Original Article

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Bi-specific ligand-controlled chimeric antigen receptor T-cell therapy for non-small cell lung cancer

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Summary **Our goal is to develop a switch-controlled approach to enable better control of reactivity and safety of chimeric antigen receptor (CAR)-T therapy for non-small-cell lung cancer (NSCLC). Lentiviral transduction was performed to generate anti-FITC CAR-T cells and target cells stably expressing either isoform of the folate receptor. Colorimetric-based cytotoxic assay, enzyme-linked immunosorbent assay, and multiparametric flow cytometry analysis were used to evaluate the specificity and activity of CAR-T cells *in vitro*. Human primary T cells stably expressing the fully human anti-FITC CAR were generated. Anti-FITC CAR-T cells displayed antigen-specific and folate-FTIC dependent reactivity against engineered A549-FRα and THP-1-FRβ. The selective activation and proliferation of anti-FITC CAR-T cells *in vitro* stringently relied on the co-existence of folate-FITC and FR-expressing target cells and was dose-titratable with the folate-FITC switch. The excellent *in vitro* efficacy and specificity of an adaptor-controlled CAR-T therapy to target both tumor cells and tumor-associated macrophages in NSCLCs were validated.**

Keywords: Non-small cell lung cancer, chimeric antigen receptor T cell, folate receptor, folate-FITC

1. Introduction

Lung cancer continues to be one of the most commonly diagnosed cancer types and the leading cause of cancer deaths in both United States and China (*1,2*). Non-small-cell lung cancer (NSCLC) accounts for 80% to 85% of all lung cancer cases (*3*). Although giant steps have been made in targeted chemotherapy and immunotherapy of NSCLC, the 5-year survival rate remains below 20% (*3,4*). Therefore, the development of new and more effective therapies for NSCLC is imperative.

Recently, chimeric antigen receptor (CAR)-T cell therapy has had great success in treatment of hematologic malignancies, most notably in B cell acute lymphoblastic leukemia (B-ALL) with up to a 90% complete remission rate (*5*). With FDA approval of Kymriah for certain pediatric and young adult patients with a form of acute lymphoblastic leukemia (ALL) (*6*), we entered a new frontier in medical innovation with the ability to genetically reprogram patients-derived T lymphocytes to attack a deadly cancer. For the treatment of NSCLC, an appropriate target is the folate receptor α (FRα), a glycosylphosphatidylinositol (GPI)-anchored cell surface protein, which binds and mediates the uptake of free folic acid as well as its drug conjugates with high affinity and specificity (*7*). Numerous studies have confirmed elevated expression of FRα in over 70% of lung adenocarcinomas and about 10% of squamous cell carcinomas by using different clones of anti-FRα antibodies (*8-12*). Notably, a high concordance of FRα expression was not only observed between biopsy and primary tumor but also seen between distant metastases of NSCLC and their corresponding local tumors (*9*).

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In contrast, the low to negligible distribution of FRα in normal human tissues is restricted to the apical (luminal) surface of cells, which reside in the bronchial epithelium, renal tubules and choroid plexus (*13,14*). Consequently, six folate-targeted drugs are currently undergoing human clinical trials (*15-19*), and one folate-vinca alkaloid conjugate (EC145) has even advanced to phase IIb clinical trials for targeted therapy of NSCLC (*18,19*). Thus, FRα has not only become an established NSCLC marker, but it could also act as a promising target for adoptive CAR-T therapy for this cancer.

However, normal tissue toxicity is an issue, which needs to be addressed especially when expression of tumor-associated antigen targeted by CAR-T is generally less restricted (*20-24*). Several strategies have been proposed to minimize CAR-T therapy-related toxicities, including the use of kill switches (*25*), splitting the chimeric receptor (*26*), and antibody-based intercellular switches to mediate the formation of immunological synapses between CAR-T cells and target cells (*27-31*).

In recognition of the fact that over 70% of lung adenocarcinomas expressing FRα also contain tumor-associated macrophages (TAMs) with high expressing levels of FRβ (*10*), we herein describe an approach using fluorescein isothiocyanate (FITC) conjugated with folate (folate-FITC) as an intermediary "switch" to induce the assembly of a pseudoimmunological synapse between anti-FITC CAR-T cells and target cells expressing either FRα or FRβ. We demonstrate potent antigen-specific and dose-dependent *in vitro* efficacies of anti-FITC CAR-T cells against both FRα-positive NSCLC cell lines and macrophage cell lines expressing the β isoform of FR. Moreover, the activation and proliferation of anti-FITC CAR-T cell was strictly dependent on the presence of the bi-specific switch molecule folate-FITC. These results suggest that CAR-T cells could be exploited to target both tumor and the tumor microenvironment in NSCLC patients by using our approach.

