

Use of a Single CAR T Cell and Several Bispecific Adapters Facilitates Eradication of Multiple Antigenically Different Solid Tumors

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Abstract

Most solid tumors are comprised of multiple clones that express orthogonal antigens, suggesting that novel strategies must be developed in order to adapt chimeric antigen receptor (CAR) T-cell therapies to treat heterogeneous solid tumors. Here, we utilized a cocktail of low-molecular-weight bispecific adapters, each comprised of fluorescein linked to a different tumor-specific ligand, to bridge between an anti-fluorescein CAR on the engineered T cell and a unique antigen on the cancer cell. This formation of an immunologic synapse between the CAR T cell and cancer cell enabled use of a single anti-fluorescein CAR T cell to eradicate a diversity of antigen-

ically different solid tumors implanted concurrently in NSG mice. Based on these data, we suggest that a carefully designed cocktail of bispecific adapters in combination with anti-fluorescein CAR T cells can overcome tumor antigen escape mechanisms that lead to disease recurrence following many CAR T-cell therapies.

Significance: A cocktail of tumor-targeted bispecific adapters greatly augments CAR T-cell therapies against heterogeneous tumors, highlighting its potential for broader applicability against cancers where standard CAR T-cell therapy has failed.

Introduction

Chimeric antigen receptor (CAR) T-cell therapies have recently demonstrated considerable potential in the treatment of a variety of hematopoietic cancers. In the case of acute lymphoblastic leukemia (ALL), infusion of anti-CD19 CAR T cells has yielded a 79% to 94% complete response rate, with a median overall survival of 7.8 to 29 months (1–4). In the treatment of refractory non-Hodgkin's lymphoma, similar anti-CD19-specific CAR T cells have been shown to achieve a >80% response rates (including a 54% complete remission; ref. 5), and upon treatment of refractory/recurrent multiple myeloma, an unrelated CART-cell preparation that recognizes B-cell maturation antigen has demonstrated an 89% overall response rate (6). Based on these remarkable results, CAR T-cell therapies are rapidly emerging as one of the most promising developments in clinical oncology in many years.

Despite the aforementioned promising outlook, a substantial fraction of CAR T-cell-treated patients may initially respond to therapy, but later recur due to rapid selection against malignant cells that express the CAR-recognized antigen (7). In four different

clinical trials of ALL patients treated with an anti-CD19 CART-cell preparation, 11% to 26% of patients suffered a subsequent recurrence of cancer cells that lacked CD19 but expressed other unrelated tumor antigens (e.g., CD22; refs. 7–11). Indeed, general tumor genome sequencing has repeatedly demonstrated that an instability in the cancer genome can lead to loss of some tumor-defining antigens and simultaneous acquisition of others, leading to evolution of an initial single malignant clone into a heterogeneous assembly of many related clones (12). Unfortunately, conventional CAR constructs are unable to target multiple tumor clones, because conventional CARs only recognize a single antigen. Given the inherent difficulty in preparing a personalized cocktail of antigen-specific CAR T cells for treatment of every patient's heterogeneous tumor, it became advisable to explore the development of a single CAR T cell that could be adapted to treat all heterogeneous tumors.

In an effort to design this universal CAR T cell, we examined the possibility of engineering a T cell whose CAR construct would contain the usual cytoplasmic activation domains found in conventional CARs (e.g., CD3 ζ fused to 4-1BB or CD28) plus an extracellular single-chain variable fragment (scFv) that was designed to recognize fluorescein (anti-FITC CAR T cell, Supplementary Fig. S1A). In this strategy, addition of a bispecific adapter comprised of fluorescein linked to a tumor-specific ligand would promote bridging of the CAR T cell to the tumor cell, leading to formation of immunologic synapse between the engineered T cell and its malignant target, triggering CAR T-cell activation and the subsequent destruction of the cancer cell. In the absence of such a bispecific adapter, CAR T-cell engagement with the cancer cell would not occur, and the consequent killing of the cancer cell would not proceed. More importantly, with the increasing availability of low-molecular-weight tumor-specific ligands (13–17), a cocktail of orthogonal fluorescein-linked bispecific adapters

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could be prepared in which each fluorescein-linked adapter was tethered to a unique tumor-specific ligand capable of binding one of the cancer cell's antigens. When coadministered with the anti-FITC CAR T cells, such a cocktail of adapters could conceivably engage all cancer cell clones in an antigenically heterogeneous tumor and lead to tumor eradication without selection for antigen-deficient resistant clones. In this article, we test the ability of a single anti-fluorescein CAR T-cell preparation to recognize and destroy multiple antigenically unique human cancer cells upon addition of the appropriate fluorescein-linked tumor-specific ligand. We demonstrate that both MDA-MB-231 and HEK293 cells transfected with a variety of tumor antigens can be rapidly killed both *in vitro* and *in vivo* upon addition of the correct antigen-matched CAR T-cell adapter molecule (CAM).

