Redirecting gene-modified T cells toward various cancer types using tagged antibodies

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Abstract

**Purpose:** To develop an adaptable gene-based vector that will confer immune cells 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 (Abs) 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 immune-compromised 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 epidermal growth factor 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 pre-established late-stage tumors. In immune-competent 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 non-specific 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.

**Clinical 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 CAR T cells can destroy non-cancerous tissue, 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 a 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.
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 CD3ζ or Fcε. 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 cancer patients (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, since 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 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 TAAAs. 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 CD3ζ (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 (anti-EGFR), trastuzumab (anti-Her2), and rituximab (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.
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 two costimulation signaling motifs, CD28 and 4-1BB, and CD3ζ signaling domains, designated as anti-FITC CAR (Figure 1A). The K_d of anti-FITC scFv to fluorescein is approximately 2.3 x 10^{-8} M (11). When human PBMC 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 (Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 2B, left panel). Appreciable cytolytic activity was detected at ratios as low as one T cell to twenty target cells. In the presence of FITC-control IgG, cytolytic activity of anti-FITC CAR cell was comparable to that of control T cells (Figure 2B, right panel). 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 non-labeled Ab (Figure 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 (Figure 2D).

Collectively, these data demonstrate anti-FITC CAR T cells’ 1) functionality, 2) specificity to kill tumor cells with FITC-Ab, and 3) 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*. Immune-deficient NSG mice were injected subcutaneously (s.c.) with SW480 colon cancer cells followed by intraperitoneal (i.p.) injection with Ctx, FITC-Ctx, or FITC-IgG. One day later, the mice were injected intravenously (i.v.) 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 (Figure 3A upper left panel). Compared to 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 (Figure 3A). In spite of 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 signals from CD28 or 41BB (2, 12). Therefore, the presence of anti-FITC CAR T cells in our model was examined in the spleen, liver, bone marrow, peripheral blood and in tumor explants. Approximately 10% of total human T cells detected in tumor explants were anti-FITC CAR T cells (Figure 3B). Variable percentages of anti-FITC CAR T cells were found in other organs in the recipient mice, while 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 non-labeled Ctx. T cells in the tumor explants demonstrated significantly enhanced proliferation (Figure 3C) and cytokine/chemokine production (Supplemental Figure 1) 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 to SW480 cells maintained in tissue culture (Figure 3D). It should be noted that EGFR expression on tissue culture SW480 was heterogeneous with some cells lacking EGFR. Thus, inability 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 homogenous EGFR (Figure 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 non-labeled 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 (Figure 4B). No tumor relapse occurred during the time of observation. In contrast, mice treated with anti-FITC CAR T cells and non-labeled Ctx succumbed to the tumor. These results demonstrate the ability of anti-FITC CAR T cells to eradicate established tumors when high and homogenous Ag is targeted.

Anti-FITC CAR T cells demonstrate therapeutic effects against syngeneic tumor in immune-competent mice. Whereas anti-FITC CAR T cells demonstrate powerful antitumor effects on human tumors in NSG mice, these models utilize immune-deficient
hosts and tumors allogeneic to CAR T cells. Therefore, we further evaluated the efficacy of CAR T cells against syngeneic tumors in immune-competent hosts. Similar to human CAR T cells, anti-FITC CAR T cells generated from mouse splenic T cells demonstrated potent functionality, as shown by a significant increase of cytokine and chemokine production by stimulation with FITC-labeled rituximab (FITC-Rtx) compared to non-labeled Rtx (Figure 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 CD3ζ are functional in mouse T cells (Supplemental Figure 2). 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 non-labeled Rtx (Figure 5B left panel). As expected, this cytotoxic activity was not observed against CD20-negative tumor (Figure 5B right panel). 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 (Figure 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 immune-competent mice was examined. Mice were inoculated i.v. with human CD20-expressing 38C13 mouse tumors as a model of Rtx insensitive B cell lymphoma (13) and treated with a transfer of anti-FITC CAR T cells in combination with either FITC-Rtx
or non-labeled Rtx. Survival of the mice treated with FITC-Rtx significantly prolonged compared to those with non-labeled Rtx (Figure 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 pre-established E0771/E2 but not Her2-negative parental E0771 tumor (Figure 6B). These results demonstrate an ability of anti-FITC CAR T cells to mediate therapeutic effects against syngeneic tumors established in immune-competent hosts.