2. Materials and Methods

2.1. *Generation of CAR-T cells*

The human anti-FITC scFv (*31*) gene was synthesized by GENEWIZ and cloned into a lentiviral vector (LV-vector) containing a CD8α hinge and transmembrane region, and 41BB and CD3ζ signaling domains. Lentiviruses were produced in HEK293T cells, and transduction of human primary T cells was performed as previously described (*31*). Briefly, 8×10^6 HEK293T cells were seeded per 10-cm tissue dish 24 hours before transfection. Cells were transfected with anti-FITC CAR plasmid and viral packaging vectors using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Waltham, MA). Supernatants were harvested 48h later. Viral particles were concentrated using an Amicon®Ultra 100K device (Merk Millipore, Worcester, MA), titrated, and stored at -80°C until use. All plasmids were purified using Endo-free Maxi prep kits (Qiagen, Valencia, CA). Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteer donors using Ficoll-Hypaque Solution (GE Healthcare, Chicago, IL). Isolated PBMCs were transferred into complete medium (CM) (AIM-V, 5% human serum AB, 1% pen-strep media) (Thermo Fisher Scientific, Waltham, MA) and incubated at 37°C for 3 hours. Suspension cells were collected and activated with anti-CD3/CD28 beads (Thermo Fisher Scientific, Waltham, MA). Activated T cells were transduced with lentivirus after 24 hours of activation in the presence of 10 μg/mL protamine sulfate (Sigma-Aldrich, St. Louis, MO). T cells were then expanded in CM with 300 IU/mL of recombinant human interleukin-2 (rhIL-2) (R&D Systems, Minneapolis, MN) after media replacement, maintaining a cell density of 0.5 - 2 × 10^6 cells/mL. Anti-FITC CAR expression was verified by flow cytometry using Alexa Fluor® 647 conjugated anti-human IgG $F(ab')_2$ antibodies (Jackson ImmunoResearch, West Grove, PA). Its binding capacity to FITC was also confirmed by using a FITC-labeled mouse IgG1 isotype control antibody (Biolegend, San Diego, CA) and folate-FITC (AdooQ, Nanjing, Jiangsu). Non-transduced T cells or T cells transduced with lentivirus expressing green fluorescent protein (GFP) stained with corresponding reagents served as background controls.

2.2. *Cell lines*

HEK293T cell line, human lung adenocarcinoma cell line A549, a human monocytic cell line THP-1, immortalized human T lymphocyte cell line Jurkat, and the HL-60 (Human promyelocytic leukemia cells) cell line were purchased from American Type Culture Collection. A549 and THP-1 cells were transduced with lentiviral vectors encoding human FRα and FRβ gene, respectively, to generate cells stably expressing FRα (A549-FRα) and FRβ (THP-1-FRβ). The expression of human FRα and FRβ was confirmed by quantitative RT-PCR. Briefly, total RNA was extracted from the corresponding cell lines. Then cDNA was generated using the Reverse Transcription System (Promega, Fitchburg, WI) and added to SYBR Green PCR Master Mix with primers specific for human FRα or FRβ, in triplicate. The relative human FRα or FRβ messenger RNA (mRNA) copy number was calculated using the standard curve method and the real-time PCR system (Bio-Rad Laboratories, Hercules, CA). A549-FRα and THP-1-FRβ cells were maintained in folate-deficient RPMI 1640 media with 10% fetal bovine serum and antibiotics. A549, THP-1, Jurkat and HL60 cells were cultured in complete media prepared with RPMI1640. HEK293T cells were cultured in complete media prepared with DMEM. All reagents were purchased

from Thermo Fisher Scientific (Waltham, MA).

2.3. *Colorimetric-based cytotoxic assays*

Anti-FITC or GFP CAR T cells were co-cultured with target cells at indicated E:T (effector-to-target) ratios in 96-well round bottom plates with 100 μL/well of complete media (folate-deficient RMPI 1640, 5% fetal bovine serum, 1% pen-strep media) containing different concentrations of folate-FITC. After incubation at 37℃ for 24 hours, cytotoxicity towards target cells was determined by measuring the amount of lactate dehydrogenase (LDH) released into culture media using CytoTox-96 nonradioactive cytotoxicity assay kit (Promega, Fitchburg, WI). The percent lytic activity was calculated with the following formula: (values used represent absorbance at 490 nM) % Cytotoxicity = 100 × [((Target cells + Effector cells + folate-FITC) - (Target cells + Effector cells only)) / ((Maximum target cell lysis) – (Target cells only))].

2.4. *T cell activation assay*

Anti-FITC or GFP CAR T cells were co-cultured with target cells as described above. Supernatants were harvested, and IFN-γ released by T cells was quantified by enzyme-linked immunosorbent assay (ELISA) using Human IFN-gamma Quantikine ELISA Kit (BD Biosciences, San Jose, CA). The expression level of T cell activation markers, CD25 and CD69, were analyzed by flow cytometry using anti-CD25 (BD Biosciences, San Jose, CA) and anti-CD69 (eBioscience, San Diego, CA). After being co-cultured with target cells for 5 hours, the ability of CAR-T cells to secrete cytolytic granules was visualized by CD107a staining using anti-CD107a antibody (eBioscience, San Diego, CA).

2.5. *T cell proliferation assay*

Anti-FITC CAR-T cells were pre-labeled with 5 mM PKH26 (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Anti-FITC CAR-T cells were cocultured with target cells at an effector to target cell ratio (E:T ratio) of 1 to 1 in triplicate in 96-well round bottom plates in 200 μL of complete media (folate-deficient RMPI 1640, 5% fetal bovine serum, 1% pen-strep media) containing different concentrations of folate-FITC. After a 3-day incubation in the presence of folate-FITC, cells were stained and analyzed for PKH26 distribution.

2.6. *Statistical analysis*

The data are reported as mean ± SD. Statistical analysis was performed using Student's *t* test or Mann-Whitney test. Statistical significance and EC_{50} values were calculated using GraphPad Prism 5.0. $P < 0.05$ was considered significant.

