

Human T cells were isolated from peripheral blood of healthy donors under an Institutional Review Board-approved protocol (95-054), using BD Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ) following the manufacturer's instructions. T cells were cultured in RPMI 1640 supplemented with 300 IU/ml IL-2

(Novartis Pharmaceuticals, Shanghai, China) and stimulated with anti-CD3 and anti-CD28 mAbs.

### Mice

Five- to six-week-old female nonobese diabetic/severe combined immunodeficiency (NOD/SCID) CB-17 mice were purchased from Beijing HFK Bio-Technology Co, Ltd (Beijing, China). Animals were kept in a specific pathogen-free facility at the Animal Experimental Center of Sichuan University (Sichuan, China). All animal studies were performed in accordance with the Institutional Animal Care guidelines.

### CAR Construction

The CAR constructs used in this study are schematically illustrated in Figure 1A, using an assembly technique as follows. Generation of the anti-EGFR-scFv DNA has recently been described in detail [25]. The hIgG2-Fc fragment was subcloned by polymerase chain reaction (PCR) from pFUSE-hIgG2-Fc1 plasmid (InvivoGen, San Diego, CA). The fused CD28 transmembrane region and a part of its intracellular region and CD3- $\zeta$  molecules were synthesized by the GenScript company. Several genetic fragments were assembled by overlapping PCR, in which the signal peptide of hIL-2 and an HA tag was also introduced through the forward primer. The expression cassettes were subcloned into the *NheI* and *BglII* sites of pmaxCloning vector. The sequence of the whole genetic fragment was confirmed by direct sequencing. The vehicle construct contained the same components in similar configuration and order, except that it lacked the anti-EGFR scFv sequence.

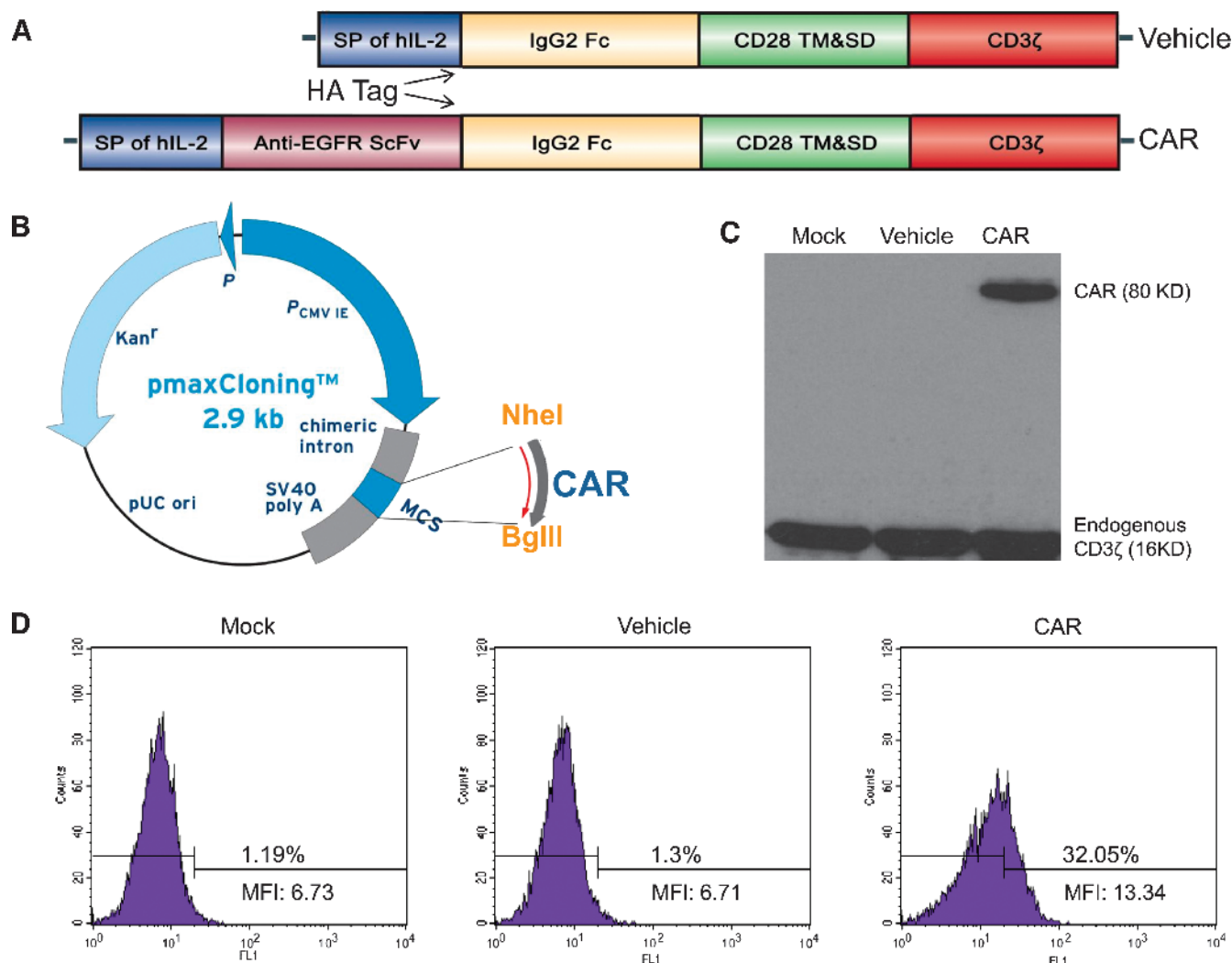
### Antigen-specific Cytotoxicity and Cytokine Secretion by CAR-Modified T Cells

The cytolytic activity of CAR-modified T cells was measured by assays of lactate dehydrogenase (LDH) release after 6 hours using a Cytotoxicity Detection Kit (Promega, Madison, WI) according to the manufacturer's instructions. The capacity of modified T cells to produce cytokines [interferon- $\gamma$  (IFN- $\gamma$ ), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, and IL-4] was determined by ELISA Kit in supernatants after 24 hours of incubation with EGFR-positive cells (Neobioscience, Shenzhen, China).

