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Redirection of Genetically Engineered CAR-T Cells Using Bifunctional Small Molecules

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Supporting Information

ABSTRACT: Chimeric antigen receptor (CAR)-engineered T cells (CAR-Ts) provide a potent antitumor response and have become a promising treatment option for cancer. However, despite their efficacy, CAR-T cells are associated with significant safety challenges related to the inability to control their activation and expansion and terminate their response. Herein, we demonstrate that a bifunctional small molecule "switch" consisting of folate conjugated to fluorescein isothiocyanate (folate-FITC) can redirect and regulate FITC-specific CAR-T cell activity toward folate receptor (FR)-overexpressing tumor cells. This system was shown to be highly cytotoxic to FR-positive cells with no activity against FR-negative cells, demonstrating the specificity of redirection by folate-FITC. Anti-FITC-CAR-T cell activation and proliferation was strictly dependent on the presence of both folate-FITC and FR-positive cells and was dose titratable with folate-FITC switch. This novel treatment paradigm may ultimately lead to increased safety for CAR-T cell immunotherapy.

Recently, immunotherapies have emerged as a powerful alternative to conventional chemotherapy for the treatment of cancer.[1] One exciting approach is the use of engineered chimeric antigen receptor (CAR) T cells (CAR-Ts).[2] In this approach, T cells are genetically modified to express a chimeric receptor that consists of an extracellular binding domain (e.g., a single-chain antibody variable region, scFv) and a combination of intracellular signaling domains (e.g., CD3ζ and CD137). After transfer back into patients, these cells robustly proliferate to mount a potent response against malignant cells. Indeed, CAR-Ts engineered to target B-cell antigen CD19 (CART-19) have demonstrated unprecedented responses in recent clinical trials in the treatment of relapsed or refractory leukemia and lymphoma.[3]

Despite these successes, there are significant safety challenges associated with the use of CAR-T cells. For example, robust T cell proliferation has caused cytokine release syndrome and CD-19 targeting has resulted in long-term B-cell aplasia.[4] These adverse effects are due to the inability to regulate persistent CAR-T activity.[2,5,6] More importantly, these safety issues pose a greater problem when this approach is used to target solid tumors, where tumor associated antigens (TAAs) are not as restricted as hematological markers and may be found on normal tissues.[7−10]

One approach to control the activity of CAR-T cells is to use an intermediate bifunctional molecule as a "switch" to redirect the specificity of CAR-T cells. Unlike conventional CAR-T cells, the extracellular domain of such a CAR does not directly bind to any TAA; thus, these CAR-T cells are essentially inert in the presence of normal and cancerous cells. However, in the presence of a bifunctional molecule that is specific for both the TAA and the CAR, CAR-T cells can be redirected specifically to target cancer cells. Moreover, the activity of these CAR-T cells can be titrated based on the dosage of switch molecule. The feasibility of this concept has been previously demonstrated in *in vitro* and in mouse models using FITC- or biotin-tagged antibodies.[11−15] More recently, Campana and co-workers have employed a similar approach using CAR-T cells that binds to the Fc portion of a full-length antibody, which enables the targeting of cancer cells in an antibody-dependent manner.[15] Herein, we show that the specificity of CAR-T cells can be redirected using a bifunctional small molecule. Small molecules have excellent tumor penetration and can be readily modified by medicinal chemistry to produce candidates with optimized biophysical and pharmacokinetic properties.[16,17]

As a proof-of-concept, we designed a heterobifunctional folate-FITC conjugate (Figure 1A) that can simultaneously bind to folate receptor (FR) overexpressed on malignant cells and to a CAR-T cell with engineered specificity for FITC. The alpha isoform of FR is expressed on nearly 50% of cancers with a particularly high incidence in breast, lung, uterus, and ovarian cancers, whereas very low levels are detectable on normal tissues.[18−20] In addition, the endogenous ligand, folate, has a high affinity ($K_d \approx 1$ nM) to FR,[21] making it an excellent cancer-targeting small molecule for use as a switch. The use of the synthetic molecule FITC as a ligand for the CAR reduces the potential for off-target cross-reactivity of CAR and eliminates competition by endogenous ligands.