3. Results

3.1. *Generation of anti-FITC CART cells*

Recently, a proof-of-concept study demonstrated that the efficacy of FITC-conjugated anti-CD19 Fab directed anti-FITC CAR-T against human $CD19^+$ B-acute lymphoblastic leukemia line was comparable to the current FDA-approved CTL019 therapy (Kymirah®) both *in vitro* and *in vivo* (*31*). More importantly, it has been reported that folate-FITC, a FRα-targeted optical contrast agent also known as EC17 (*32*), was 100% accurate in identifying lung adenocarcinomas with very limited toxicities in a phase I clinical trial of targeted intraoperative molecular imaging for surgical resection (*33*). Therefore, we plan to evaluate if folate-FITC would be able to elicit a specific reactivity of anti-FITC CAR-T cells against FRα-expressing lung cancer cells (Figure 1A). To create a FITC-specific CAR, a fully human anti-FITC scFv clone (*31*) was subcloned into a lentiviral vector containing a second-generation CAR (from N-terminus: anti-FITC scFv, CD8α hinge and transmembrane domain, the cytoplasmic domains of 4-1BB and CD3ζ), using GFP CAR as a control (Figure 1B). Lentiviral particles were produced in HEK293T cells and used to transduce Jurkat cells. A transduction efficiency of 69.3% was observed by flow cytometry after staining for scFv surface expression using mouse anti-human IgG, $F(ab')_2$ polyclonal antibody (Figure 1C, left panel). To further confirm that the anti-FITC scFv was properly folded on the cell surface and retained its affinity for FITC, a flow cytometry binding assay was performed using folate-FITC and a commercially available FITC-labeled mouse IgG1 isotype control antibody. Similar to the transduction efficiency measured as stated above, we observed the binding of FITC anti-mouse IgG antibody (62.7% positive) (Figure 1C, middle panel) and folate-FITC (64.7% positive) (Figure 1C, right panel) to Jurkat cells.

3.2. *Anti-FITC CAR-T cells display antigen-specific and switch-dependent reactivity against engineered A549-FRα*

To evaluate the ability of folate-FITC to redirect the anti-FITC CAR-T cells toward FRα-positive NSCLC cells, we first engineered A549, an FR-negative human lung adenocarcinoma cell line, to constitutively overexpress human FRα (A549-FRα). Surface expression of functional FRα was assessed, by labeling with folate-FITC, using flow cytometry (Figure 1D), and the selective expression of FRα but not FRβ mRNA was confirmed with quantitative RT-PCR (Figure 1E). Moreover, results from Western Blot with anti-human FRβ polyclonal antibody also demonstrated that A549-

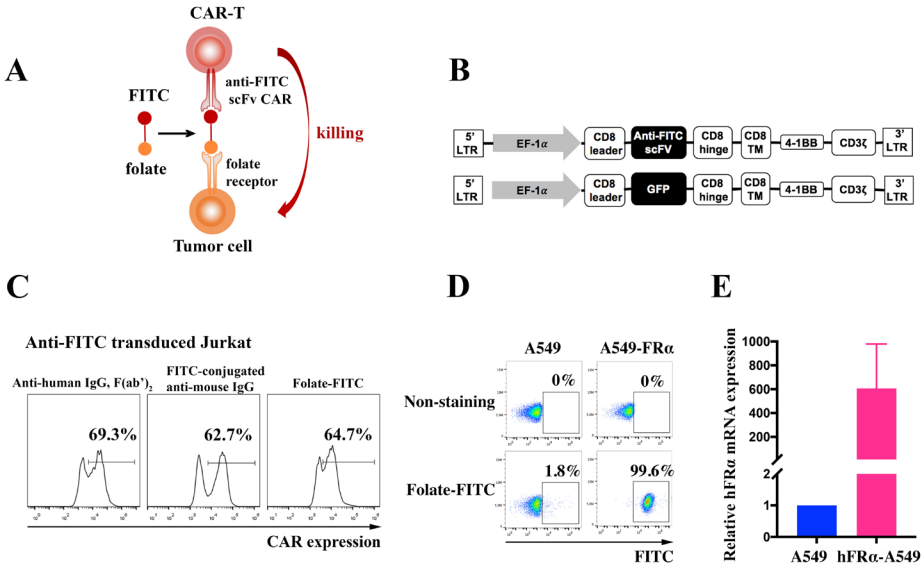

Figure 1. Schematic representation of the killing mechanism, lentivrial vector and CAR-modified Jurkat cells, and engineered A549-FRα cell line. (A) Schematic diagram of folate-FITC mediated CAR-T cytotoxicity to tumor cells. **(B)** Anti-FITC and GFP CAR lentiviral construct. **(C)** Anti-FITC CAR expression on Jurkat cells was assessed by staining with AlexaFlour647® mouse anti-human IgG F(ab′)$_2$ antibody (Jackson ImmunoResearch, West Grove, PA), a FITC-conjugated anti-mouse IgG1 isotype control antibody (Biolegend, San Diego, CA), or folate-FITC (AdooQ, Nanjing, Jiangsu). **(D)** Assessment of folate-FITC binding to A549 and A549-FRα cell lines by flow cytometry. **(E)** Relative FRα mRNA expression was confirmed using quantitative RT-PCR. Indicated mRNA expression is shown relative to A549. Results shown represent findings observed in three independent experiments.