### Mouse Models

**Tumorigenicity model.** CAR-transfected lymphocytes and A549 tumor cells were washed with serum-free, antibiotic-free medium and the cell concentrations were adjusted to  $2 \times 10^8$ /ml and  $2 \times 10^7$ /ml, respectively. Ten mice per group were used and each mouse was inoculated subcutaneously (s.c.) in the scapular region with lymphocyte/tumor cell mixture in 100  $\mu$ l of serum-free, antibiotic-free medium at a ratio of 10:1. All animals were monitored for a total of 60 days; animals were weighed and observed for clinical signs every 3 days. The tumor volume was estimated using two measurements of the nodules, with the formula  $V = 0.52 \times \text{length} \times \text{width}^2$ .

**S.c. model.** Log-phase A431 or A2780 cells ( $5 \times 10^6$  cells per mouse) were injected s.c. into the scapular region. Once tumors were grown to 200 to 500 mm<sup>3</sup> in size, animals were administered CAR-modified T cells [ $1 \times 10^7$  cells per mouse; intravenously (i.v.) through the tail vein] at 24, 48, and 72 hours after mice were grouped.



**Figure 1.** Construction and expression of the EGFR-specific CAR in transduced human T lymphocytes. (A and B) Schematic representation of the construction of EGFR-specific CAR. This CAR consisted of the hIL-2 signal peptide, anti-EGFR scFv, HA tag, IgG2 Fc, the transmembrane region, and part of the cytoplasmic region of the human CD28 signaling chain, fused to the cytoplasmic region of the CD3 $\zeta$  chain. This *CAR* gene was cloned into *NheI* and *BglII* sites of a CMV promoter-based nonviral vector, pmaxCloning. (C) Western blot analysis of CAR expression in T lymphocytes at 16 hours after transduction. PVDF membranes were labeled with an anti-human CD3 $\zeta$  antibody and a goat anti-rabbit IgG HRP-conjugated second antibody. (D) Flow cytometric analysis of CAR expression in T lymphocytes at 16 hours after transduction. The transduced cells were stained with anti-EGFR scFv antiserum and goat anti-rabbit IgG FITC-conjugated secondary antibody and were then detected by a BD FACSCalibur flow cytometer. MFI is the abbreviation of mean fluorescence intensity. Results are representative of three experiments.

**Lung metastasis model.** A549 cells ( $2 \times 10^6$  cells) were administered i.v. to mice on day 0. Then, tumor-bearing mice were randomly assigned to one of the four groups: 1) mice treated with normal saline (NS), 2) mice treated with Mock T cells, 3) mice treated with Vehicle T cells, and 4) mice treated with CAR T cells. CAR-modified T cells ( $2 \times 10^6$ ) were administered i.v. at days 3, 6, 9, 12, 15, and 18 after tumor injection. Five mice per group were sacrificed at day 30, and the lung metastasis index of each group was statistically determined as described in our previous report [26].

#### Analysis of Toxic Effects Induced by Systemic Administration in Mice

NOD/SCID mice (five mice per group) were studied for evidence of acute toxic effects induced by a single i.v. injection of CAR-

modified T cells ( $2 \times 10^7$  cells per mouse). Forty-eight hours later, the mice were sacrificed and serum was separated. Some of the mice from the lung metastasis model were also sacrificed 48 hours after the last cell administration, and serum was separated. Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured at the Biochemistry Laboratory in the National Chengdu Center for Safety Evaluation of Drugs (Chengdu, China). After sacrifice of the mice in the lung metastasis model at 30 days, the heart, liver, spleen, and kidney tissues were used for morphologic analysis and hematoxylin and eosin (H&E) staining.

#### Statistical Analysis

All statistical analyses were performed using SPSS 19.0 software. Data are presented as means  $\pm$  SD. Statistical analysis was performed

using Student's *t* test for comparing two groups and by analysis of variance for multiple group comparisons. *P* values less than .05 were considered statistically significant.

## Results

### *Efficient Generation of CAR-Modified T Cells Using a Nucleofection Gene Transfer System*

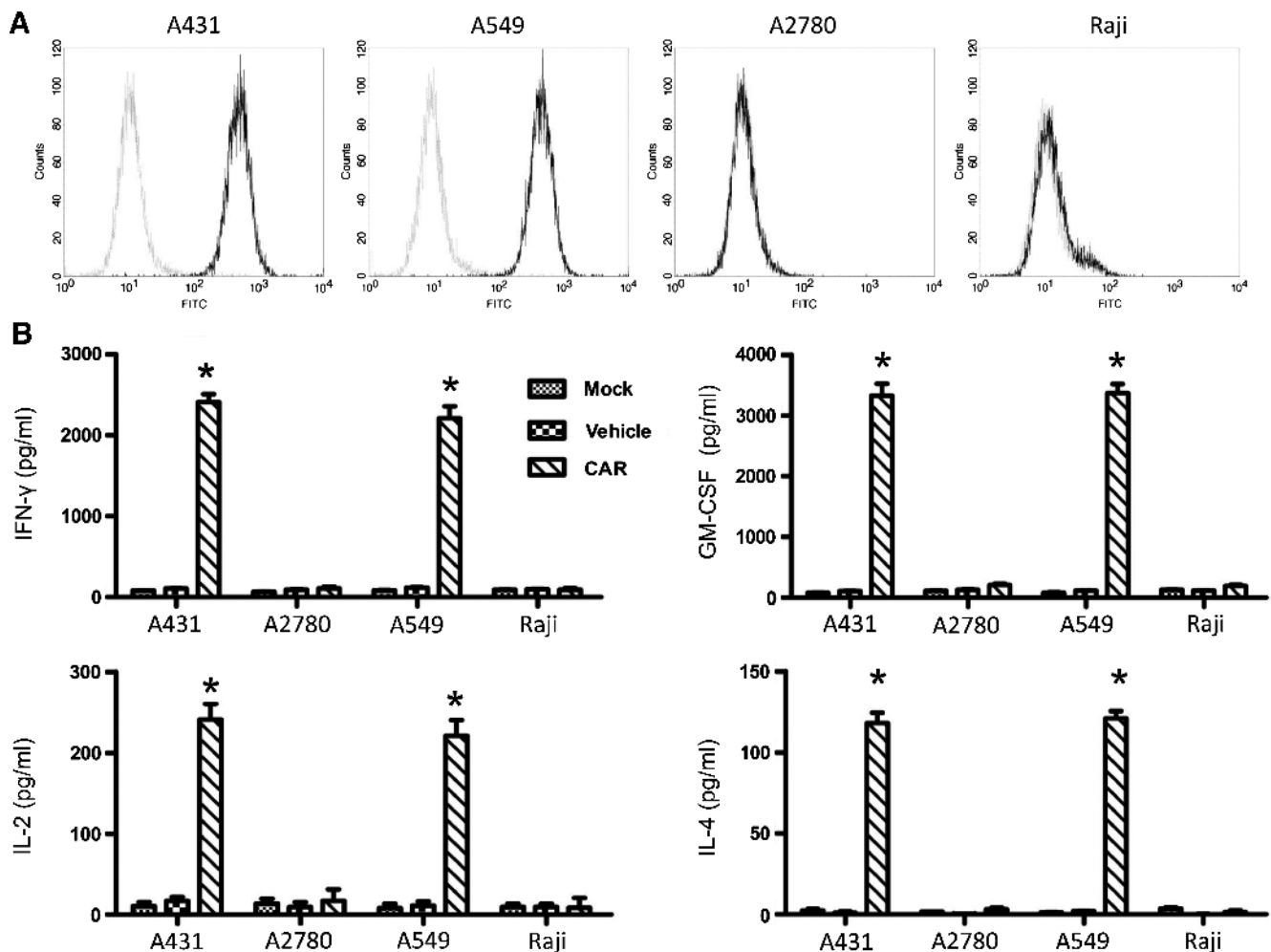
To investigate the therapeutic potential of primary human T lymphocytes genetically modified to recognize and kill tumors that express EGFR, an EGFR-specific single-chain antibody sequence was selected due to its excellent specificity and binding affinity, as shown in our previous report [25]. In this study, an EGFR-based CAR, consisting of scFv of mAb 108 linked to CD28 and CD3 signaling moieties, was constructed and cloned into a nonviral vector, pmaxCloning (Figure 1, *A* and *B*). On the basis of the protocol of the Nucleofector System (Lonza, Walkersville, MD), optimized nuclear transfer of lymphocytes was performed in a preliminary experiment. We found that the best balance between transfection efficiency and cell survival could be achieved using  $8 \times 10^6$  T cells and 3  $\mu$ g of plas-

