

Redirecting Gene-Modified T Cells toward Various Cancer Types Using Tagged Antibodies

Koji Tamada^{1,2,3}, Degui Geng¹, Yukimi Sakoda^{1,3}, Navneeta Bansal¹, Ratika Srivastava¹, Zhaoyang Li¹, and Eduardo Davila^{1,2}

Abstract

Purpose: To develop an adaptable gene-based vector that will confer immune cell specificity to various cancer types.

Experimental Design: Human and mouse T cells were genetically engineered to express a chimeric antigen receptor (CAR) that binds a fluorescein isothiocyanate (FITC) molecule, termed anti-FITC CAR T cells. Various antibodies (Ab) currently in clinical use including cetuximab (Ctx), trastuzumab (Her2), and rituximab (Rtx) were conjugated with FITC and tested for their ability to bind tumor cells, activate T cells, and induce antitumor effects *in vitro* and *in vivo*.

Results: Anti-FITC CAR T cells recognize various cancer types when bound with FITC-labeled Abs resulting in efficient target lysis, T-cell proliferation, and cytokine/chemokine production. The treatment of immunocompromised mice with human anti-FITC CAR T cells plus FITC-labeled cetuximab (FITC-Ctx) delayed the growth of colon cancer but unexpectedly led to the outgrowth of EGFR receptor (EGFR)-negative tumor cells. On the other hand, in a human pancreatic cancer cell line with uniform EGFR expression, anti-FITC CAR T cells plus FITC-Ctx eradicated preestablished late-stage tumors. In immunocompetent mice, anti-FITC CAR T cells exhibited potent antitumor activity against syngeneic mouse breast cancer expressing Her2 and B-cell lymphoma expressing CD20 by combining with FITC-Her2 and FITC-Rtx, respectively. In addition, the activity of anti-FITC CAR T cells could be attenuated by subsequent injections of nonspecific FITC-IgG.

Conclusion: These studies highlight an applicability of anti-tag CAR technology to treat patients with different types of cancers and a possibility to regulate CAR T-cell functions with competing FITC molecules. *Clin Cancer Res*; 18(23); 6436–45. ©2012 AACR.

Introduction

The generation of a versatile system that affords T cells an ability to recognize various types of cancers has major clinical ramifications. One of the strategies to achieve this goal is genetic engineering to express a chimeric antigen receptor (CAR) on T cells (1–3). The extracellular domain of a CAR contains single-chain fragment variable (scFv) of V_L and V_H domains derived from the antigen (Ag)-binding

fragment of monoclonal antibody (mAb) against cancer cells. The scFv portion is linked to a transmembrane domain followed by a tyrosine-based activation motif such as that from CD3 ζ or Fc ϵ . The so-called third generation CAR includes additional activation domains from costimulatory molecule such as CD28 and CD137 (4-1BB) which serve to enhance T-cell proliferation and survival (1, 2, 4). CAR T cells seek out and destroy cancer cells by recognizing tumor-associated Ag (TAA) expressed on their surface in an MHC-independent fashion. Various preclinical and early-phase clinical trials highlight the efficacy of CAR T cells to treat patients with cancer (5–8).

Despite the promise of CAR T cells in cancer therapy, current methods encompass several limitations in its development as generalized clinical application. First, as no single TAA is expressed by all cancer types, scFv encoded by CAR genes need to be constructed for each potential TAA. The inability of current CAR to target more than one Ag on tumors could promote preferential growth of more aggressive, tumor Ag-negative cancer cells. Second, there also exists a major financial cost and labor-intensive procedures associated with identifying and engineering scFv against a variety of TAAs. Therefore, generation of flexible CAR

Authors' Affiliations: ¹Marlene and Stewart Greenebaum Cancer Center, ²Department of Otorhinolaryngology-Head and Neck Surgery, University of Maryland, Baltimore, Maryland; and ³Department of Cellular Signal Analysis, Yamaguchi University Graduate School of Medicine, Ube, Japan

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

D. Geng and Y. Sakoda share first co-authorship of this study.

Corresponding Authors: Eduardo Davila and Koji Tamada, Greenebaum Cancer Center, University of Maryland Baltimore, 655 W. Baltimore St., Baltimore, MD 21201. Phone: 410-706-5051; Fax: 410-706-2484; E-mail: edavila@som.umaryland.edu and ktamada@som.umaryland.edu

doi: 10.1158/1078-0432.CCR-12-1449

©2012 American Association for Cancer Research.

Translational Relevance

While T cells capable of recognizing and destroying cancer cells expressing tumor-associated antigens show promise in treating patients, several limitations preclude the widespread use of this form of therapy. These obstacles include the fact that chimeric antigen receptor (CAR) T cells can destroy noncancerous tissue and can only recognize a single tumor-associated antigen, and labor-intensive and high financial cost associated with generating engineered T cells. We improve the existing CAR technology by developing a versatile system that grants T cells specificity to a tag conjugated with antibodies. These studies emphasize the potential of using a single-gene vector to treat patients with different types of cancers and an ability to modulate CAR T-cell responses if needed.

platforms that can confer T cells specificity against multiple TAA is highly desired.

In this report, we describe a new generation of CARs in which a single construct is unlimitedly adaptable to recognize a variety of TAAs. Our novel CAR consists of scFv derived from an anti-fluorescein isothiocyanate (FITC) mAb, which is connected with signaling motifs of CD28, 4-1BB, and CD3 ζ (referred to as anti-FITC CAR). FITC is a fluochrome dye that can be cheaply and easily conjugated with Ab and safely used in human body (9, 10). Specific interaction between FITC-labeled Ab and anti-FITC CAR enables T cells to target cancers in combination with a variety of tumor-reactive mAbs, including those already applied in clinic such as cetuximab (Ctx; anti-EGFR), trastuzumab (anti-Her2), and rituximab (Rtx; anti-CD20) as shown in this study, as well as those currently under development for future application. The current studies address the use of the anti-FITC CAR platform in recognizing various types of TAA for cancer immunotherapy.

Materials and Methods

Mice and cell lines

Male or female 6- to 10-week-old NOD-scid IL-2R γ null (NSG), DBA/2, C57BL/6, and C3H/HeN mice (Jackson Laboratory) were used for experiments under approval by the Institutional Animal Care and Use Committee. The human EGF receptor (EGFR)-positive colon cancer SW480 and Her2-positive AU565 breast cancer cell lines were obtained from American Type Culture Collection. Panc 6.03, a human pancreatic adenocarcinoma cell line expressing both EGFR and Her2, was provided by Dr. Elizabeth Jaffee (Johns Hopkins University, Baltimore, MD; ref. 11). The mouse B-cell lymphoma line 38C13 expressing human CD20 was provided by Dr. John Timmerman (University of California Los Angeles, Los Angeles, CA; ref. 12). The P815-TGL, a mouse mastocytoma cell line, was provided by Dr. Marcel R. van den Brink (Memorial Sloan-Kettering Cancer

Center, New York, NY; ref. 13). P815-TGL that stably expresses high-level human CD20 was established in our laboratory by gene transfection. Human Her2-expressing mouse mammary tumor cell lines D2F2/E2 and E0771/E2 were provided by Dr. Wei-Zen Wei (Wayne State University, Wayne, PA; ref. 14) and Dr. Qiao Li (University of Michigan, Ann Arbor, MI; ref. 15), respectively. The Phoenix Ampho and Eco packaging cell lines were purchased from Orbigen Inc.