A folate-FITC conjugate has been previously reported by Endocyte (clinically as EC-17) for use in image-guided surgery[22] and hapten conjugate-targeted immunotherapy[23−25] in FR-

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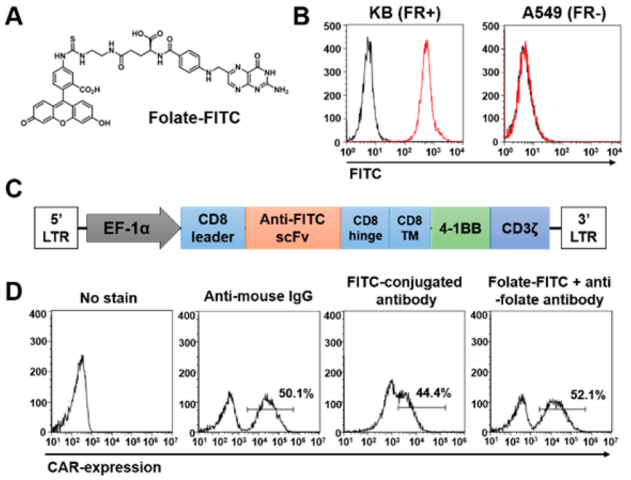

Figure 1. (A) Structure of folate-FITC. (B) Assessment of folate-FITC binding to KB (FR+) and A549 (FR-) cell lines by flow cytometry. The cells were labeled with (red line) or without (black line) 50 nM folate-FITC and analyzed by flow cytometry in the FITC channel. (C) Anti-FITC CAR lentiviral construct. (D) Anti-FITC CAR expression on human T cells was assessed by direct staining with antimouse IgG F(ab′)2 antibody (Jackson Immuno Research) or a FITC-conjugated isotype antibody (eBiosciences). Expression was also detected by primary staining with folate-FITC and secondary staining with an antifolate antibody (Novus Biologicals) conjugated with Alexa Fluor 647 dye. Results shown represent findings observed in seven (B) and three (D) experiments.

overexpressing cancer patients. EC-17 has been thoroughly characterized in preclinical and clinical studies. In addition, a recently published crystal structure of human FR in complex with folate suggests that the FITC conjugation site in folate-FITC is solvent-exposed and will not likely interfere with FITC binding to CAR-T cells.[21] To synthesize the folate-FITC switch, we followed established synthetic schemes[25] (Supplementary Figure 1) and confirmed the purity (>95%) using high-resolution mass spectrometry and NMR. The selectivity of folate-FITC was verified by flow cytometry using FR-positive cervical carcinoma (KB) and FR-negative lung carcinoma (A549) cell lines (Figure 1B).

To create a FITC-specific CAR, the anti-FITC scFv clone 4M5.3[26] was subcloned into a lentiviral vector containing a second generation CAR (from C-terminus: CD3ζ, 4-1BB, CD8 transmembrane domain, and anti-FITC scFv; Figure 1C).[27] Lentiviral particles were generated in HEK293FT cells and used directly to transduce human T cells (Supporting Information). Transduction efficiencies of 40−50% were measured by flow cytometry by staining for scFv surface expression using antimouse IgG antibody (Figure 1D and Supplementary Figure 2). To confirm that the scFv was properly folded on the cell surface and retained its affinity for FITC, flow cytometry was performed using a commercially available FITC-conjugate antibody. We observed binding of FITC-conjugated antibody (44.4% positive, Figure 1D) that was similar to the transduction efficiency measured by the antimouse IgG antibody described above. In addition, the binding of an antifolate antibody to folate-FITC labeled CAR-T cells (52.1% positive, Figure 1D) indicates that the folate group of the folate-FITC is solvent-exposed and is accessible for the simultaneous binding to FR-positive cells.

Next, we evaluated the ability of folate-FITC to redirect anti-FITC CAR-T cells to FR-positive KB cell lines (~1.5 × 10^6 FR/cell),[28] using FR-negative A549 cell lines as a control. At an effector to target cell ratio of 10 to 1 in folate deficient media, folate-FITC demonstrated highly potent (EC_{50} of ~9 pM, Figure 2A) redirection of anti-FITC CAR-T cells to KB cells over 24 h.