mid per electroporation cuvette and the T-23 program (data not shown). This method was thus used in the subsequent experiments.

The expression of the CAR was first confirmed by staining with anti-CD3 $\zeta$  antibody in Western blot analysis. As shown in Figure 1C, a protein of the expected molecular mass (80 kDa) was detected. The surface expression of the CAR on T cells was further identified by staining with anti-EGFR scFv serum. Cells were then analyzed by flow cytometry; we found that only 1.19% and 1.3% of the cells showed binding of anti-EGFR scFv serum, while 32.05% of T cells expressed the CAR (Figure 1D).

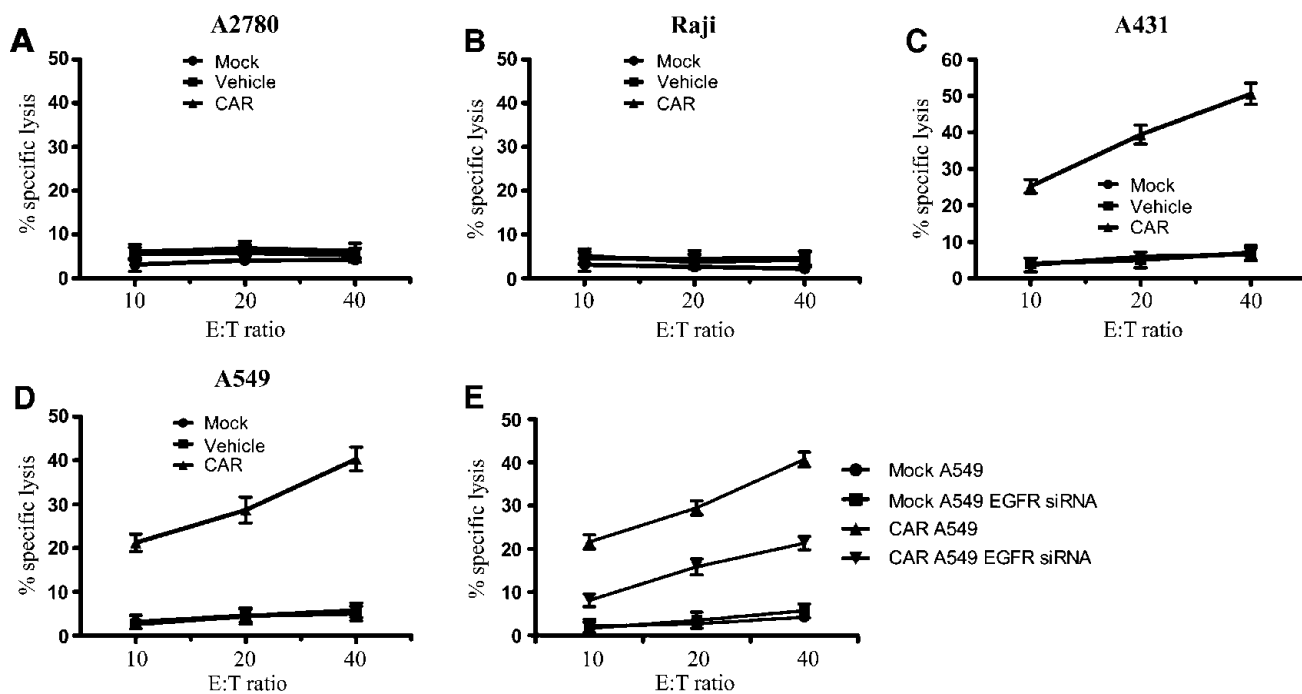
### *Functional Characterization of Anti-EGFR CAR-Expressing Primary Human T Cells*

To determine whether CAR-modified lymphocytes could specifically target and kill tumor cells, we first determined the expression of EGFR in several cell lines using flow cytometry, employing staining with the anti-EGFR antibody. Two EGFR overexpression cell lines (A431 and A549) and two EGFR-negative cell lines (A2780 and Raji) were selected for use in the subsequent experiments (Figure 2A). To investigate whether the CAR-modified T cells were capable of



**Figure 2.** Cytokine production triggered by EGFR-specific CAR. (A) The expression of EGFR of four tumor cells was detected by flow cytometric analysis. A431, A549, A2780, and Raji cells were washed once by phosphate-buffered saline and then incubated with anti-EGFR antibody and goat anti-rabbit IgG FITC-conjugated antibody. (B) CAR-modified T lymphocytes were cultured with A431, A549, A2780, and Raji tumor cells for 24 hours. Supernatants were harvested and the production of IFN- $\gamma$ , GM-CSF, IL-2, and IL-4 were detected by ELISA. Results were expressed as means  $\pm$  SD of triplicate samples and representative of three experiments (\**P* < .01).