The potential for CAR T cells to attack non-cancerous 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. Since 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 non-specific 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 non-specific IgG. While the regular CAR T cell therapy achieved complete tumor regression, additional injections of FITC-labeled control IgG permitted eventual tumor growth (Figure 6C). These results suggest that the in vivo effects of anti-FITC CAR T cells can be attenuated by injecting FITC-labeled non-specific IgG probably because of competitive interference of CAR T cell-tumor interaction.
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 demonstrated 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 immune-deficient mice inoculated with human tumor as well as immune competent mice bearing a syngeneic tumor. Importantly, injections of FITC-labeled non-specific 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 CAR T 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 (Figure 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, while 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, in order to target multiple TAAs on a single tumor cell type, mAbs against such TAAs must be available. The number and type of FDA-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 \textit{in vivo}. Indeed, we detected anti-FITC Ab in the sera of the mice treated with FITC-Rtx (\textbf{Supplemental Figure 3}). Anti-FITC immunity could shorten an \textit{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 (Figure 6A) might be due to the emergence of anti-FITC immunity, while a loss of targeted Ag would be also a possible mechanism as shown in Figure 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 immune competent 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 CAR T 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 cancer patients with mutated signal transduction proteins. For example, 30-60\% colon cancer patients express activating \textit{Kras} mutations and do not gain therapeutic benefits from treatment with
cetuximab due to a loss of its effect to block EGFR signal (14, 15). Accordingly, the current American Society of Clinical Oncology guidelines recommend that colorectal cancer patients should be screened for Kras mutations prior to being offered cetuximab, and those with Kras mutations should not be given cetuximab treatment (16). By combining with anti-FITC CAR technology, however, cetuximab could provide therapeutic benefits even in Kras-mutated cancer, as our studies demonstrated the antitumor effects in SW480, a colon cancer harboring a homozygous mutation in codon 12 of Kras (17). 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 non-cancerous cells expressing target Ags, so-called on-target off-tumor toxicity (18). 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 et al. 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 (19). In our current studies, on the other hand, we highlighted that anti-FITC CAR T cell activity can be attenuated by injecting FITC-labeled non-specific 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 non-specific 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 this manuscript, Urbanska et al. reported a method using biotin-conjugated Ab together with CAR expressing dimeric form of avidin (20). Our studies demonstrate complete tumor regression using both human tumor models in immune-compromised NSG mice and syngeneic mouse tumor models in immune-competent 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 non-specific 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.
Materials and methods