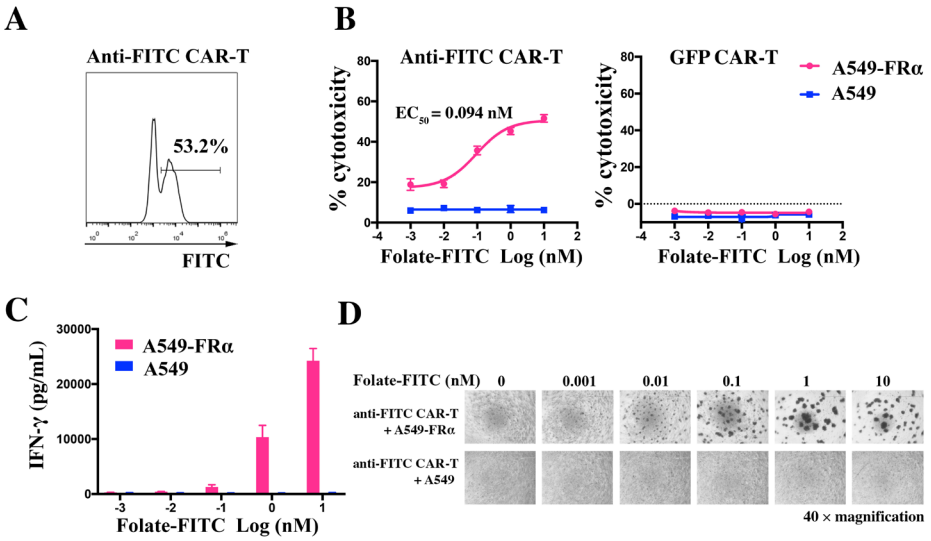

Figure 2. Folate-FITC can specifically and dose-dependently redirect anti-FITC CAR-T cells to target A549-FRα cells. (A) Anti-FITC CAR expression on human T cells was detected by a FITC-conjugated anti-mouse IgG1 isotype control antibody. **(B)** 24 hours co-culture of anti-FITC CAR-T or GFP CAR-T cells with A549 and A549-FRα cells in the presence of serial dilutions of folate-FITC at an effector to target cell ratio of 5:1 (5×10^4 to 1×10^4 cells per well). Cytolytic activity was determined by measuring the amount of LDH released into culture media using CytoTox 96 Nonradioactive cytotoxicity assay kit (Promega). Each concentration was carried out in triplicate and error bars represent SD. **(C)** Quantification of IFN-γ levels in cultures at a E:T ratio of 5:1 by ELISA. Error bars represent SD derived from triplicate samples. **(D)** Microscopic images of cytotoxicity assay at a E:T ratio of 5:1.

FRα cells do not express FRβ (data not shown). Then, lentiviral particles were used to transduce activated peripheral human blood mononuclear cells (PBMCs), and 7 days post viral transduction, more than 50% of the primary human T cells expressed CAR as determined by flow cytometry after labeling with folate-FITC (Figure 2A).

As shown in Figure 2B, at an effector to target cell ratio (E:T ratio) of 5 to 1 in folate-deficient media, highly potent cytotoxic activity of anti-FITC CAR-T cells was induced by folate-FITC (EC_{50} = 0.094 ± 0.116 nM) against A549-FRα. These results were reproducible across multiple T cell donors with similar efficacies. In contrast, folate-FITC did not induce lytic activity towards A549-FRα cells in the presence of GFP CAR-T cells (Figure 2B, right panel). Furthermore, no significant