**Figure 3.** Cytotoxicity of the EGFR-specific CAR to EGFR overexpression tumor cells. (A–D) The CAR-modified T cells were co-cultured with A431, A549, A2780, or Raji tumor cells at various effector-to-target (E:T) ratios at 37°C for 6 hours. The cytotoxicity of CAR-modified T cells to target cell lysis was assessed by LDH release assay. Results were expressed as means  $\pm$  SD of triplicate samples and were representative of three experiments. (E) Decreased cytotoxicity of the CAR to EGFR silencing A549 cells. A549 cells were transfected with EGFR siRNA using Lipofectamine 2000 reagent following the manufacturer's instruction for 24 hours. The cytotoxicity of CAR-modified T cells to EGFR silencing A549 cells was performed similarly as in A to D.

specifically recognizing tumor cells, a panel of tumor cell lines and the amount of secreted effector cytokines, IFN- $\gamma$ , GM-CSF, IL-2, and IL-4, were determined. The CAR-modified T cells produced high levels of Tc1 cytokines IFN- $\gamma$ , GM-CSF, and IL-2 as well as the Tc2 cytokine IL-4 when they were co-cultured with EGFR-overexpressing tumor cells, whereas the CAR-modified T cells co-cultured with EGFR-negative tumor cells showed no significant cytokine secretion. In addition, the mock-treated (Mock) and vehicle-treated (Vehicle) T cells co-cultured with either EGFR-overexpressing or EGFR-negative tumor cell lines secreted no measurable cytokines (Figure 2B).

The cytotoxicity of CAR-modified lymphocytes was next evaluated by an LDH release assay. As shown in Figure 3, A to D, the CAR-modified T cells were able to lyse A431 and A549 tumor cells but not A2780 and Raji cells. Down-regulation of EGFR expression in A549 cells by siRNA was also performed to consequently verify the EGFR-mediated killing by CAR T cells. In addition, the results showed that cytotoxicity of CAR-modified lymphocytes was significantly decreased in EGFR silencing A549 cells (Figures W1 and 3E). The apoptosis staining was used to visualize the binding capacity of CAR-modified lymphocytes to tumor cells and the killing of tumor cells depending on EGFR expression. Sixteen hours after the co-cubation with T lymphocytes, apoptosis or already dead A549 cells were identified by propidium iodide (PI) staining. The ratio of apoptotic and necrotic EGFR silencing A549 cells decreased significantly compared with normal A549 cells after the treatment of CAR T cells (Figure W2A). To quantitatively measure cell apoptosis, the cells are double stained with annexin V and PI. The annexin V-positive and PI-negative A549 cells were also obviously reduced when EGFR was

silenced (Figure W2A). Taken together, the above data showed that CAR-modified T cells could effectively mediate antigen-specific cytokine release and cytolytic activity.

T cell proliferative ability is also a very important aspect for the antitumor immune response, in addition to their cytotoxicity and cytokine production. *In vitro* expansion of lymphocytes was performed to determine whether CAR-modified T cells retained the same cell proliferative ability as control T cells. About 50-fold expansion of CAR-modified T cells could be achieved under standard culture conditions on day 12 (Figure 4A). Next, we assessed whether the proliferation of CAR-modified T cells was an antigen-dependent or antigen-independent effect *in vitro*. As shown in Figure 4B, CAR-modified T cells exhibited significantly greater proliferation compared to Mock and Vehicle T cells after stimulation with A431 cells. However, no significant proliferation was observed when these different T cells were stimulated with EGFR-negative A2780 cells (Figure 4C). Another experiment was designed to verify whether CAR-modified T cells could also retain favorable proliferation *in vivo*. Three weeks after establishment of an A549 lung metastasis tumor model in NOD/SCID mice, the same numbers of Vehicle and CAR-modified T cells were *i.v.* co-injected. The sequences of the vector and anti-EGFR scFv were used to monitor the persistence of these cells. The results suggested that CAR-modified T cells had a higher *in vivo* persistence than Vehicle T cells (Figure 4D).

#### Evaluating Antitumor Responses of CAR-Modified Human Primary T Cells *In Vivo*

The *in vivo* antitumor efficacy of CAR-modified T cells was first evaluated in an A431 tumorigenicity model. Animals were monitored

for a total of 60 days after co-inoculation of lymphocyte/tumor cell mixture. The mice infused with CAR-modified lymphocytes displayed a smaller tumorigenic proportion and lower tumor growth (Figure 5A). The tumor volume of the mice treated with CAR-modified lymphocytes was only half of that of the control groups on day 60 (Figure 5, B and C). The antitumor activity of CAR-modified T cells against an established A431 and A2780 s.c. tumor model was next evaluated. The mice were administered (i.v. through tail vein) CAR-modified T cells at days 23, 24, and 25, when tumors had grown to 200 to 500 mm<sup>3</sup> in size. A431 tumor-bearing mice treated with CAR-modified T cells showed significant inhibition of tumor growth and prolonged survival of animals compared with mice treated with Mock and Vehicle T cells (Figure 5, D and E). In contrast, all of the mice treated with Mock, Vehicle, and CAR-modified T cells showed no specific antitumor effect above that observed with NS in A2780 tumor model (Figure 5, F and G).

Advanced tumor is usually combined with occasional metastatic spread to the lung. A xenogeneic model of advanced lung metastatic A549 cancer was established through tail vein injection to evaluate the functional activity of CAR-modified T cells. CAR-modified T cells were administered at days 3, 6, 9, 12, 15, and 18 after tumor injection (Figure 6A). As shown in Figure 6, B and C, A549-derived tumor lung metastasis was significantly decreased when mice were treated with CAR-modified T cells. The mice treated with CAR-modified T cells had a very low tumor metastasis index ( $P < .05$  compared with all other groups), whereas the majority of mice treated with NS or Mock or Vehicle T cells had a higher tumor index (Figure 6C). Lung weights were also significantly different ( $P < .05$