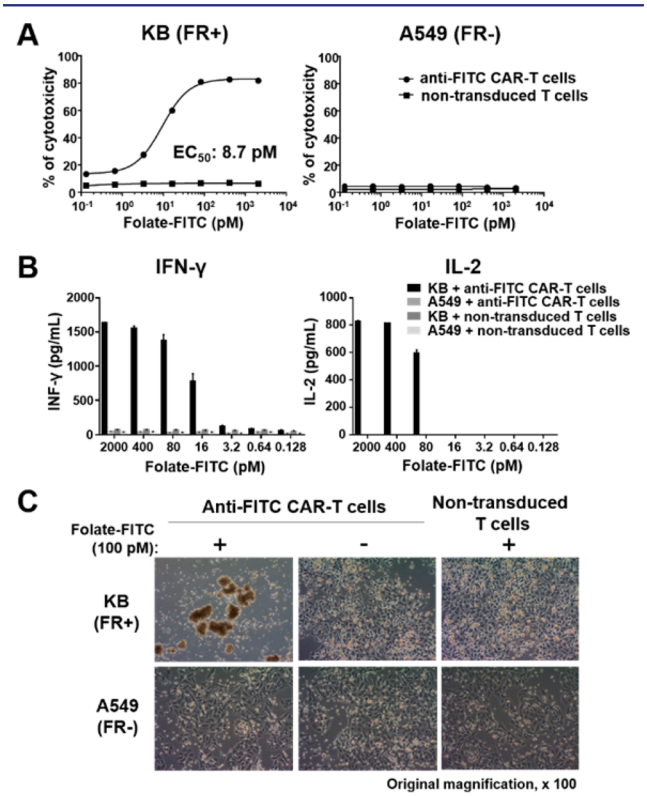

Figure 2. (A) Twenty-four hours cytotoxicity assay of anti-FITC CAR-T cells targeting KB (FR+) and A549 (FR-) cancer cells cultured with serial dilutions of folate-FITC at an effector to target cell ratio of 10:1 (1 × 10^5 to 1 × 10^4 cells per well). Cytolytic activity was determined by measuring the amount of LDH released into cultured media using CytoTox 96 Nonradioactive cytotoxicity assay kit (Promega). Each concentration was carried out in triplicate and error bars represent SEM. (B) Quantification of cytokine (IFN-γ and IL-2) levels in cultures described in panel A by ELISA. Error bars represent SEM derived from triplicate samples. (C) Microscopic images of cytotoxicity assay described in panel A obtained on Zeiss Axio Observer A1.

These results were reproducible across multiple T cell donors with similar efficacies (average EC_{50} = 9.7 ± 3.2 pM, Supplementary Figure 2). Folate-FITC failed to induce lysis of KB cells in the presence of nontransduced T cells. Furthermore, no significant cytotoxic activity was observed with 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 and IL-2 cytokines released during the 24 h assay positively correlated with the concentration of FITC-folate and was dependent on the presence of FR-positive cells (Figure 2B). Lastly, selective formation of aggregates, indicative of cross-linking between T cells and target cells, was only visible in cocultures of anti-FITC CAR and KB cells with folate-FTIC at 100 pM, but not in any of the controls (Figure 2C). Overall, our findings demonstrate that folate-FITC, as a small molecule "switch", can specifically and dose-dependently redirect anti-FITC CAR-T cells to target FR-positive cells.

We next confirmed the specificity of anti-FITC CAR-T cell activation by monitoring expression of cell surface activation markers, CD25 and CD69, on CAR-T cells in coculture with KB cells. After a 24 h incubation in the presence (2.5 nM) or absence

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of folate-FITC, the anti-FITC CAR-T cells were labeled with anti-CD25 and anti-CD69 antibodies and analyzed by flow cytometry. As anticipated, folate-FITC increased CD25 and CD69 expression levels on anti-FITC CAR-T cells compared to nontransduced T cells in the presence of FR-positive cells (Figure 3A and Supplementary Figure 3). In addition to T cell

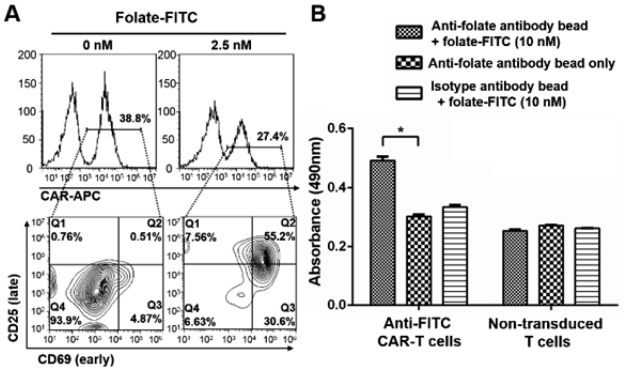

Figure 3. (A) Folate-FITC dependent activation of anti-FITC CAR-T cells. Anti-FITC CAR-T cells (1×10^5 cells) were cocultured with KB cells (1×10^5 cells) for 24 h in the presence (2.5 nM) or absence of folate-FITC, and subsequently stained with APC-conjugated antimouse IgG F(ab′)2 antibody followed by PE-conjugated anti-CD25 and PerCP-Cy5.5-conjugated anti-CD69 antibodies (eBioscience). (B) T cell proliferation assay. Folate-FITC (10 nM) and beads (Dynabeads, Invitrogen) conjugated with the indicated antibodies were added to anti-FITC CAR-T or nontransduced T cells (1×10^5 cells) and cultured for 72 h. T cell proliferation was measured using the CellTiter 96 AQueous One Solution Assay kit (Promega). Results are shown as an average absorbance at 490 nm. Error bars represent SEM derived from triplicate samples. *$P \leq 0.05$ was determined by 2-paired Student t test. Results represent findings from three independent experiments.