Materials and Methods

Cell lines and human T cells

Folic acid-free RPMI 1640 (Gibco) containing 10% heat-inactivated FBS and 1% penicillin-streptomycin was used to maintain folate receptor-positive cell lines (e.g., FR α -expressing HEK and MDA-MB-231). All other cancer cell lines and derived clones were maintained in RPMI 1640 containing 10% heat-inactivated FBS and 1% penicillin-streptomycin. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation (GE Healthcare Lifesciences) from human whole blood obtained from healthy volunteers. Pure CD3⁺ T cells were enriched from PBMCs using an EasySep Human T Cell Isolation Kit (STEM CELL Technologies) and then cultured in TexMACS medium (Miltenyi Biotech Inc.) containing 1% penicillin and streptomycin sulfate and 2% human serum (Valley Biomedical) in the presence of human IL2 (100 IU/mL; Miltenyi Biotech Inc.). Human T cells were counted every 2 to 3 days and maintained at 0.5×10^6 cells/mL. MDA-MB-231 was obtained from the ATCC, and authentication of MDA-MB-231 was carried out by short-tandem repeat analysis based on the ATCC. All cells were maintained in 5% CO₂ at 37°C and were regularly tested for contamination of *Mycoplasma*.

Preparation and use of lentiviral vector encoding anti-FITC CAR

An scFv with high affinity for fluorescein (18) was synthesized (GeneScript), and plasmids encoding human CD8 α , 4-1BB, and CD3 ζ chain were purchased from GeneScript. Overlapping PCR method was then used to generate the final 1551 bp anti-FITC CAR construct and inserted into a pCDH-EF1-MCS-(PGK-GFP) lentiviral expression vector (System Biosciences). The sequence of the anti-FITC CAR construct was confirmed by DNA sequencing (Purdue Genomic Core Facility). Purified human T cells were first activated using Dynabeads coupled to anti-CD3/CD28 antibodies (Life Technologies) for 12 to 24 hours in the presence of human IL2 (100 IU/mL) and then infected with the aforementioned lentivirus. After 3 to 5 days of transduction, T cells were harvested and analyzed for GFP fluorescence by flow cytometry to determine transduction efficiency.

Generation of antigenically heterogeneous cancer cells

In order to try to mimic human tumor heterogeneity in an animal model, MDA-MB-231 cells (FR α -expressing human breast cancer cell line) were transduced with lentivirus encoding a gene for prostate-specific membrane antigen (PSMA; NM_004476.1) or carbonic anhydrase IX (CA IX; NM_001216.2). For a second

heterogeneous tumor model, HEK 293 cells were also transduced with lentivirus encoding FR α (NM_000802.3), PSMA, CA IX, or NK1R (NM_001058.3). Expression of each receptor on the desired cancer cell line was confirmed by flow cytometry after staining with fluorochrome-conjugated antibodies or the appropriate FITC-labeled CAM.

Binding of CAMs to anti-FITC CAR and cancer cell receptors

To evaluate the binding affinity of each CAM for both the anti-FITC scFv on the CAR T cell and its tumor antigen on the cancer cell, FITC-folate, FITC-DUPA, FITC-AZA, or FITC-NKRL were prepared and characterized as previously described (13, 14, 16, 19). Because our transduced CAR T cells expressed GFP (as a means of evaluating CAR transduction efficiencies), we quantitated CAM binding to the CAR T cells by analyzing displacement of FITC-AlexaFluor647 (FITC-Alexa647) from the anti-FITC CAR T cells by each FITC-labeled CAM. For this purpose, CAR T cells were incubated with various concentrations of FITC-Alexa647 for 1 hour at room temperature, followed by washing 3 \times with PBS and measurement of cell-associated AlexaFluor647 fluorescence by flow cytometry. To confirm the specificity of CAM binding to anti-FITC CAR T cells, CAR T cells were also incubated with FITC-Alexa647 in the absence or presence of excess (1 μ mol/L) competitive ligands (i.e., FITC-biotin, FITC-Folate, FITC-PSMA, or FITC-AZA; Supplementary Fig. S1B) for 1 hour at room temperature.