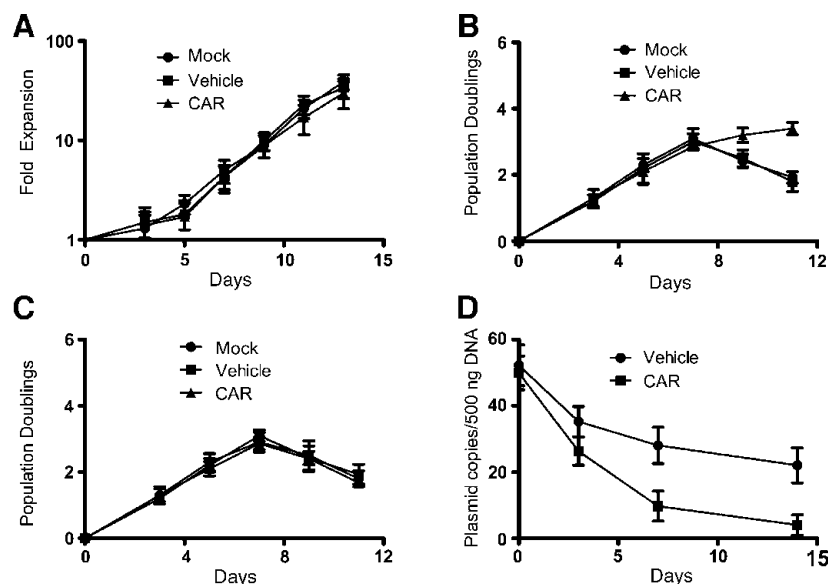
between mice treated with CAR-modified T lymphocytes and the other control groups (Figure 4D).

#### Analysis of Toxic Effects Induced by Systemic Administration in Mice

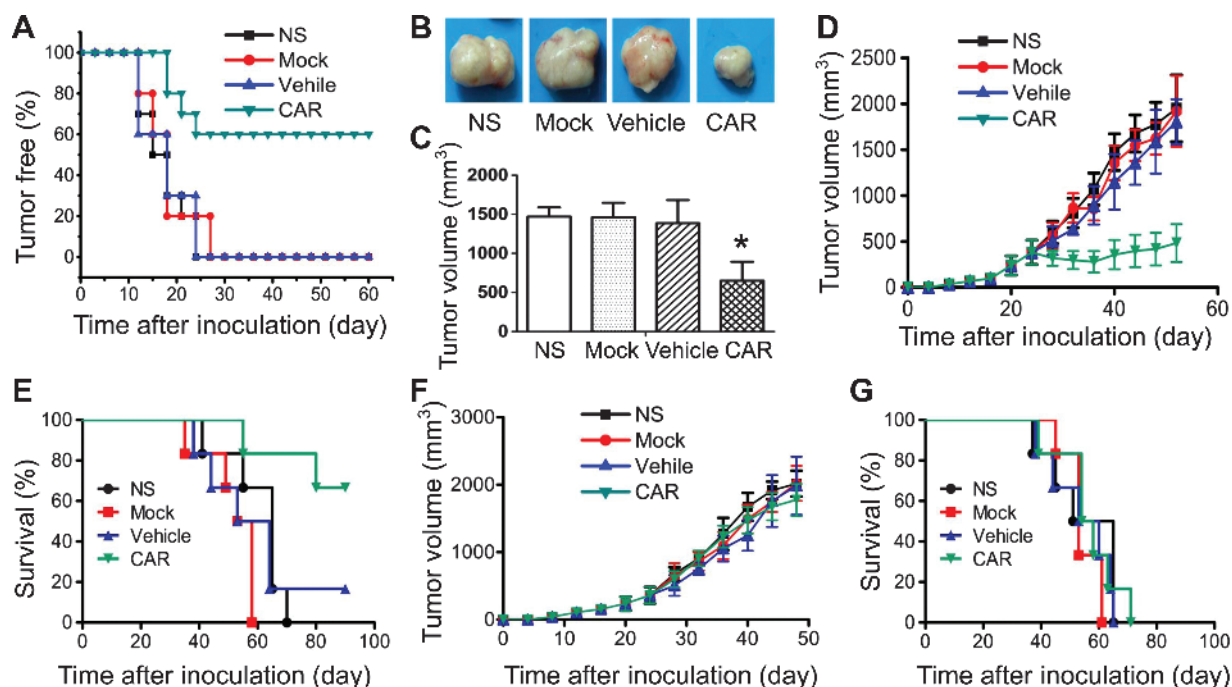
Systemic toxic effects in the mice were evaluated to characterize whether treatment with CAR-modified T lymphocytes is safe in pre-clinical experiments. Single doses of  $2 \times 10^7$  CAR-modified T cells per mouse were given by tail vein injection. Blood samples were collected from the mice after treatment, and serum levels of liver AST and serum ALT were measured. However, treatment with CAR-modified T cells did not increase the levels of AST and ALT, indicating an absence of significant liver toxicity in the mice (Figure 7A). Some of the mice from the lung metastasis model experiment were also sacrificed 48 hours after the last T cell administration, and serum was separated; the same results as in Figure 7A were obtained (Figure 7B). The results of H&E staining showed that mice treated with CAR-modified T cells had no pathologic changes in major organs compared with NS mice (Figure 7C). Collectively, these data suggested that CAR-modified T cells have no or only minimal systemic acute toxicity.

#### Discussion

The long-term goal of tumor immunotherapy is to enhance the targeting ability of the immune system to the cancer antigen. However, this is very challenging, because most tumor-specific antigens are autologous proteins and have a low immunogenicity. Moreover, tumors