Antibodies and reagents

Cetuximab (anti-EGFR; ImClone LLC), trastuzumab (anti-Her2; Genentech), rituximab (anti-CD20; Genentech), and control human IgG (Invitrogen) were conjugated with FITC by using a FITC-labeling kit (Thermo Scientific). An average of 3 FITC molecules were conjugated per one molecule of antibody. FITC-labeled dextran beads and phycoerythrin (PE)-labeled CD8 mAb were purchased from Sigma-Aldrich and eBioscience, respectively. Flow cytometric analysis of cell surface molecule expression was conducted by LSR-II (BD Biosciences).

Retroviral vector generation and transduction of human and mouse T cells

The retroviral vector backbone pMSGV1 was a kind gift from Dr. Richard Morgan (National Cancer Institute, Baltimore, MD) and is derived from pMSGV (16). The mouse scFv against FITC was generated according to the previous report (17) and linked to the hinge and transmembrane regions of the human CD8 α chain and the cytoplasmic regions of the human CD28, 4-1BB, and CD3 ζ molecules. Transduction of human and mouse T cells was conducted as previously described (18, 19). Briefly, retroviruses were produced by transfection of anti-FITC CAR plasmid into Phoenix Eco and Ampho packaging cell lines for transduction of mouse and human T cells, respectively. Supernatants containing retroviruses were harvested 48 hours after transfection. For transduction of human T cells, 3×10^6 peripheral blood mononuclear cells (PBMC) were cultured in 24-well plates in the presence of OKT3 (50 ng/mL) and interleukin (IL)-2 (50 IU/mL) for 48 hours. For transduction of mouse T cells, 3×10^6 spleen and lymph node cells were activated with plate-bound anti-CD3 mAb (2.5 μ g/mL), anti-CD28 mAb (1.2 μ g/mL), and IL-2 (100 IU/mL) for 48 hours. Supernatants containing retroviruses were mixed with the activated human or mouse T cells (1×10^6 /mL) in the presence of retronectin (10 μ g/mL) and centrifuged for 2 hours at 3,000 rpm followed by incubation for 48 hours in the presence of IL-2 (100 IU/mL). The surface expression of anti-FITC CAR on transduced T cells was determined by flow cytometry after staining with FITC-Ctx or FITC-conjugated dextran beads.

T-cell culture for proliferation, cytokine production, and cytotoxicity assay

For proliferation assay, 1×10^5 to 3×10^5 anti-FITC CAR or control T cells were cultured in 96-well tissue culture

plates in the presence of FITC-labeled Abs, nonlabeled Abs, or FITC-Dex beads for 72 hours. In some experiments, anti-FITC CAR or control T cells were co-cultured with irradiated (100 Gy) SW480 cells in the presence of FITC-labeled or nonlabeled cetuximab. Proliferative activity of T cells was assessed by incorporation of ^3H -thymidine during the last 16 hours of 3-day culture. Cytotoxic activity of CAR T cells against tumors was assessed by a standard 4-hour ^{51}Cr -release assay. Radioactivity of ^3H and ^{51}Cr was measured by Wallac Microbeta TriLux counter (PerkinElmer Inc). Concentration of cytokines and chemokines produced in the culture supernatants was measured by Bio-Plex Pro system (Bio-Rad) or Milliplex kit (Millipore). All tests were conducted in triplicate wells and results are shown as mean \pm SD.

***In vivo* models to assess antitumor therapeutic effects of CAR T cells**

In prophylactic tumor models, NSG mice were injected subcutaneously in the rear leg flank with 1×10^6 to 2×10^6 SW480 tumor cells and treated intraperitoneally (i.p.) with FITC-Ctx or nonlabeled Ctx (25 $\mu\text{g}/\text{mouse}$) 1 day later. One day after Ab injection, the mice were injected intravenously with 5×10^6 anti-FITC CART cells generated from PBMCs of healthy donors. In the therapeutic tumor model, NSG mice were injected s.c. with 1×10^6 to 2×10^6 Panc 6.03 and kept untreated until tumor grow up to 500 mm^3 . Then, the mice were treated i.p. with FITC-Ctx or nonlabeled Ctx weekly for 3 times and injected i.v. with 5×10^6 anti-FITC CART cells 1 day after the first Ab injection. In mouse models using syngeneic mouse-derived tumors, C3H/HeN mice were injected i.v. with 2×10^4 human CD20-expressing 38C13 and treated i.p. with 25 μg FITC-Rtx or nonlabeled Rtx starting 4 days after tumor inoculation and repeated weekly for 3 times. Anti-FITC CAR T cells generated from T cells of syngeneic mice were transferred i.v. into the mice 1 day after the first Ab injection. In another set of experiments, C57BL/6 mice were inoculated s.c. with human Her2-positive E0771/E2 or Her2-negative E0771 tumor and kept untreated until tumor grow up to approximately 500 to 800 mm^3 . Thereafter, the mice were exposed to sublethal irradiation (4 Gy) and 1 day later treated i.p. with FITC-Her2 or nonlabeled Her2 (25 $\mu\text{g}/\text{mouse}$) in conjunction with i.v. transfer of 5×10^6 anti-FITC CAR T cells. Injection of Ab was repeated every 5 days for total 3 times. A cohort of mice treated with FITC-Her2 was further injected i.p. with FITC-labeled nonspecific human IgG (100 $\mu\text{g}/\text{mouse}$) every 5 days for total 4 times. In all experiments, tumor size was measured every 3 to 4 days by digital calipers in a blinded manner. The survival of the treated mice was also assessed.

Statistical analysis

The 2-tailed Student *t* test was used to compare 2 groups. For survival data, Kaplan–Meier survival curves were prepared, and statistical differences were analyzed using the log-rank (Mantel–Cox) test. Differences were considered to be significant at *P* values less than 0.05.

Results

Anti-FITC CAR T cells proliferate and kill target cells following recognition of FITC-labeled Abs

We generated a CAR construct composed of anti-FITC scFv fused with 2 costimulation signaling motifs, CD28 and 4-1BB and CD3 ζ signaling domains, designated as anti-FITC CAR (Fig. 1A). The K_d of anti-FITC scFv to fluorescein is approximately 2.3×10^{-8} mol/L (20). When human PBMCs were transduced with anti-FITC CAR retrovirus, roughly 60% of total T cells expressed anti-FITC CAR as analyzed by staining with FITC-labeled cetuximab (FITC-Ctx) or FITC-labeled dextran (FITC-Dex) beads (Fig. 1B). To confirm their functionality and specificity, anti-FITC CAR T cells were cultured in the presence of titrating doses of immobilized FITC-Ctx, Ctx, FITC-Dex beads, or soluble FITC-IgG. As a control, T cells were transduced with empty vector. Anti-FITC CAR T cells proliferated vigorously following stimulation with immobilized FITC-Ctx and FITC-Dex in a dose-dependent manner (Fig. 1C). In sharp contrast, neither immobilized Ctx nor soluble FITC-IgG induced detectable proliferation, highlighting a necessity of cross-linking the anti-FITC CAR on T cells. Control T cells did not proliferate with FITC-Ctx or FITC-Dex (Fig. 1C).