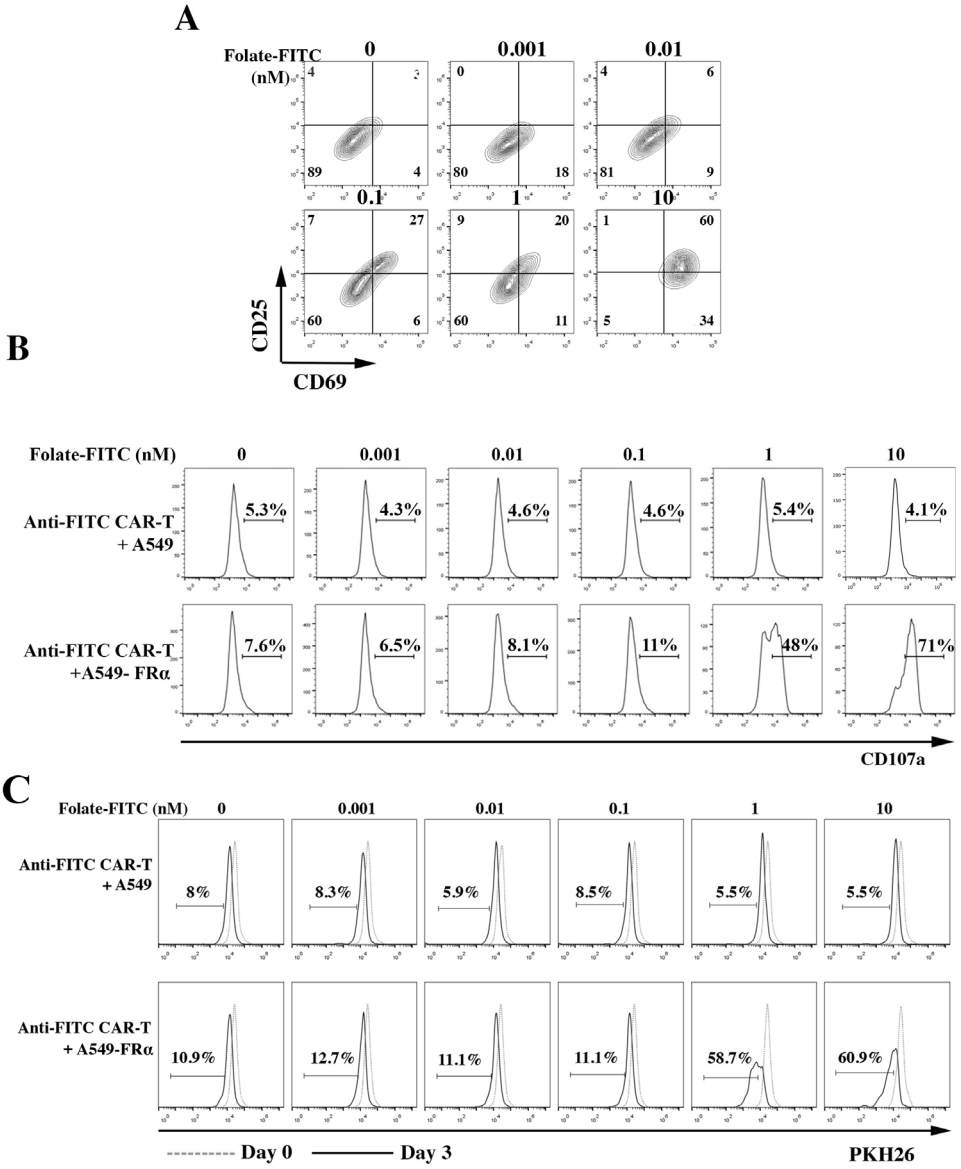

Figure 3. Activation, degranulation and proliferation of anti-FITC CAR-T cells co-cultured with A549-FRα in the presence of Folate-FITC. (A) Folate-FITC dependent titratable activation of anti-FITC CAR-T cells. Anti-FITC CAR-T cells (5×10^4 cells) were co-cultured with A549- FRα cells (1×10^4 cells) for 24 h with serial dilutions of folate-FITC, and subsequently stained with AlexaFlour647 mouse anti-human IgG $F(ab')_2$ antibody followed by PE-conjugated anti-CD25 (BD Biosciences, San Jose, CA) and FITC-conjugated anti-CD69 antibody (eBioscience, San Diego, CA). **(B)** Folate-FITC dependent degranulation of Anti-FITC CAR-T cells. Anti-FITC CAR-T cells (1×10^5 cells) were co-cultured with A549 and A549-FRα cells (1×10^5 cells) in the presence of folate-FITC and PE-conjugated anti-human CD107a antibody (eBioscience, San Diego, CA). After 5 hours of co-culture, CD107a surface expression was measured following the staining of AlexaFlour647 mouse anti-human IgG F(ab′)2 antibody. **(C)** Folate-FITC dependent proliferation of Anti-FITC CAR-T cells. Anti-FITC CAR-T cells were pre-labeled with 5 mM PKH26 (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Anti-FITC CAR T cells (1×10^4 cells) were co-cultured with A549 and A549-FRα cells (1×10^4 cells). After 3-day incubation in the presence of folate-FITC, cells were stained and analyzed for PKH26 distribution.

cytotoxic activity was observed with FRα-negative A549 cells at concentrations of folate-FITC up to 10 nM, demonstrating the high selectivity of folate-FITC and anti-FITC CAR-T cells. The amount of IFN-γ released from anti-FITC CAR-T cells after 24-hour co-culture with A549-FRα was positively correlated with the dose of folate-FITC put into the assay media (Figure 2C). Moreover, formation of aggregates, indicative of cross-linking between T cells and target cells, was visible in cocultures of anti-FITC CAR-T cells and A549-FRα cells with folate-FTIC, but not in any other controls (Figure 2D). Overall, our findings demonstrate that folate-FITC, as a bispecific small molecule, can specifically and dose-dependently redirect anti-FITC CAR-T cells to target FRα-positive cells.

3.3. *Folate-FITC mediates activation and proliferation of FITC-specific CAR-T for A549-FRα*

Next, we confirmed the stringent dependence of anti-FITC CAR-T cell activation on folate-FITC by monitoring their expression of cell surface activation