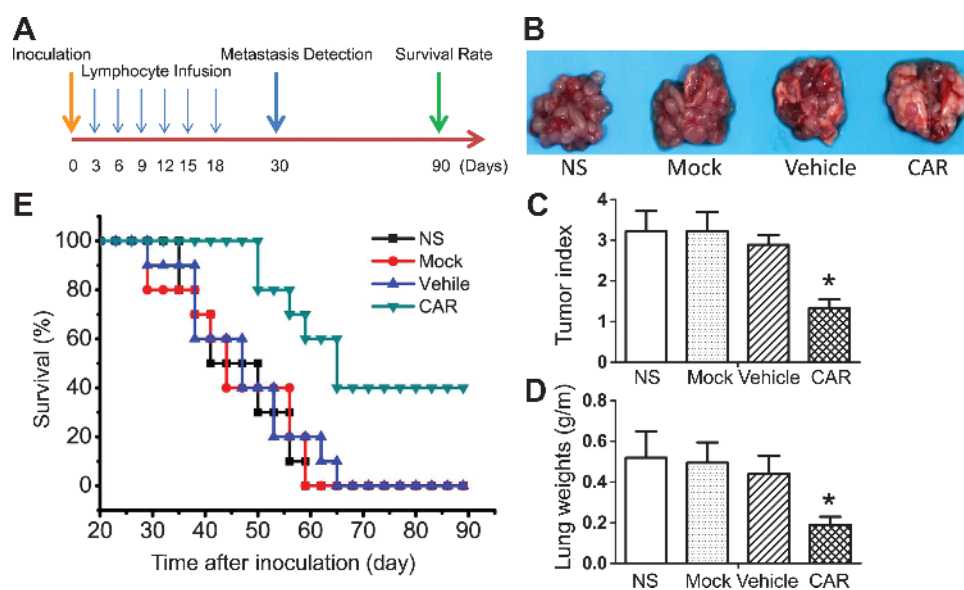
**Figure 4.** Proliferation characteristics of EGFR-specific CAR-modified T cells. (A) *In vitro* expansion of human T lymphocytes following different transduction conditions. Twelve hours after nucleofection, exogenous IL-2 was added to the cultures, every other day, at 300 IU/ml. Data are representative of three separate experiments. (B and C) *In vitro* expansion of CAR-modified T lymphocytes with antigen-dependent effect. CAR-modified T lymphocytes were cultured under the same condition just as in A. Cultures were restimulated with either A431 cells (B) or A2780 cells (C) at 7 days. Positive T cells were stained with anti-HA tag antibody and counted by flow cytometry. Data are representative of at least two separate experiments. (D) *In vivo* persistence of EGFR-specific CAR-modified T cells. The same numbers of Vehicle and CAR T cells were co-injected (i.v.) into NOD/SCID mice bearing a A549 lung metastasis model. The number of copies of Vehicle and CAR vectors in the spleen DNA from mice was evaluated, at various times following T cell injection, by quantitative PCR. Bars, means  $\pm$  SD.



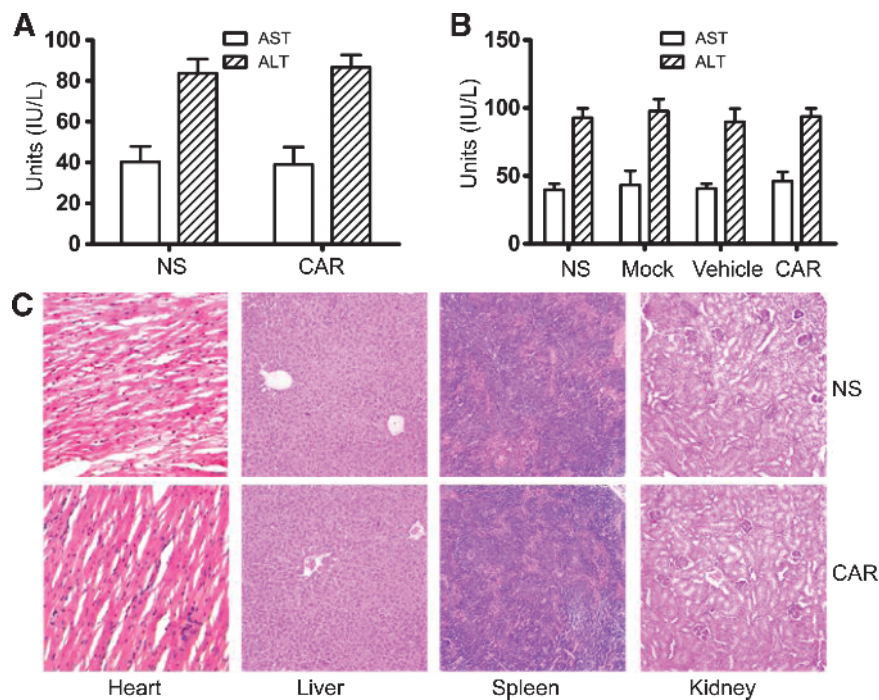
**Figure 5.** Efficient inhibition of s.c. tumor growth by CAR-modified T cells *in vivo*. (A–C) The suppression of tumorigenicity of A549 cells by CAR-modified T cells. CAR-transfected lymphocytes and A549 tumor cells were co-inoculated s.c. at a ratio of 10:1. Tumorigenicity of the animals was monitored and calculated every 3 days (A). All of the mice were sacrificed and the mean tumor volume was calculated. Bars, means  $\pm$  SD (\* $P$  < .05) (B and C). (D–G) Inhibition of the tumor growth by CAR-modified T lymphocytes. A431 or A2780 tumor cells were implanted s.c. into NOD/SCID mice at day 0. Once tumors had grown to 200 to 500 mm<sup>3</sup>, animals were grouped and i.v. injected with CAR-modified T cells on days 23, 24, and 25. (D) Tumor volume of A431 tumors. Bars, means  $\pm$  SD. (E) Survival of animals bearing A431 tumors. (F) Tumor volume of A2780 tumors. Bars, means  $\pm$  SD. (G) Survival of animals bearing A2780 tumors.

can also downregulate the expression of the MHC and immunosuppressive molecules and cytokines to escape the attacks of the immune system [27]. To overcome this difficulty, many researchers have tried a variety of immunotherapeutic strategies, including tumor vaccine

[28,29], mAb [30,31], and ACT [15,32]. Genetically modified T lymphocytes, which can express a CAR to recognize tumor antigens, hold great potential as a treatment strategy in the field of cancer immunotherapy [33].



**Figure 6.** CAR-modified T cells inhibit pulmonary metastases in A549 mouse model. NOD/SCID mice received i.v. injections of A549 tumor cells and were randomized into four groups before beginning therapy with CAR-modified T cells. Treatment strategy of A549 tumor-bearing mice as shown in A. (B) Kaplan-Meier survival of tumor-bearing mice. (C–E) Five mice per group were sacrificed at day 30, and the mean lung metastases index and lung weights of each group were calculated. Bars, means  $\pm$  SD (\* $P$  < .05)



**Figure 7.** Systemic toxicity evaluation of CAR-modified T cells. (A) Acute toxic effects induced by a single i.v. injection of CAR-modified T cells ( $2 \times 10^7$  cells per mouse). Forty-eight hours later, the mice were sacrificed and serum was separated. Levels of serum ALT and AST were measured. Bars, means  $\pm$  SD. (B and C) Acute toxic effects induced by multiple i.v. injections of CAR-modified T cells. Some of the mice from the lung metastasis model were sacrificed 48 hours after the last cell administration, and several organs and serum were separated. (B) Levels of ALT and AST were measured using the same method as in A. Bars, means  $\pm$  SD. (C) The heart, liver, spleen, and kidney tissues were used for morphologic analysis and H&E staining (original magnification,  $\times 100$ ).