Anti-FITC CAR T cells also showed robust proliferation after co-culture with EGFR-positive SW480 colon cancer cells in the presence of FITC-Ctx (Fig. 2A). Anti-FITC CAR T cells effectively lysed FITC-Ctx-stained SW480 colon cancer cells at various effector to target ratios as compared with control T cells (Fig. 2B, left). Appreciable cytolytic activity was detected at ratios as low as 1 T cell to 20 target cells. In the presence of FITC-control IgG, cytolytic activity of anti-FITC CAR cell was comparable with that of control T cells (Fig. 2B, right). To test an ability to recognize different types of cancers when bound with different Abs, cytolytic activity of anti-FITC CAR T cells was assessed against Panc 6.03 pancreatic cancer cells, which express both EGFR and Her2, in the presence of FITC-Ctx or FITC-trastuzumab (FITC-Her2) or against Her2-positive AU565 breast cancer cells in the presence of FITC-Her2. Anti-FITC CAR T cells efficiently and specifically lysed tumors in the presence of FITC-Ctx or FITC-Her2 but showed minimal cytolytic activity with nonlabeled Ab (Fig. 2C). Anti-FITC CAR T cells lysed target tumor cells in the presence of as little as 0.01 ng/mL FITC-Her2 (10:1 effector to target), highlighting their remarkable potential to become activated by low levels of FITC-Ab bound to tumor cells (Fig. 2D). Collectively, these data show anti-FITC CAR T cells' (i) functionality, (ii) specificity to kill tumor cells with FITC-Ab, and (iii) ability to target different FITC-tagged Abs and a diverse set of tumor cell types.

Anti-FITC CAR T cells delay tumor growth but result in the development of Ag-negative tumor

We next examined an ability of anti-FITC CAR T cells to eliminate tumors *in vivo*. Immunodeficient NSG mice were injected subcutaneously with SW480 colon cancer cells followed by intraperitoneal injection with Ctx, FITC-Ctx, or FITC-IgG. One day later, the mice were injected

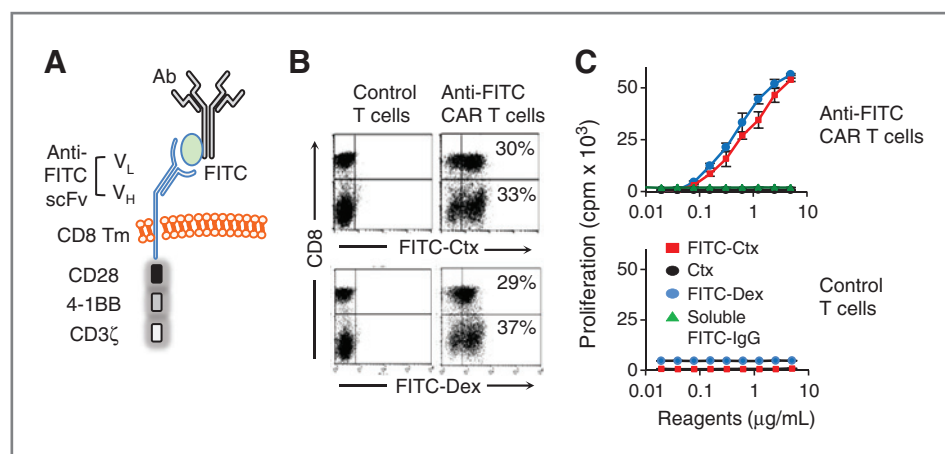


Figure 1. Generation and functional characterization of anti-FITC CAR T cells. A, schematic representation of anti-FITC scFv CAR structure containing CD28, 4-1BB, and CD3 ζ signaling domains. V_L indicates variable region of light chain, V_H indicates variable region of heavy chain, and Tm indicates transmembrane region. B, two days after retroviral transduction, expression of anti-FITC CAR on human T cells was stained by FITC-Ctx (top) or FITC-Dex beads (bottom) in conjunction with PE-conjugated anti-CD8 mAb staining and analyzed by flow cytometry. C, anti-FITC CAR and control T-cell proliferation in response to the indicated doses of plate-bound FITC-Ctx, FITC-Dex beads, Ctx, or soluble FITC-control IgG was assessed by ³H-thymidine incorporation.

intravenously with anti-FITC CAR T cells. Tumor growth kinetics was similar among mice receiving anti-FITC CAR T cells plus Ctx, anti-FITC CAR T cells plus FITC-IgG, or Ctx alone (Fig. 3A, top left). Compared with these groups, the mice receiving anti-FITC CAR T cells plus FITC-Ctx significantly delayed the growth of SW480 tumor and prolonged tumor-free period and overall survival (Fig. 3A). Despite

this survival advantage, however, mice treated with anti-FITC CAR T cells plus FITC-Ctx eventually succumbed to the tumor.

The mechanisms contributing to the failure of anti-FITC CAR T cells in long-term treatment were further investigated. CAR T cells can display a shortened life when activated with CD3 mAb plus IL-2, despite receiving prosurvival

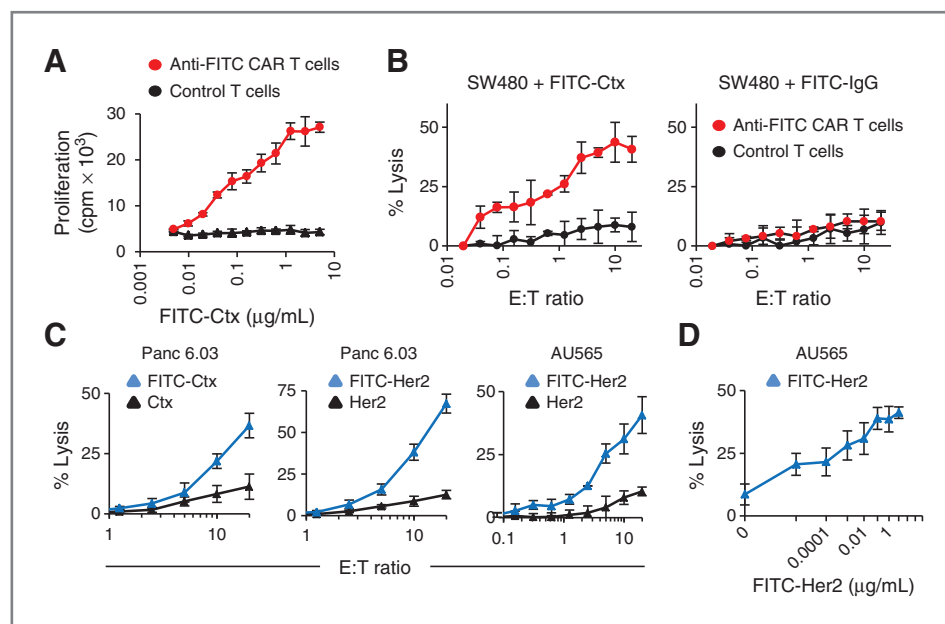


Figure 2. Proliferation and killing activity of anti-FITC CAR T cells in response to tumor cells. A, anti-FITC CAR and control T cells were incubated with irradiated SW480 tumor cells which were pulsed with indicated doses of FITC-Ctx. Proliferative activity of T cells was examined by ³H-thymidine incorporation. B, cytotoxic activity of anti-FITC CAR and control T cells against SW480 in the presence of FITC-Ctx or FITC-IgG was assessed by ⁵¹Cr-release assay at the indicated effector to target (E:T) ratios. C, cytotoxic activity of anti-FITC CAR T cells against Panc 6.03 or AU565 tumors in the presence of FITC-Ctx, nonlabeled Ctx, FITC-Her2, or nonlabeled Her2 was examined at the indicated E:T ratios. D, anti-FITC CAR T-cell cytotoxicity against AU565 in the presence of titrated doses of FITC-Her2 was also examined (E:T ratio at 10). The data are representative of at least 2 independent experiments. All data are shown as mean \pm SD.