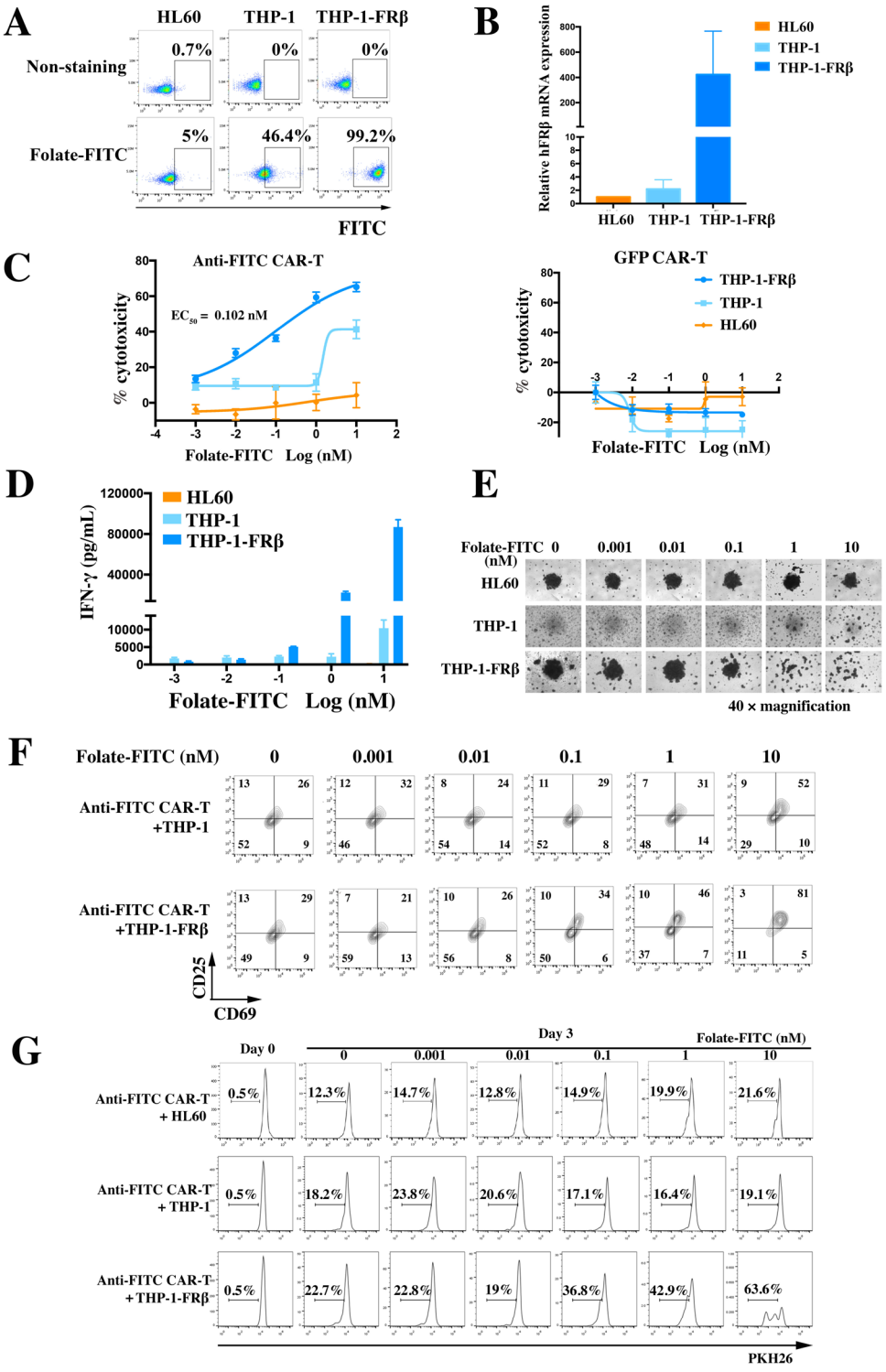

Figure 4. Folate-FITC can specifically and dose-dependently redirect anti-FITC CAR-T cells to target FRβ-positive cells. **(A)** Assessment of folate-FITC binding to HL60, THP-1 and THP-1-FRβ cell lines by flow cytometry. **(B)** Relative FRβ mRNA expression was confirmed using quantitative RT-PCR. Indicated mRNA expression is shown relative to HL60. Results shown represent findings observed in three experiments. **(C)** 24 hours co-culture of anti-FITC CAR-T or GFP CAR-T cells with HL60, THP-1 and THP-1-FRβ cells in the presence of serial dilutions of folate-FITC at an effector to target cell ratio of 2:1 (4×10^4 to 2×10^4 cells per well). Cytolytic activity was determined by measuring the amount of LDH released into culture media using CytoTox 96 Nonradioactive cytotoxicity assay kit (Promega). Each concentration was carried out in triplicate and error bars represent SD. **(D)** Quantification of IFN-γ levels in cultures at a E:T ratio of 2:1 by ELISA. Error bars represent SD derived from triplicate samples. **(E)** Microscopic images of cytotoxicity assay at a E:T ratio of 2:1. **(F)** Titratable activation of anti-FITC CAR-T cells co-cultured with THP-1-FRβ at a E:T ratio of 2:1 in the presence of Folate-FITC. CD25 and CD69 expression was measured following the staining of AlexaFlour647 mouse anti-human IgG F(ab′)$_2$ antibody. **(G)** Folate-FITC dependent proliferation of Anti-FITC CAR-T cells. Anti-FITC CAR-T cells were pre-labeled with 5 mM PKH26 (Sigma-Aldrich, St. Louis, MO). Anti-FITC CAR T cells (1×10^4 cells) were co-cultured with HL60, THP-1 and THP-1-FRβ cells (1×10^4 cells). After 3-day incubation in the presence of folate-FITC, cells were stained and analyzed for PKH26 distribution.

markers, CD25 and CD69, after 24-hour co-culture with A549-FRα cells. As expected, the increase of CD25 and CD69 expression levels on anti-FITC CAR-T cells was correlated with the concentration of folate-FITC in the assay media (Figure 3A). Degranulation, as quantified by increased cell surface CD107a expression, is an established surrogate for T-cell lytic function. After 5-hour co-culture, we observed specific degranulation in anti-FITC CAR-T cells only in the presence of A549-FRα, and surface mobilization of CD107a was also dependent on the concentration of folate-FITC (Figure 3B). Besides, folate-FITC dependent proliferation of anti-FITC CAR-T cells was also confirmed by detecting the PKH26 dilution of PKH26 labelled anti-FITC CAR-T cells after 3-day co-culture with A549-FRα cells (Figure 3C).

3.4. *Anti-FITC CAR-T cells exhibit specific cytotoxicity against FRβ-positive macrophage cell line*

To evaluate the ability of folate-FITC to redirect the anti-FITC CAR-T cells toward FRβ-positive macrophage cells, we first engineered THP-1-FRβ cell line, which stably overexpresses human FRβ. Surface expression of functional FRβ on THP-1 and THP-1-FRβ was assessed by labeling with folate-FITC using flow cytometry (Figure 4A). FRβ mRNA expression on THP-1 and THP-1-FRβ were measured by quantitative RT-PCR (Figure 4B). Both quantitative RT-PCR and Western Blot were performed to show that THP-1 and THP-1-FRβ do not express FRα. Besides, protein level of FRβ on THP-1 and THP-1-FRβ was also measured by Western Blot with anti-human FRβ polyclonal antibody (data not shown).