activation, we also assessed the proliferative response of the anti-FITC CAR-T cells in the presence of folate-FITC using antifolate antibody-conjugated beads to mimic FR-overexpressing cells. Anti-FITC CAR-T cells cultured in the presence of folate-FITC (10 nM), and these beads induced significant CAR-T cell proliferation over 72 h (Figure 3B, $p < 0.01$).

Lastly, to determine whether the mechanism of *in vitro* cytotoxic activity of CAR-T cells is strictly dependent upon the bispecific binding of folate-FITC to both FR and anti-FITC scFv, we carried out competition assays using excess amount of nonconjugated folate or fluorescein. As shown in Figure 4A, the presence of a 1000-fold molar excess of fluorescein reduced the lysis of KB cells from 64 to 24%. Furthermore, these results suggest that administration of free fluorescein molecule may be used to attenuate anti-FITC CAR-T cell activity in the case of an adverse event, thereby providing an additional method of regulating CAR-T cell activity. Unlike fluorescein, folate is normally present in blood and thus may compete with folate-FITC for FR binding, which may reduce the therapeutic efficacy of CAR-T cells. To assess this possibility, we examined the cytotoxic activity of CAR-T cells in the presence of folate concentrations relevant to physiologically relevant folate levels in human (20 nM) and rodent (200 nM) serum.[29] Notably, certain cell culture media, such as RPMI-1640, contains up to 2 μM folate. Indeed, as shown in Figure 4B, the presence of folate in media reduced the potency of folate-FITC; however, folate-FITC still remained highly potent at concentrations of folate, which are physiologically relevant in human (EC_{50} = 9.3 ± 1.5 pM) and rodents (EC_{50} = 66.9 ± 11.6 pM). This minimal

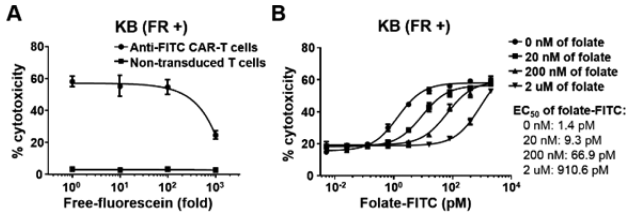

Figure 4. (A) Twenty-four hours of cytotoxicity assay of anti-FITC CAR-T cells (1×10^5) targeting KB cells (1×10^4) in the presence of 200 pM folate-FITC and varied concentrations of free fluorescein (0−1000-fold of folate-FITC). Each data point represents a mean of triplicates, and error bars represent SEM. (B) Twenty-four hours of cytotoxicity assay of anti-FITC CAR-T cells (1×10^5) targeting KB (1×10^4) cells in the presence of different concentrations of folate-FITC in folate-free media supplemented with the indicated concentrations of free folate. Each data point represents a mean of triplicate samples, and error bars represent SEM.

decrease in potency likely results from multivalent binding of folate-FITC to anti-FITC CAR-T cells in the presence of KB cells, which provides sufficient avidity to overcome competition from physiological levels of folate. At supra-physiological concentrations of folate (2 uM), the EC_{50} of folate-FITC is reduced 600-fold, further confirming that the activity of anti-FITC CAR-T is mediated by the binding of folate-FITC. Further evaluation by flow cytometry revealed that excess folate and fluorescein inhibited folate-FITC binding to KB and ant-FITC CAR-T cells, respectively, in a concentration-dependent manner (Supplementary Figure 4), suggesting that the attenuated activity of anti-FITC CAR-T shown in Figure 4 is a result of reduced binding of folate-FITC.

In conclusion, we have demonstrated a novel therapeutic approach that combines the dose titratability of small molecules with the potency of CAR-T cell therapy. Future studies will evaluate the *in vivo* efficacy of folate-FITC-controlled anti-FITC CAR-T cells in FR-positive xenograft tumor models. This switchable platform may overcome some of the safety hurdles associated with current CAR-T cell approaches and provide a promising immunotherapeutic for FR-positive cancer patients.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary Figures S1−S4 and detailed materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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