In contrast to tumor infiltrating lymphocytes (TIL) or cytokine-induced killer (CIK), CAR is used to overcome MHC restriction on the inherent antigen presentation of T lymphocytes. The core part of this strategy is the generation of genetically modified T lymphocytes. An effective T lymphocyte must meet the following rigorous conditions: capacity to specifically recognize tumor targets, long time *in vivo* persistence, low immunogenicity risk, and it should be easily obtained. In the present study, we have generated a new kind of EGFR-specific CAR gene-modified T lymphocytes using a nonviral vector delivered by nucleofection technology. The results of Western blot and flow cytometry experiments showed that the CAR was efficiently expressed on the cell membrane. Moreover, this CAR-modified T cells demonstrated good antitumor effects both *in vitro* and *in vivo*, without MHC restriction. Moreover, no marked systemic toxicity was detected during the treatment process.

The selection of the target antigen is an important aspect for CAR gene-mediated cell therapy. EGFR is overexpressed on the cell surface of a wide variety of solid tumors [34]. It plays crucial roles in cell signaling, cell proliferation, regulation of apoptosis and angiogenesis, and tumor occurrence and development. Several targeting agents directed at tumors that express high levels of EGFR have been approved for clinical treatment [5,6,35]. Prerequisites for effective CAR-modified lymphocytes are the target specificity and antigen affinity of the scFv. An anti-EGFR single-chain antibody gene was previously obtained by ribosome display technology, and an anti-EGFR immunotoxin was generated in our previous study [25]. The recombinant anti-EGFR immunotoxin showed high specificity, affinity, and effective cytotoxicity to EGFR-positive cells, both *in vitro* and *in vivo*. However, higher affinity is not always advantageous because

of the potential toxicity (on target/off tumor) to normal tissue. The EGFR antigen is also weakly expressed in some normal tissues. If the affinity of the anti-EGFR scFv is too high, CAR-modified lymphocytes may bind to normal tissues before it arrives at the tumor tissue and thus cause serious side effects [22,36,37]. Therefore, we have examined the potential systemic toxicity of CAR-modified lymphocytes; our results showed that it may be a safe tumor therapy strategy.

*In vivo* persistence of CAR-modified lymphocytes has also great impact on therapy efficacy, in addition to targeting specificity. Injection or expressions of cytokines, immune clearance, and addition of gene fragments that can extend the life of CAR-modified T lymphocytes to the intracellular domain of CAR have been used extensively. Several researchers have reported that IL-2, IL-7, IL-15, and IL-21 could improve *in vivo* persistence and antitumor effects [38–41]. However, expression of these cytokines will be regulated by many factors, such as Treg cells [42]. It is hard to judge to what extent these cytokines will promote tumor therapeutic efficacy. In the present study, *IL15* was designed to be co-expressed with the CAR in the T lymphocytes. However, this strategy did not significantly improve the therapeutic effectiveness of CAR-modified lymphocytes. In addition, some researchers found that Treg cells soon have a marked negative regulatory effect following injection of therapeutic lymphocytes [43]. Rosenberg et al. have improved the therapeutic response, to more than 70%, using a sublethal dose of radiation before applying ACT. However, many older patients are not able to tolerate sublethal doses of immune clearance strategies, and high-dose IL-2 can cause significant side effects, such as renal toxicity [44]. CAR construction has improved from the first to the third generation in recent years. Some studies have shown that the first-generation CARs only had



basic antitumor effects, and there was insufficient evidence to prove that the CAR-modified lymphocytes could significantly improve antigen-mediated cell proliferation. The main design of the second generation of CARs was the insertion of the CD28 or 4-1BB intracellular signaling domains between the transmembrane region and the CD3 $\zeta$  domain. Experimental results have proven that second-generation CARs can significantly enhance lymphocyte proliferation, while still maintaining the same antitumor effects as first-generation CARs [45,46]. Third-generation CARs were produced by connecting a third intracellular signal component to the second-generation CAR. These modified CARs can further amplify the signal transduction triggered by antigen binding by the CAR, thus markedly enhancing its antitumor effects. However, it also increases the risk in clinical treatment because of the potential of producing a high level of inflammatory cytokines in a short time, termed a “cytokine storm” [47,48]. On the basis of this concern, we designed the CAR used in this study following a second-generation approach, hoping in so doing to keep the balance between sufficient antitumor effects and low systemic toxicity.

Gene transfection of lymphocytes is also a major problem in immunology research. The most popular strategy used in transfection of lymphocytes employs an integrated virus, such as yielded by lentiviral and retroviral vectors. These viruses can randomly integrate into the genome of lymphocytes, which may lead to the activation of some unknown cancer genes and other disease genes, resulting in a latent hazard [49]. In this study, we designed the CAR construct using a nonviral vector and achieved efficient transfection efficiency using the Nucleofector System after optimizing the electric transfer parameters. Cell viability could be maintained at more than 85%, and transfection efficiency of lymphocytes achieved was 25% to 35%. Although there is still a big gap of the transfection efficiency of nucleofector compared with viral vectors, it is rare in the nonviral vector system and has a higher safety. The nonviral vector-based CAR in our study could be continually expressed *in vivo* in 2 weeks, which ensured a sufficient time for its antitumor effect (Figure 4D).

Taken together, we have investigated the anticancer ability of the EGFR-targeting scFv in genetically modified EGFR-targeting lymphocytes. The EGFR-targeted CAR-modified T lymphocytes demonstrated specific cytotoxicity and expressed cytokines efficiently after stimulation with the tumor antigen. Through the recognition of EGFR, our ACT method directly inhibited tumor formation, growth, and metastasis, and the CAR-modified lymphocyte treatment strategy demonstrated good safety. Our study provides an experimental basis for the clinical application of genetically engineered EGFR-targeted lymphocytes; moreover, we also evaluated a new and interesting therapy protocol.

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## Supplementary Materials and Methods

### Antibody

Anti-EGFR and anti-CD3 $\zeta$  antibodies were purchased from Cell Signaling Technology (Danvers, MA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Trevigen (Gaithersburg, MD). To prepare anti-EGFR scFv serum, New Zealand white rabbits (Laboratory Animal Center of Sichuan University, Chengdu, China) were immunized s.c. three times with a mixture of EGFR scFv protein/complete and incomplete Freund's adjuvant; then, the rabbits were sacrificed and serum collected and stored at  $-80^{\circ}\text{C}$ .