Mice and cell lines
Male or female 6-10 weeks old NOD-scid IL-2Rgamma\textsuperscript{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 EGFR-positive colon cancer SW480 and Her2-positive AU565 breast cancer cell lines were obtained from ATCC. Panc 6.03, a human pancreatic adenocarcinoma cell line expressing both EGFR and Her2, was provided by Dr. Elizabeth Jaffee (Johns Hopkins University) (21). The mouse B cell lymphoma line 38C13 expressing human CD20 was provided by Dr. John Timmerman (University of California Los Angeles) (22). The P815-TGL, a mouse mastocytoma cell line, was provided by Dr. Marcel R. van den Brink (Memorial Sloan-Kettering Cancer Center) (23). P815-TGL that stably expresses high level human CD20 was established in our laboratory by gene transfection. Human Her2-expressing mouse mammary tumor cell line D2F2/E2 and E0771/E2 were provided by Dr. Wei-Zen Wei (Wayne State University) (24) and Dr. Qiao Li (University of Michigan) (25), 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 was conjugated per one molecule of antibody. FITC-labeled dextran beads and PE-labeled
CD8 mAb were purchased from Sigma-Aldrich and eBioscience, respectively. Flow cytometric analysis of cell surface molecule expression was performed 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) and is derived from pMSGV (26). The mouse scFv against FITC was generated according to the previous report (27) and linked to the hinge and transmembrane regions of the human CD8α chain and the cytoplasmic regions of the human CD28, 4-1BB, and CD3ζ molecules. Transduction of human and mouse T cells was conducted as previously described (28, 29). 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 hrs after transfection. For transduction of human T cells, $3 \times 10^6$ PBMC were cultured in 24-well plates in the presence of OKT3 (50 ng/ml) and IL-2 (50 IU/ml) for 48 hours. For transduction of mouse T cells, $3 \times 10^6$ spleen and lymph node cells were activated with plate-bound anti-CD3 mAb (2.5 μg/ml), anti-CD28 mAb (1.2 μg/ml) and IL-2 (100 IU/ml) for 48 hours. Supernatants containing retroviruses were mixed with the activated human or mouse T cells ($1 \times 10^6$/ml) in the presence of retronectin (10 μg/ml) and centrifuged for 2 hours at 3000 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, 1-3 x 10^5 anti-FITC CAR or control T cells were cultured in 96-well tissue culture plates in the presence of FITC-labeled Abs, non-labeled Abs, or FITC-Dex beads for 72 hrs. 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 non-labeled Ctx. Proliferative activity of T cells was assessed by incorporation of ³H-thymidine during the last 16 hrs of 3-days culture. Cytotoxic activity of CAR T cells against tumors was assessed by a standard 4-hr ⁵¹Cr-release assay. Radioactivity of ³H and ⁵¹Cr 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 (Milliipore). All tests were performed in triplicate wells and results are shown as a mean ± SD.

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

In prophylactic tumor models, NSG mice were injected subcutaneously (s.c.) in the rear leg flank with 1-2 x 10^6 SW480 tumor cells and treated intraperitoneally (i.p.) with FITC-Ctx or non-labeled Ctx (25 µg/mouse) one day later. One day after Ab injection, the mice were injected intravenously (i.v.) with 5 x 10^6 anti-FITC CAR T cells generated from PBMC of healthy donors. In the therapeutic tumor model, NSG mice were injected s.c. with 1-2 x 10^6 Panc 6.03 and kept untreated until tumor grow up to 500 mm³. Then the mice were treated i.p. with FITC-Ctx or non-labeled Ctx weekly for three times and injected i.v. with 5 x 10^6 anti-FITC CAR T cells one day after the first Ab injection.
mouse models using syngeneic mouse-derived tumors, C3H/HeN mice were injected i.v. with $2 \times 10^4$ human CD20-expressing 38C13 and treated i.p. with 25 µg FITC-Rtx or non-labeled 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 one 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 500-800 mm$^3$. Thereafter, the mice were exposed to sub-lethal irradiation (4 Gy), and one day later treated i.p. with FITC-Her2 or non-labeled Her2 (25 µg/mouse) in conjunction with i.v. transfer of $5 \times 10^6$ 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 non-specific human IgG (100 µg/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**

Two-tailed student’s $t$ test was used to compare two 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.
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References


Figure legends

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 CD3ζ 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 (upper panels) or FITC-Dex beads (lower panels) 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 \(^3\)H-thymidine incorporation.

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 \(^3\)H-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 \(^{51}\)Cr-release assay at the indicated 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, non-labeled Ctx, FITC-Her2, or non-labeled 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 two independent experiments. All data are shown as mean ± 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, non-labeled Ctx, or FITC-IgG one day later. Then, the mice were injected i.v. with anti-FITC CAR T cells generated from healthy donor PBMC one 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 30-35 days after tumor inoculation. Tumor tissue, PBMC, 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 ± SD (n=3). (C) T cells extracted from tumor explants were cultured with irradiated SW480 in the presence of FITC-Ctx or non-labeled Ctx. Proliferative activity of T cells was assessed by ³H-thymidine incorporation. Data from 3 individual mouse sample (ms1, 2, and 3) are shown. (D) EGFR expression on SW480 tumor explants and SW480 cells from tissue culture was examined by flow cytometry.
Figure 4. Therapeutic effects of anti-FITC CAR T cells against pre-established 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 non-labeled Ctx weekly for 3 times, and further transferred i.v. with anti-FITC CAR T cells one day after the first Ab injection. Tumor growth (left panel) and mouse survival (right panel) were assessed.