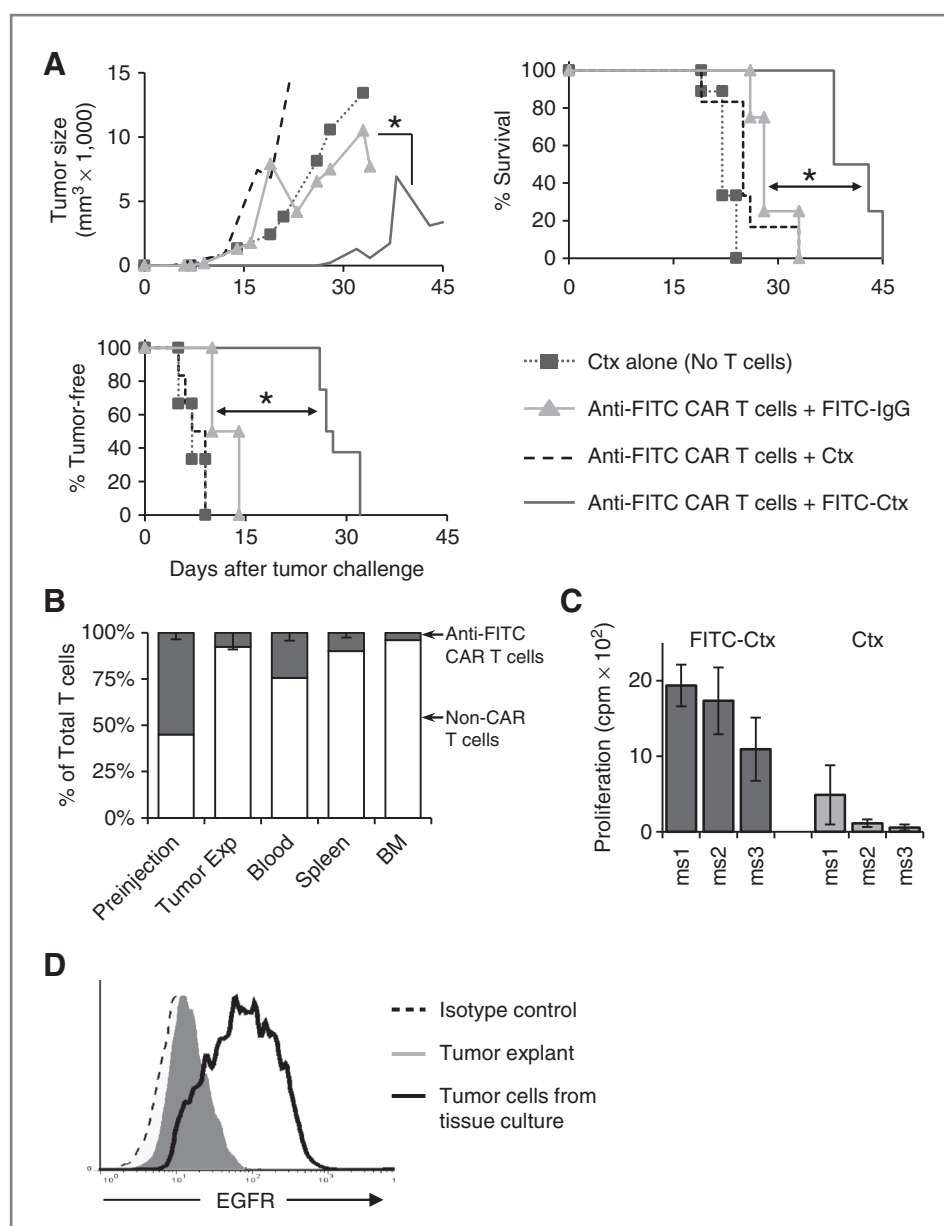


Figure 3. *In vivo* antitumor effects of anti-FITC CAR T-cell therapy and selective growth of Ag loss tumor. A, NSG mice were inoculated s.c. with SW480 and injected i.p. with FITC-Ctx, nonlabeled Ctx, or FITC-IgG 1 day later. Then, the mice were injected i.v. with anti-FITC CAR T cells generated from healthy donor PBMCs 1 day after Ab injection or left untreated. Tumor size, tumor-free period, and overall mouse survival were assessed. *, $P < 0.05$. B, the mice inoculated with SW480 and treated with FITC-Ctx and anti-FITC CAR T cells as A were sacrificed 30 to 35 days after tumor inoculation. Tumor tissue, PBMCs, spleen, and bone marrow cells were harvested and assessed for the presence of anti-FITC CAR T cells by flow cytometric analysis. The percentage of anti-FITC CAR-positive and -negative T cells in total human T cells is shown as average \pm SD ($n = 3$). C, T cells extracted from tumor explants were cultured with irradiated SW480 in the presence of FITC-Ctx or nonlabeled Ctx. Proliferative activity of T cells was assessed by ³H-thymidine incorporation. Data from 3 individual mouse samples (ms1, 2, and 3) are shown. D, EGFR expression on SW480 tumor explants and SW480 cells from tissue culture was examined by flow cytometry.

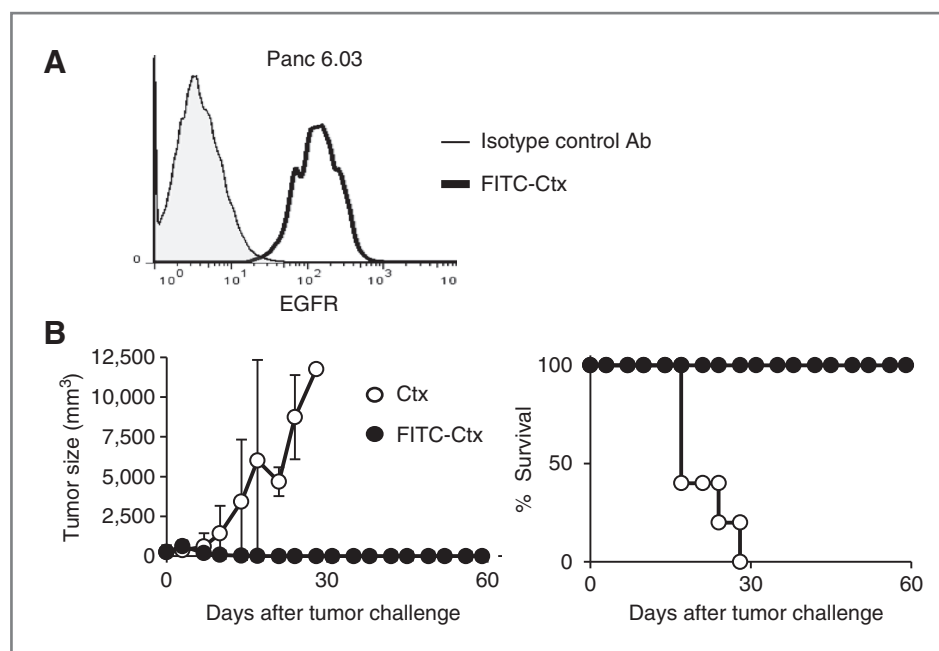
signals from CD28 or 41BB (2, 21). Therefore, the presence of anti-FITC CAR T cells in our model was examined in the spleen, liver, bone marrow, peripheral blood, and tumor explants. Approximately 10% of total human T cells detected in tumor explants were anti-FITC CAR T cells (Fig. 3B). Variable percentages of anti-FITC CAR T cells were found in other organs in the recipient mice, whereas it should be noted that these percentages were lower than the starting percentage of 60% at infusion. These results indicate a gradual loss of anti-FITC CAR T cells *in vivo*.