For analysis of the binding affinity of each CAM for its tumor antigen on the cancer cells, increasing concentrations of each of CAM were incubated with the desired cancer cells for 1 hour at room temperature. After incubation, cancer cells were washed 3 \times with PBS and analyzed by flow cytometry. The GraphPad Prism version 7 software was used to analyze binding affinity.

Analysis of cytotoxic activity of anti-FITC CAR T cells *in vitro*

Antigen-expressing MDA-MB-231 or HEK293 cells were seeded at a density of 10^4 cells/100 μ L into 96-well plates and grown overnight. Anti-FITC CAR T cells (5×10^4) were added to each well in the absence or presence of the desired CAM(s). After incubation for 18 to 24 hours, plates were centrifuged at $350 \times g$ for 10 minutes to remove debris, and supernatants were analyzed for lactate dehydrogenase release (cell death analysis) using Pierce LDH cytotoxicity assay kit (Thermo Fisher Scientific) and IFN γ levels using human IFN γ ELISA kit (Biolegend).

Analysis of antitumor activity of anti-FITC CAR T cells *in vivo*

Immunodeficient NSG mice (Jackson Laboratory) were implanted subcutaneously with MDA-MB-231 cells or HEK293 cells that expressed FR α -, PSMA-, CA IX-, or NK1R tumor antigens. After allowing tumors to grow to approximately 100 mm³, mice were injected intravenously with 10^7 CAR T cells and then with increasing doses of the appropriate CAMs (as indicated in the figure legends). A schedule of escalating CAM doses (i.e., 5 nmol/kg on days 1 and 2, 50 nmol/kg on days 4 and 6, 100 nmol/kg on days 8 and 10, and 500 nmol/kg from day 12 onward; Supplementary Table S1) was invariably used, because it was found from a systematic analysis of various dosing concentrations and frequencies to yield complete tumor elimination with no apparent toxicity. Analysis of the pharmacokinetic of FITC-folate in both animals and humans has been reported elsewhere (20), and an evaluation of the pharmacokinetics of the PSMA, CA IX, and NK1R ligands has also been reported (21–23).

Because the high levels of folate present in normal rodent chow raise the serum folate levels to many times the normal physiological range (24), mice to be treated with an FITC-folate CAM were maintained on folic acid-deficient diet (Envigo) in order to lower their serum folate levels to concentrations found in humans. Otherwise, all mice were maintained on normal chow.

In order to evaluate whether a single anti-FITC CAR T cell might be exploited to eliminate antigenically heterogeneous tumors, two different animal models were used. First, PSMA- and CA IX-expressing MDA-MB-231 were implanted at different locations (neck and flank) on the same immunodeficient NSG mice. Then, when tumors reached approximately 100 mm³ in size, 10⁷ anti-FITC CAR T cells were intravenously injected into the mice followed by either (1) PBS, (2) FITC-DUPA alone, (3) FITC-AZA alone, or (4) a cocktail of FITC-DUPA and FITC-AZA. As a second animal model, instead of growing the antigenically different clones at the separate locations on the same mouse, PSMA- and CA IX-expressing MDA-MB-231 cells were mixed and then implanted into NSG mice, thereby generating a single solid tumor comprised of two antigenically distinct subclones. Following treatment as described above, the change in clonal composition of the solid tumor was analyzed by digesting the tumor with digestion cocktail (i.e., 1 mg/mL of collagenase IV + 0.1 mg/mL of hyaluronidase from bovine test + 0.2 mg/mL of deoxyribonuclease) to obtain single cells and immunostaining with fluorochrome-conjugated antibodies to quantitate the distribution of cells in the final mixture. All tumors were measured every other day with calipers, and tumor volumes were calculated according to equation: Tumor volume = $\frac{1}{2} (L \times W^2)$, where *L* is the longest axis of the tumor, and *W* is the axis perpendicular to *L*. Systemic toxicity was monitored by measuring bodyweight loss. All animal care and use were followed by NIH guidelines, and all experimental protocols were approved by the Purdue Animal Care and Use Committee.