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 non-labeled Rtx. After 3 days, concentration of 23 types of cytokines and chemokines in the culture supernatants was measured. Data was shown as mean ± SD. (B) Anti-FITC CAR T cells generated from mouse T cells were assessed for cytotoxic activity against human CD20-positive P815-TGL (left panel) or CD20-negative P815-TGL (right panel) in the presence of FITC-Rtx or non-labeled 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 non-labeled Her2. Cytotoxicity at 20:1 E/T ratio with the titrated doses of Ab was tested by ⁵¹Cr-release assay.
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 non-labeled 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 500-800 mm³, the mice were exposed to sub-lethal irradiation, and one day later treated i.p. with FITC-Her2 or non-labeled Her2 (25 μg/mouse) together with i.v. transfer of 5 x 10⁶ anti-FITC CAR T cells generated from C57BL/6 spleen T cells. Injection of Ab was repeated every 5 days for 3 times. In (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 non-specific human IgG (100 μg/mouse; open square) or unlabeled human IgG (filled circle) starting one day after the last injection of FITC-Her2 and repeated every 5 days additional 3 times. Tumor size is shown as mean ± SD.
Figure 1

A

Anti-FITC scFv

CD8 Tm

CD28

4-1BB

CD3ζ

B

Control T cells

Anti-FITC CAR T cells

30%

25%

50%

FITC-Ctx

FITC-Dex

33%

29%

37%

C

Proliferation (cpm x 10³)

Anti-FITC CAR T cells

FITC-Ctx

Ctx

FITC-Dex

Soluble FITC-IgG

Control T cells

reagents (μg/ml)

0.01 0.1 1 10

0 25 50

0 25 50

0 0.1 1 10
Figure 2

A. Proliferation of Anti-FITC CAR T cells and Control T cells with varying concentrations of FITC-Ctx. 
B. Lysis of SW480 cells with FITC-Ctx or FITC-IgG by Anti-FITC CAR T cells and Control T cells. 
C. Lysis of Panc 6.03 cells with FITC-Ctx, FITC-Her2, or Ctx by Anti-FITC CAR T cells and Control T cells. 
D. Lysis of AU565 cells with FITC-Her2 with varying concentrations of FITC-Her2 by Anti-FITC CAR T cells and Control T cells.
Figure 3

A

Tumor size (mm$^3 \times 10^3$) vs. % Survival

- Ctx alone (No T cells)
- Anti-FITC CAR T cells + FITC-IgG
- Anti-FITC CAR T cells + Ctx
- Anti-FITC CAR T cells + FITC-Ctx

Days post tumor challenge

B

% of Total T cells

- Anti-FITC CAR T cells
- Non-CAR T cells

Proliferation (cpm $\times 10^3$)

FITC-Ctx

C

Ctx

D

EGFR

- Isotype control
- Tumor explant
- Tumor cells from tissue culture
Figure 4

Panel A: Panc 6.03

- Isotype Control Ab
- FITC-Ctx

Panel B: Tumor size vs. Survival

- Ctx
- FITC-Ctx

Days post tumor challenge

% Survival

Tumor size (mm$^3$)
Figure 6

A

% Survival

Days post tumor challenge

B

Her2-positive tumor

Her2-negative tumor

Tumor size (mm³)

Days post tumor challenge

C

Tumor size (mm³)

Days post tumor challenge
Redirecting gene-modified T cells toward various cancer types using tagged antibodies

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