As shown in Figure 4C, at an effector to target cell ratio (E:T ratio) of 2 to 1 in folate-deficient media, highly potent cytotoxic activity of anti-FITC CAR-T cells was induced by folate-FITC (EC_{50} = 0.102 ± 0.272 nM) against THP-1-FRβ. Notably, anti-FITC CAR-T cells also exhibited specific reactivity against endogenous FRβ on THP-1 cells in the presence of 10 nM Folate-FITC. In contrast, no lytic activity of GFP CAR-T cells was induced towards THP-1 and THP-1-FRβ by Folate-FITC. For all the cytotoxicity experiments, HL60 cells were used as a control because none of the HL60 cells expressed FRβ as determined by both folate-FITC binding and quantitative RT-PCR (Figure 4A, 4B and 4C). These results were reproducible across multiple T cell donors with similar efficacies. Herein, the cytolytic effect of anti-FITC CAR-T on target cells was FRβ dependent and relied on the concentration of Folate-FITC. The amount of IFN-γ released from anti-CAR-T cells after 24-hour co-culture with THP-1-FRβ was positively correlated with the dose of folate-FITC in the assay media (Figure 4D). The reduction of cell aggregation in the bottom indicated the lytic reactivity of T cells to target cells, which was visible in co-cultures of anti-FITC CAR T cells and THP-1-FRβ cells with 1 nM and 10 nM folate-FTIC. Meanwhile, cell aggregation in co-cultures of anti-FITC CAR T cells and THP-1 cells with 10 nM folate-FTIC was also visible though slightly decreased (Figure 4E). Overall, our findings demonstrate that folate-FITC, as a bispecific small molecule, can redirect anti-FITC CAR-T cells to target FRβ-positive cells in an antigen-specific and dose-dependent manner. In addition, cell surface expression of CD25 and CD69 were detected after 24-hour co-culture with THP-1 and/or THP-1-FRβ cells to confirm the strict dependence of anti-FITC CAR-T cell activation on folate-FITC. As expected, the increase of CD25 and CD69 expression levels on anti-FITC CAR-T cells was not only correlated with concentration of folate-FITC in the assay media but also correlated with relevant surface expression levels of FRβ in target cells (Figure 4F). Furthermore, folate-FITC dependent proliferation of anti-FITC CAR-T cells was also confirmed by detecting the PKH26 dilution of PKH26 labelled anti-FITC CAR-T cells after 3-day co-culture with THP-1-FRβ cells (Figure 4G).

4. Discussion

The morbidity and mortality of lung cancer both rank first in all malignancies worldwide, and NSCLC is the most common form of lung cancer. Although targeted therapy such as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) and activated lymphocyte kinase (ALK)-TKI can prolong the survival of NSCLC patients with such mutations, drug resistance almost inevitably occurs (*4*). In recent years, immunotherapy, a promising field that harnesses the power of the immune system as a therapeutic agent, has been revolutionizing the lung cancer treatment paradigm. Unfortunately, checkpoint blockade therapy using PD-L1/PD-1 antibodies like Nivolumab and Pembrolizumab only works in a small percentage of NSCLC patients (*34*). Another potentially more potent approach being investigated is adoptive transfer of chimeric antigen receptor T-Cell (CAR-T) with high binding affinity and specificity to the tumor associated antigen (TAA). CAR-T cell immunotherapy has shown tremendous success in the treatment of acute lymphocytic leukemia (ALL) and refractory large B-cell lymphoma demonstrated by the FDA approval of Kymriah and YESCARTA, respectively (*35,36*). However, there are several obstacles to overcome in the process of translation of CAR T-cell therapy to solid tumors.

First and foremost, an ideal tumor specific antigen target is required for generating high affinity scFv, which is the most commonly used ectodomain for CAR. Previous studies have proved that FRα is highly expressed in NSCLC cells and to a large extent in lung adenocarcinoma (*8-10*). Moreover, several studies have

found that its expression levels are associated with tumor stage and survival in lung adenocarcinoma (*11-13*). With the advent of targeted therapies towards FRα for NSCLC treatment and with several such agents, i.e. folate-conjugated small molecule drug and humanized anti-FRα monoclonal antibody, in late stage clinical development (*15-18*), FRα has long been appreciated as a promising target for NSCLC therapy.

Another limitation endowed with current approved CAR-T therapy is treatment-related toxicities. Although the safety and efficiency of Kymriah and YESCARTA were both demonstrated in multicenter clinical trials, unique acute toxicities, which can be severe or even fatal, have been brought to the forefront. The two most commonly observed toxicities with CAR-T therapy are cytokine release syndrome (CRS), which is triggered by the activation and proliferation of CAR-T cells causing high fever, flu-like symptoms, and/or multi-organ toxicity; and a CAR-T cell-related encephalopathy syndrome (CRES), which can sometimes lead to lethal cerebral edema.