Eventual growth of tumor may have also resulted from intrinsic dysfunctions of anti-FITC CAR T cells, such as *in vivo* induction of anergy and exhaustion. To address this, T cells were enriched from the tumor explants and immediately stimulated *in vitro* with SW480 cells from tissue culture

in the presence of FITC-Ctx or nonlabeled Ctx. T cells in the tumor explants showed significantly enhanced proliferation (Fig. 3C) and cytokine/chemokine production (Supplementary Fig. S1) by stimulation with FITC-Ctx. These results suggest that anti-FITC CAR T cells in the tumor site maintain an ability to proliferate and release soluble factors in response to FITC-Ab bound to tumor cells.

On the basis that anti-FITC CAR T cells were present in the mice and responsive to FITC stimulation, we next examined a possibility that tumor cells downregulate EGFR expression *in vivo*. Indeed, all tumor explants from the treated mice almost completely lost EGFR expression, as compared with SW480 cells maintained in tissue culture (Fig. 3D). It should be noted that EGFR expression on tissue culture SW480 was heterogeneous with some cells lacking EGFR. Thus, inability

Figure 4. Therapeutic effects of anti-FITC CAR T cells against preestablished Panc 6.03 pancreatic tumor. **A**, EGFR expression on Panc 6.03 pancreatic tumor cell line was analyzed by flow cytometry. **B**, Panc 6.03 was inoculated s.c. into NSG mice and allowed to grow up to approximately 500 mm³. Then, the mice were injected i.p. with FITC-Ctx or nonlabeled Ctx weekly for 3 times and further transferred i.v. with anti-FITC CAR T cells 1 day after the first Ab injection. Tumor growth (left) and mouse survival (right) were assessed.



of anti-FITC CAR T cells to mediate long-term regression of SW480 was probably due to preferential survival and expansion of EGFR-negative tumors, which escaped from T-cell-mediated eradication *in vivo*.

Anti-FITC CAR T cells mediate tumor regression of Ag-positive pancreatic tumor

Given the heterogeneity of EGFR expression on SW480 tumor cells, the therapeutic ability of anti-FITC CAR T cells was further investigated in Panc 6.03 pancreatic cancer cells which express high and homogeneous EGFR (Fig. 4A). The mice inoculated with Panc 6.03 were kept untreated until a formation of vascularized late-stage tumor at approximately 500-mm³ size and then treated with anti-FITC CAR T cells plus either FITC-Ctx or nonlabeled Ctx. Injection of anti-FITC CAR T cells and FITC-Ctx induced the complete regression of tumors and achieved long-term survival of the recipient mice (Fig. 4B). No tumor relapse occurred during the time of observation. In contrast, mice treated with anti-FITC CAR T cells and nonlabeled Ctx succumbed to the tumor. These results show the ability of anti-FITC CAR T cells to eradicate established tumors when high and homogeneous Ag is targeted.

Anti-FITC CAR T cells demonstrate therapeutic effects against syngeneic tumor in immunocompetent mice

Whereas anti-FITC CAR T cells show powerful antitumor effects on human tumors in NSG mice, these models use immunodeficient hosts and tumors allogeneic to CAR T cells. Therefore, we further evaluated the efficacy of CAR T cells against syngeneic tumors in immunocompetent hosts. Similar to human CAR T cells, anti-FITC CAR T cells generated from mouse splenic T cells showed potent functionality, as shown by a significant increase of cytokine and chemokine production by stimulation with FITC-labeled

rituximab (FITC-Rtx) compared with nonlabeled rituximab (Fig. 5A). Mouse T cells expressing anti-FITC CAR devoid of intracellular signaling domains lost their ability to become activated by FITC-Ab, indicating that the signaling motifs derived from human CD28 and 4-1BB and CD3 ζ are functional in mouse T cells (Supplementary Fig. S2). Among 23 types of cytokines and chemokines tested by a multiplex assay, no specific polarization pattern, such as Th1, Th2, and Th17, was observed. Anti-FITC CAR T cells generated from mouse spleen cells exhibited profound lysis of human CD20-expressing P815-TGL tumor in the presence of FITC-Rtx but not nonlabeled rituximab (Fig. 5B, left). As expected, this cytotoxic activity was not observed against CD20-negative tumor (Fig. 5B right). Anti-FITC CAR T cells which lysed CD20-expressing tumor cells in the presence of FITC-Rtx also lysed human Her2-expressing mouse mammary tumors in the presence of FITC-labeled trastuzumab (Fig. 5C). Taken together, these results indicate that anti-FITC CAR T cells have an ability to recognize and kill syngeneic tumor cells in an Ag-specific manner.

Next, the potential for anti-FITC CAR T cells to eradicate a syngeneic tumor in immunocompetent mice was examined. Mice were inoculated i.v. with human CD20-expressing 38C13 mouse tumors as a model of rituximab-insensitive B-cell lymphoma (22) and treated with a transfer of anti-FITC CAR T cells in combination with either FITC-Rtx or nonlabeled rituximab. Survival of the mice treated with FITC-Rtx was significantly prolonged compared with those with nonlabeled rituximab (Fig. 6A; $P < 0.001$). The therapeutic effect of anti-FITC CAR T cells was further examined in another model using E0771/E2, a mouse breast cancer line expressing human Her2. Transfer of anti-FITC CAR T cells generated from syngeneic C57BL/6 mice and concurrent administration of FITC-Her2 induced rejection of