### CAR Gene Transduction of Human T Cells

After stimulating with anti-CD3 and anti-CD28 mAbs for 5 days, human primary T cells were washed once in RPMI 1640 medium without FBS and were resuspended in 100  $\mu\text{l}$  of nucleofactor solution; 3  $\mu\text{g}$  of plasmid DNA (viz., EGFR-specific CAR vector or an equal amount of the vehicle vector, in moles) was immediately added and mixed well with the cell solution. The cell/DNA mixture was subsequently transferred to an electroporation cuvette and placed in the Nucleofector device (Lonza). Nucleofection of the cells was accomplished using the T-23 program, and samples were immediately transferred to six-well plates containing 2 ml of prewarmed media without hIL-2. Cells were incubated in a humidified  $37^{\circ}\text{C}/5\% \text{CO}_2$  incubator. Medium that contained IL-2 was changed 12 hours after the transfection.

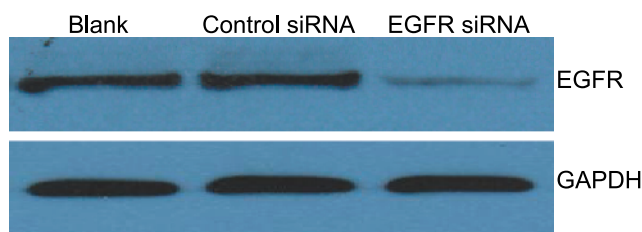
### Detection of CAR Expression on Human T Cells

For Western blot analysis, human T cells transfected with the CAR construct were lysed in RIPA buffer and the cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 12% acrylamide gel. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which was then incubated first with a rabbit anti-human CD3 zeta antibody and then with a goat anti-rabbit IgG HRP-conjugated antibody. Antibody binding was revealed using an Enhanced Chemiluminescent Kit (Santa Cruz Biotechnology Inc, Santa Cruz, CA).

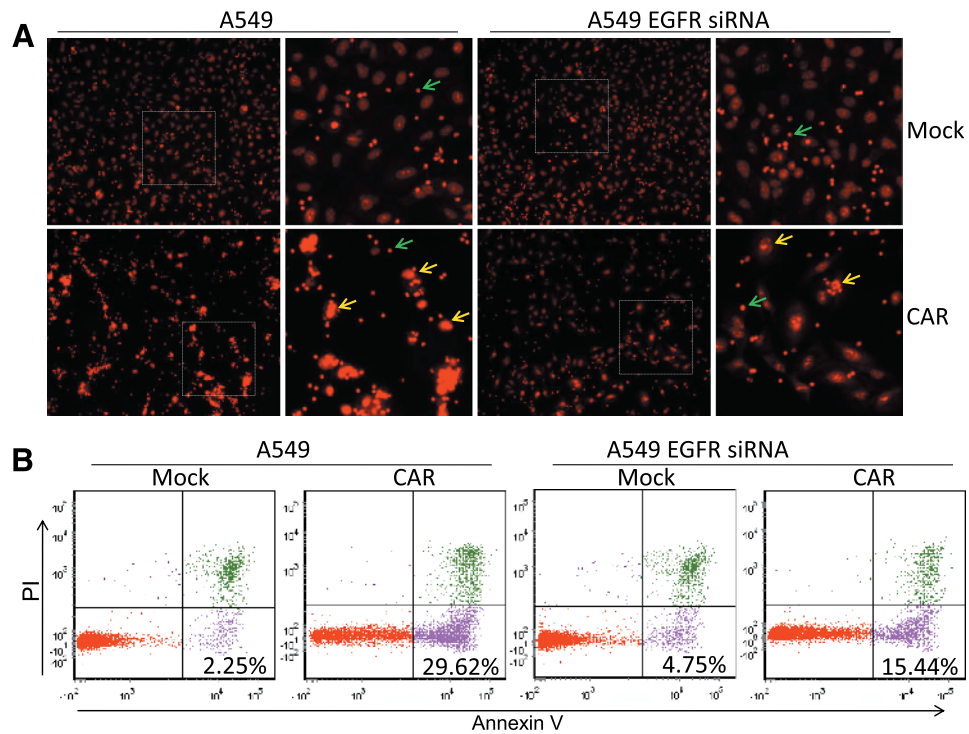
For flow cytometric analysis, CAR construct–transfected human T cells were washed once with phosphate-buffered saline and then incubated with anti-EGFR scFv serum and subsequently with a goat anti-rabbit IgG fluorescein isothiocyanate (FITC)–conjugated antibody. Positive cells were detected using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

### Competitive In Vivo Persistence Experiment with CAR T Cells and Vehicle T Cells

Mice were engrafted with  $2 \times 10^6$  A549 cells, followed 3 weeks later by escalating doses of a 1:1 mixture of CAR T cells and Vehicle T cells, after adjusting according to the transfection efficiency. Some of the animals were sacrificed and spleen samples were collected 0, 7, 14, and 28 days after T cell injection. The biodistribution of the CAR T cells and Vehicle T cells were investigated using quantitative PCR for the vector and anti-EGFR scFv sequence.



**Figure W1.** Down-regulation of EGFR expression in A549 cells by siRNA. A549 cells were transfected with EGFR siRNA using Lipofectamine 2000 reagent following the manufacturer's instruction for 24 hours. The transfected A549 cells were lysed in RIPA buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 12% acrylamide gel. After transfer to a PVDF membrane, this was incubated with anti-EGFR or anti-GAPDH primary antibody and goat anti-rabbit IgG HRP-conjugated second antibody. Antibody binding was revealed by using the Enhanced Chemiluminescent Kit.



**Figure W2.** Visualization of the binding capacity and cytotoxicity of CAR-modified lymphocytes to tumor cells. A549 cells were transfected with EGFR siRNA using Lipofectamine 2000 reagent following the manufacturer's instruction for 24 hours. Then, the A549 cells were co-incubated with T lymphocytes. (A) Sixteen hours later, apoptosis or already dead A549 cells were identified by PI staining. A549 cells (yellow arrow) with bigger nuclei can be differed from lymphocytes (green arrow; original magnification,  $\times 100$ ). (B) Four hours later, the cells are double stained with annexin V and PI and detected by flow cytometry.