Besides administration of anti-IL-6 antibodies and steroids, various strategies are being pursued to rein in the side effects of engineered T cells. One approach to mitigate adverse effects is by incorporating an inducible safety switch into the T-cell product. Mechanisms which are being explored include: inducible caspase 9 system where a small molecule (AP1903) drug dimerizes caspase-9 pro-molecules so that apoptotic pathways in CAR-T cells are activated (*25,37*); transducing genes for CD20 or EGFR so that antibody therapy with rituximab or cetuximab respectively depletes CAR-T cells; HSV thymidine kinase incorporation so that the pro-drug ganciclovir leads to elimination of CART cells (*38*); transient CAR expression by using mRNA (*39*). Undoubtedly, these methods would improve our ability to manage toxicity when it occurs, but they would also affect the persistence of CAR-Ts. Therefore, engineering CAR-Ts to further improve specificity to avoid "on-target, off-tumor" effects is another way that has been proceding. One such solution would be requiring CAR-Ts to recognize combinatorial antigens by using a synthetic NOTCH receptor for one antigen which then drives the inducible expression of another CAR specific antigen, so that target cell must express two antigens to 'arm and activate' the CAR-Ts (*40*). Other strategies have looked to the use of 'tumor sensing' where T cells are transduced with a CAR that only provides suboptimal activation after binding to its antigen, but a chimeric co-stimulatory receptor that recognizes a second antigen leads to full activation (*41*). Lastly, using a CAR-T that requires a small molecule drug to activate an 'ON-switch' so that antigen binding and intracellular signaling components only assemble in its presence is an interesting approach (*42*). While such approaches have shown improved specificity in preclinical settings, several immunotherapy groups are also working to develop a new generation of therapies that are inherently safer, yet just as efficacious. One of these technologies uses an intermediate bispecific molecule as a "switch" to redirect the specificity of CAR-Ts and control their activation, expansion, and termination. Unlike conventional CAR-T cells, the scFv portion of the CAR is not raised against any TAA, thus, these CAR-T cells do not recognize endogenous antigen or normal tissue. However, in the presence of an adaptor molecule that is recognized by the CAR extracellular domain and binds specifically to a TAA, CAR-T cells can be redirected specifically to target cancer cells. Moreover, the activity of these CAR-T cells can be regulated in a switch dose-dependent manner. More recently, proof-of-principle studies have been achieved using FITC-, biotin-, or peptide-tagged antibodies (*27-31*).

In this study, we generated CAR T-cells that express a fully humanized scFv for FITC with high affinity. Their potent antigen-specific and folate-FITC dependent *in vitro* cytotoxicity against a FRα-positive cancer cell line was demonstrated. Moreover, the activity, cytokine release ability, activation phenotype and proliferation of anti-FITC CAR-T cells could be controlled or titrated based on the dosage of folate-FITC. Furthermore, we demonstrate that this approach is readily adaptable to target FRβ on a macrophage cell line using the same anti-FITC CAR-T cells.

Folate-FITC, also known as EC17, has been evaluated clinically for use in image-guided surgery for NSCLCs and displayed almost exclusively grade I/II toxicity (*43*). Moreover, its safety has also been approved in a phase I clinical trial for kidney cancer (*44,45*). One additional advantage of using folate-FITC as a switch is the high affinity and specificity of folate, the endogenous ligand, to FRα. In addition to safety, it has been reported that folate-FITC was extremely accurate in identified pulmonary adenocarcinomas in clinical settings (*33*), which further corroborates its specificity. Therefore, the use of folate-FITC as an adaptor to redirect the reactivity of CAR-T cells almost certainly would reduce the potential for off-target cross-reactivity of CAR.

Among the four isoforms of FRs, two forms (FRα and FRβ) bind folate with high affinity (K_d ~ 1nM) (*14*). Previous studies showed that over 70% of human lung adenocarcinomas contain FRβ-positive tumor associated macrophages in regions with high immune cell infiltration (*10,33*). With the ability to use folate-FITC to target FRβ-expressing macrophages in the immunotherapy of rheumatoid arthritis that has been demonstrated in animal models, it has been suggested that folate-FITC might likewise efficiently bind to FRβ-expressing TAMs accumulated in the tumor mass of human lung adenocarcinomas. More importantly, higher abundance of TAMs in the stroma of lung adenocarcinoma was observed in patients with more

progressive disease, poorer prognosis, and a lower response rate of targeted chemotherapy (*46,47*). Given that folate-FITC directed anti-FITC CAR-T cells could effectively kill not only FRα-positive tumor cells but also FRβ-expressing macrophages, this strategy might be feasible to improve the of immunosuppressive tumor microenvironment.

Admittedly, there are several limitations to our study. First, given the exploratory nature of this initiative study, the *in vivo* efficacy of folate-FITC controlled anti-FITC CAR-T therapy towards FRα-positive NSCLCs has not been assessed. Second, the plasma clearance half-life of the folate-FITC is relatively short. After a single *i.v.* or *s.c.* injection of ^{3}H-folate-FITC (500 nmol/kg), the majority of radioactivity was cleared from the mouse plasma within 1 to 2 hours (*48*). This may affect the *in vivo* persistence of anti-FITC CAR-T cells. However, the pre-clinical potency of recruiting cytotoxic T cells to FRα-positive ovarian cancer cells for lysis using conjugates of folate and anti-CD3 Fab is promising, even if the serum half-life of folate-conjugate anti-CD3 Fab (a single *i.v.* dose of 1mg/kg) in mouse was about 60 minutes (*49*). In addition, in one previous study, we did not observe a significant difference between Fab and IgG switch mediated cytotoxicity against CD19^{+} B cell malignance in animal studies. In contrast, Fab-based switch enabled better temporal control over the reactivity due to its shorter pharmacokinetic half-life (*50*). Third, not all NSCLCs, especially squamous cell carcinomas, express FRα. Therefore, use of folate-FITC as a switch is not applicable to other NSCLCs when FRα overexpression is not present. Nevertheless, this shortcoming may also be compensated by the other aspect of this switch-base using the CAR-T strategy. By simply conjugating FITC into another antibody or ligand that targets another well-established NSCLC-specific antigen, the anti-FITC CAR-T cells are readily available to attack those tumor antigen escape variants (*31,50*).

5. Conclusion

We established that *in vitro* anti-FITC CAR-T cell activity can be controlled with a switch, *i.e.* folate-FITC. This bispecific chemical ligand mediated anti-FRα-positive lung cancer cell activity, activation, proliferation and cytokine release ability of CAR-T cells was dose-titratable with a folate-FITC switch. We have also demonstrated that use of folate-FITC as a switch intermediate allows one to target both tumor and tumor-associated macrophages with a single CAR construct. Future studies need to be performed to evaluate the *in vivo* feasibility of this approach.

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