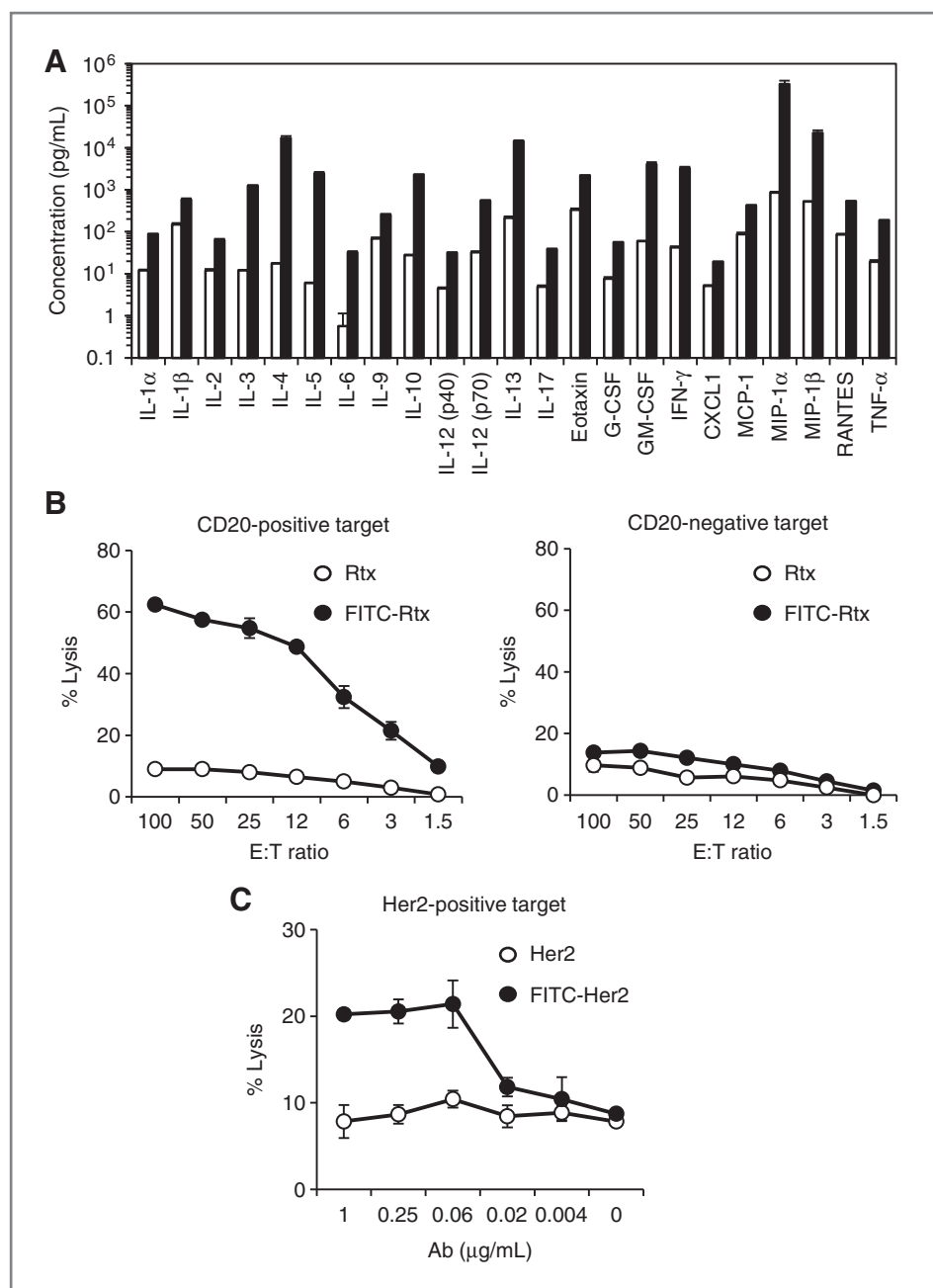


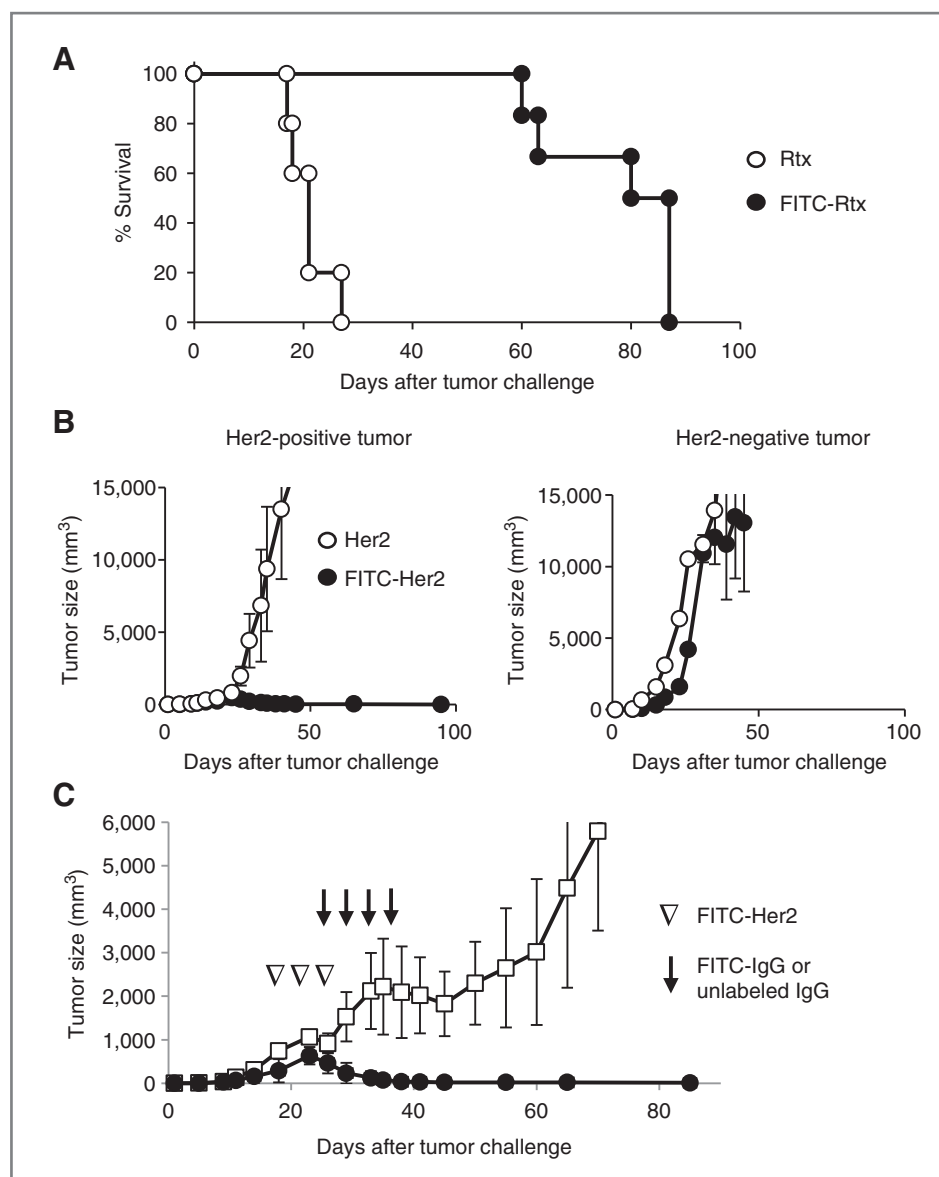
Figure 5. Cytokine/chemokine production and killing activity of mouse anti-FITC CAR T cells. **A**, anti-FITC CAR T cells generated from mouse spleen T cells were cultured in the presence of plate-bound FITC-Rtx or nonlabeled Rtx. After 3 days, concentration of 23 types of cytokines and chemokines in the culture supernatants was measured. Data were shown as mean \pm SD. **B**, anti-FITC CAR T cells generated from mouse T cells were assessed for cytotoxic activity against human CD20-positive P815-TGL (left) or CD20-negative P815-TGL (right) in the presence of FITC-Rtx or nonlabeled Rtx. Cytotoxicity at the indicated E:T ratios was tested by ⁵¹Cr-release assay. **C**, anti-FITC CAR T cells generated from mouse T cells were tested for the cytotoxic activity against human Her2-positive D2F2/E2 in the presence of FITC-Her2 or nonlabeled Her2. Cytotoxicity at 20:1 E:T ratio with the titrated doses of Ab was tested by ⁵¹Cr-release assay.

preestablished E0771/E2 but not Her2-negative parental E0771 tumor (Fig. 6B). These results show an ability of anti-FITC CAR T cells to mediate therapeutic effects against syngeneic tumors established in immunocompetent hosts.

The potential for CAR T cells to attack noncancerous tissues is referred to as "on-target off-tumor toxicity" and represents a major limitation of CAR T-cell therapy. Thus, it is in great demand to develop methods to terminate CAR T-cell responses to avoid the adverse effects. Because the effects of anti-FITC CAR T cells are exclusively dependent on binding with FITC-labeled Ab, we hypothesized that administration of excess amount of FITC-labeled nonspe-

cific Ab would diminish anti-FITC CAR T-cell function by interfering with tumor cell recognition. To address this possibility, mice inoculated with E0771/E2 tumor were treated with anti-FITC CAR T cells plus FITC-Her2 and subsequently injected with FITC-labeled nonspecific IgG. While the regular CAR T-cell therapy achieved complete tumor regression, additional injections of FITC-labeled control IgG permitted eventual tumor growth (Fig. 6C). These results suggest that the *in vivo* effects of anti-FITC CAR T cells can be attenuated by injecting FITC-labeled nonspecific IgG probably because of competitive interference of CAR T cell-tumor interaction.

Figure 6. Therapeutic effects of anti-FITC CAR T cells against syngeneic tumor in immune competent mice. **A**, C3H/HeN mice were inoculated i.v. with human CD20-positive 38C13 tumor cells. After 4 days, the mice were treated i.p. with FITC-Rtx or nonlabeled Rtx repeatedly every week for 3 times. One day after the first Ab injection, the mice were injected i.v. with anti-FITC CAR T cells generated from C3H/HeN spleen T cells. Survival of the recipient mice was assessed. **B** and **C**, C57BL/6 mice were inoculated s.c. with human Her2-positive E0771/E2 or Her2-negative E0771 tumor. When tumor size reached 500 to 800 mm³, the mice were exposed to sublethal irradiation, and 1 day later treated i.p. with FITC-Her2 or nonlabeled Her2 (25 µg/mouse) together with i.v. transfer of 5×10^6 anti-FITC CAR T cells generated from C57BL/6 spleen T cells. Injection of Ab was repeated every 5 days for 3 times. **C**, mice inoculated with E0771/E2 were treated with FITC-Her2 and anti-FITC CAR T cells as **B** and further injected i.p. with FITC-labeled nonspecific human IgG (100 µg/mouse; open square) or unlabeled human IgG (filled circle) starting 1 day after the last injection of FITC-Her2 and repeated every 5 days for an additional 3 times. Tumor size is shown as mean \pm SD.



Discussion

In the current studies, we developed a novel and adaptable approach of CAR therapy in which gene-engineered T cells acquire specificity to FITC-labeled Abs and eliminate cancers by recognition of various TAAs via Ab-dependent interactions. Our results showed that anti-FITC CAR T cells undergo significant proliferation and cytokine production by stimulation with immobilized FITC and mediate tumor lysis in the presence of FITC-labeled antitumor Abs. Transfer of anti-FITC CAR T cells together with FITC-labeled antitumor Abs prevents tumor growth and induces regression of established tumors in immunodeficient mice inoculated with human tumor as well as immunocompetent mice bearing a syngeneic tumor. Importantly, injections of FITC-labeled nonspecific Ab subsequent to FITC-labeled antitumor Ab extinguish an ability of anti-FITC CAR T cells

to attack target cells. Thus, our studies develop a new platform of CART-cell therapy which can recognize a variety of targets via FITC-labeled Abs while equipped with a system to attenuate its functions.

In this study, we observed that CAR T-cell therapy targeting a single TAA can result in transient antitumor effect and potentially lead to eventual outgrowth of TAA-negative variants when treating tumors expressing heterogeneous TAA (Fig. 3). This result underscores an importance of using CAR T cells which can target more than one TAA. In conventional CAR gene therapy, preparation of multiple CAR T cells with distinct specificity is a time-consuming and labor-intensive task. With our technology, on the other hand, it is feasible to attack multiple TAAs by transferring anti-FITC CAR T cells in conjunction with multiple FITC-labeled antitumor Abs. As intratumoral heterogeneity has

been highlighted in recent studies (1), our method to target multiple TAAs could augment therapeutic effects of CAR T cells, whereas direct demonstration of such effects needs to await our further studies. For clinical translation of our method, there are still several hurdles to overcome. First, to target multiple TAAs on a single tumor cell type, mAbs against such TAAs must be available. The number and type of U.S. Food and Drug Administration–approved anti-tumor Abs are still limited at present, and our method relies on future development of clinical-grade antitumor Abs. Second, as FITC is an immunogenic molecule, injection of FITC-labeled Abs could induce anti-FITC immune responses *in vivo*. Indeed, we detected anti-FITC Ab in the sera of the mice treated with FITC-Rtx (Supplementary Fig. S3). Anti-FITC immunity could shorten an *in vivo* half-life of FITC-Abs and weaken antitumor effects of anti-FITC CAR T cells. Eventual death of the mice in our model (Fig. 6A) might be due to the emergence of anti-FITC immunity, whereas a loss of targeted Ag would be also a possible mechanism as shown in Fig. 3D. Identification of tags with a lower immunogenicity is crucial for success of our anti-tag CAR T-cell method in cancer therapy. Finally, in immunocompetent models, we used mouse tumor cells expressing human-derived Ag including CD20 and Her2, which could be highly immunogenic for mouse immune system. To rigorously assess anti-tag CART cells for clinical application, its antitumor effects need to be examined in fully syngeneic models, in which T cells, tumor cells, tumor Ag, and the host are all derived from MHC-matched animals.

Additional advantage of anti-FITC CAR technology is its potential to restore usefulness of mAbs attenuating growth factor receptors in patients with cancer with mutated signal transduction proteins. For example, 30% to 60% patients with colon cancer express activating *Kras* mutations and do not gain therapeutic benefits from treatment with cetuximab due to a loss of its effect to block EGFR signal (23, 24). Accordingly, the current American Society of Clinical Oncology guidelines recommend that patients with colorectal cancer should be screened for *Kras* mutations before being offered cetuximab and those with *Kras* mutations should not be given cetuximab treatment (25). By combining with anti-FITC CAR technology, however, cetuximab could provide therapeutic benefits even in *Kras*-mutated cancer, as our studies showed the antitumor effects in SW480, a colon cancer harboring a homozygous mutation in codon 12 of *Kras* (26). A role of FITC-labeled antitumor mAbs in our approach is to guide gene-modified T cells to target cells, and the elimination of tumor cells is mediated by the killing functions of CAR T cells. Thus, the antitumor effects of anti-FITC CAR therapy are independent of mutations in signaling proteins including *Kras*.

While the anti-FITC CAR technology described in this study circumvents a major hurdle for individualized T-cell-based therapy, another significant challenge in CAR T-cell approach is its potential to destroy noncancerous cells expressing target Ags, so-called on-target off-tumor toxicity (27). To avoid this adverse effect, novel technologies that attenuate the effector functions of CAR T cells according to

the need are in great demand. One potential approach developed by Di Stasi and colleagues is modification of CAR T cells to express an inducible caspase-9, so as to quickly eliminate them following the administration of synthetic dimerizing drug that induces caspase-9 activation (28). In our current studies, on the other hand, we highlighted that anti-FITC CAR T-cell activity can be attenuated by injecting FITC-labeled nonspecific IgG Ab. Mechanistically, excess FITC quenched the anti-FITC CAR on T cells and therefore competitively inhibited T-cell activation and effector functions. Thus, our technology might make it possible to inactivate anti-FITC T cells when adverse effects occur by ceasing the administration of FITC antitumor Ab and by injecting FITC (or FITC-labeled nonspecific Abs), while reactivating anti-tag T cells by re-injecting FITC-antitumor Ab when needed. We propose that the next generation of CARs should be equipped with improved abilities to recognize various cancer types and allow more precise on/off regulation.

In summary, we consider that our anti-tag CAR platform provides substantial advances in the CAR technology through its potential to redirect T cells to target cells via FITC-labeled antitumor Abs. It is worth highlighting that the tag used in such platform is not limited to FITC but can be applied to any types of reagents mediating protein-protein interaction and being conjugated with antitumor Abs. Indeed, during preparation of the manuscript, Urbanska and colleagues reported a method using biotin-conjugated Ab together with CAR expressing dimeric form of avidin (29). Our studies show complete tumor regression using both human tumor models in immunocompromised NSG mice and syngeneic mouse tumor models in immunocompetent hosts, emphasizing the antitumor effects mediated by this type of approach. In addition, our studies using FITC/anti-FITC scFv exemplify a possible method to avoid nonspecific interaction between avidin and endogenous biotin or biotin-like molecules *in vivo*. In summary, the studies serve as a proof of concept highlighting the potential use of anti-tag T cells to treat patients with different types of cancers as well as the possibility to regulate CAR T-cell functions with competing tag molecules.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Tamada, D. Geng, E. Davila
Development of methodology: D. Geng, Y. Sakoda, E. Davila
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Tamada, D. Geng, Y. Sakoda
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Geng, E. Davila
Writing, review, and/or revision of the manuscript: K. Tamada, D. Geng, E. Davila
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Geng, N. Bansal, R. Srivastava
Study supervision: K. Tamada, E. Davila

Acknowledgments

The authors thank Dr. Scott E Strome for reviewing the manuscript, Dr. Elizabeth Jaffee (Johns Hopkins University) for providing Panc 6.03, Dr. John Timmerman for providing human CD20-expressing 38C13, Dr. Wei-

Zen Wei for providing D2F2/E2, and Dr. Koh-Hei Sonoda and Yukari Mizuno for technical supports for the experiments.

Grant Support

This study is supported by the NIH grants R01CA140917 (E. Davila) and R01HL088954 (K. Tamada) and the University of Maryland Marlene and Stewart Greenebaum Cancer Center.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 9, 2012; revised August 24, 2012; accepted September 16, 2012; published OnlineFirst October 2, 2012.

References

- Ertl HC, Zaia J, Rosenberg SA, June CH, Dotti G, Kahn J, et al. Considerations for the clinical application of chimeric antigen receptor T cells: observations from a recombinant DNA Advisory Committee Symposium held June 15, 2010. *Cancer Res* 2011;71:3175–81.
- Sadelain M, Brentjens R, Riviere I. The promise and potential pitfalls of chimeric antigen receptors. *Curr Opin Immunol* 2009;21:215–23.
- Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc Natl Acad Sci U S A* 1989;86:10024–8.
- Cartellieri M, Bachmann M, Feldmann A, Bippes C, Stamova S, Wehner R, et al. Chimeric antigen receptor-engineered T cells for immunotherapy of cancer. *J Biomed Biotechnol* 2010;2010:956304.
- Lipowska-Bhalla G, Gilham DE, Hawkins RE, Rothwell DG. Targeted immunotherapy of cancer with CAR T cells: achievements and challenges. *Cancer Immunol Immunother* 2012;61:953–62.
- Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* 2011;3:95ra73.
- Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, et al. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* 2006;12:6106–15.
- Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, et al. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* 2008;14:1264–70.
- van Dam GM, Themelis G, Crane LM, Harlaar NJ, Pleijhuis RG, Kelder W, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor-alpha targeting: first in-human results. *Nat Med* 2011;17:1315–9.
- McKinney R, Thacker L, Hebert GA. Conjugation methods in immunofluorescence. *J Dent Res* 1976;55:A38–44.
- Thomas AM, Santarsiero LM, Lutz ER, Armstrong TD, Chen YC, Huang LQ, et al. Mesothelin-specific CD8(+) T cell responses provide evidence of *in vivo* cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. *J Exp Med* 2004;200:297–306.
- Betting DJ, Yamada RE, Kafi K, Said J, van Rooijen N, Timmerman JM. Intratumoral but not systemic delivery of CpG oligodeoxynucleotide augments the efficacy of anti-CD20 monoclonal antibody therapy against B cell lymphoma. *J Immunother* 2009;32:622–31.
- Terwey TH, Kim TD, Kochman AA, Hubbard VM, Lu S, Zakrzewski JL, et al. CCR2 is required for CD8-induced graft-versus-host disease. *Blood* 2005;106:3322–30.
- Pilon SA, Piechocki MP, Wei WZ. Vaccination with cytoplasmic ErbB-2 DNA protects mice from mammary tumor growth without anti-ErbB-2 antibody. *J Immunol* 2001;167:3201–6.
- Jacob JB, Quagliano E, Radkevich-Brown O, Jones RF, Piechocki MP, Reyes JD, et al. Combining human and rat sequences in her-2 DNA vaccines blunts immune tolerance and drives antitumor immunity. *Cancer Res* 2010;70:119–28.
- Hughes MS, Yu YY, Dudley ME, Zheng Z, Robbins PF, Li Y, et al. Transfer of a TCR gene derived from a patient with a marked antitumor response conveys highly active T-cell effector functions. *Hum Gene Ther* 2005;16:457–72.
- Shusta EV, Raines RT, Pluckthun A, Wittrup KD. Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. *Nat Biotechnol* 1998;16:773–7.
- Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 2009;114:535–46.
- Geng D, Zheng L, Srivastava R, Riker AI, Velasco-Gonzales C, Markovic SN, et al. Amplifying TLR-MyD88 signals within tumor-specific T-cells enhances antitumor activity to suboptimal levels of weakly-immunogenic tumor-antigens. *Cancer Res* 2010;70:7442–54.
- Jung S, Pluckthun A. Improving *in vivo* folding and stability of a single-chain Fv antibody fragment by loop grafting. *Protein Eng* 1997;10:959–66.
- Brentjens RJ, Latouche JB, Santos E, Marti F, Gong MC, Lyddane C, et al. Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. *Nat Med* 2003;9:279–86.
- Xuan C, Steward KK, Timmerman JM, Morrison SL. Targeted delivery of interferon-alpha via fusion to anti-CD20 results in potent antitumor activity against B-cell lymphoma. *Blood* 2010;115:2864–71.
- Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008;359:1757–65.
- Messersmith WA, Ahnen DJ. Targeting EGFR in colorectal cancer. *N Engl J Med* 2008;359:1834–6.
- Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, et al. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J Clin Oncol* 2009;27:2091–6.
- Lee J, Lee I, Han B, Park JO, Jang J, Park C, et al. Effect of simvastatin on cetuximab resistance in human colorectal cancer with KRAS mutations. *J Natl Cancer Inst* 2011;103:674–88.
- Park TS, Rosenberg SA, Morgan RA. Treating cancer with genetically engineered T cells. *Trends Biotechnol* 2011;29:550–7.
- Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* 2011;365:1673–83.
- Urbanska K, Lanitis E, Poussin M, Lynn RC, Gavin BP, Kelderman S, et al. A universal strategy for adoptive immunotherapy of cancer through use of a novel T-cell antigen receptor. *Cancer Res* 2012;72:1844–52.