Results

To begin to mimic an antigenically heterogeneous human cancer, it was necessary to create a set of human cancer cell clones that would differ primarily in their expression of unique tumor-specific antigens. For this purpose, we transformed a human breast cancer cell line (MDA-MB-231 cells) with two orthogonal antigens that are commonly found in human tumors. CA IX has been reported to be overexpressed in cancers of the kidney, lung, breast, colon, and hypoxic regions of all other solid tumors (25). PSMA has been similarly observed not only in cancers of the prostate, but also in the neovasculature of most solid tumors (26). Together with folate receptor alpha (FR α) that is overexpressed in approximately 40% of human cancers (27), these three tumor-enriched antigens allow for construction of a tumor model that should enable testing of the ability of a single CAR T cell to eradicate a heterogeneous tumor that has mutated to express multiple orthogonal antigens. As shown in Fig. 1A, MDA-MB-231 cells that naturally express FR α (left plot) were successfully transformed to also express either PSMA (center plot) or CA IX (right plot). In the studies below, we use these three MDA-MB-231 clones to explore whether the same CAR T cell can be exploited to eradicate multiple antigenically distinct clones of a parent cancer cell upon addition of the appropriate CAMs.

Construction and characterization of CAR T cells and bispecific adapters for use in evaluation of CAR T-cell universality

To promote engagement of our anti-FITC CAR T cell with multiple tumor-specific antigens, different CAMs had to be synthesized that contained fluorescein at one end linked via a short hydrophilic spacer to a tumor-specific ligand at the other. The structures of the CAMs selected for this study are shown in Fig. 1B, with fluorescein positioned invariably on the left and the tumor-targeting ligand on the right. The affinities of these CAMs for their respective tumor receptors are evaluated in the binding curves of Fig. 1C, and their association with the anti-FITC CAR of the CAR T cell is documented in Fig. 1D ($K_d = 30$ pmol/L). Not surprisingly, the different CAMs bind their respective tumor receptors with different affinities, ranging from $K_d = 2$ to 24 nmol/L, depending on the ligand. Because all of these CAMs bind with very high affinity, one would anticipate that formation of multiple CAM-mediated bridges between a CAR T cell and its targeted tumor cell should promote a sufficiently stable interaction to facilitate CAR T-cell activation and subsequent cancer cell killing to occur. Moreover, because the CAMs are all small molecules, their abilities to penetrate solid tumors and their capacities to engage virtually all cancer cells and CAR T cells in a tumor mass should be relatively unimpeded (28).

Evaluation of antigen-specific CAM-mediated CAR T-cell engagement with cancer cells *in vitro*

To determine the ability of each CAM to promote formation of a functional immunologic synapse between the CAR T cell and its targeted cancer *in vitro*, we cocultured each antigen-expressing MDA-MB-231 cell clone with our anti-FITC CAR T-cell preparation and then added the appropriate CAM to assess CAR T-cell activation and cancer cell killing. As shown in Fig. 2, administration of the correct adapter enabled both CAR T-cell activation (as evidenced by their release of IFN γ , top plots of Fig. 2A–C) and cancer cell killing (bottom plots), whereas incubation with the wrong CAM induced neither of these responses (see below). Moreover, as anticipated from mechanistic considerations, the concentration dependence of both CAR T-cell activation and tumor cell killing was roughly bell-shaped, i.e., consistent with the fact that at very low CAM concentrations, insufficient intercellular bridges will be formed to induce CAR T-cell activation, whereas at very high CAM concentrations, intercellular bridging will be blocked due to monovalent saturation of ligand binding sites on both cell types with the excess CAMs.

Evaluation of antigen-specific CAM-mediated CAR T-cell engagement with cancer cells *in vivo*

To evaluate the ability of the bispecific CAMs to promote anti-FITC CAR T-cell killing of antigenically different MDA-MB-231 clones *in vivo*, we subcutaneously implanted MDA-MB-231 cell clones expressing each of the aforementioned tumor antigens (i.e., FR α , PSMA, or CA IX) in NSG mice, and after allowing tumors to reach 100 mm³, we treated the mice with anti-FITC CAR T cells plus the antigen-matched CAM shown in Fig. 1. As revealed in Fig. 3A, the anti-FITC CAR T-cell preparation was able to quantitatively eliminate the natural FR α -expressing MDA-MB-231 clone in the presence of the FITC-folate CAM, but not in its absence. Moreover, in the absence of the CAR T cells but in the presence of FITC-folate, no therapeutic efficacy was again

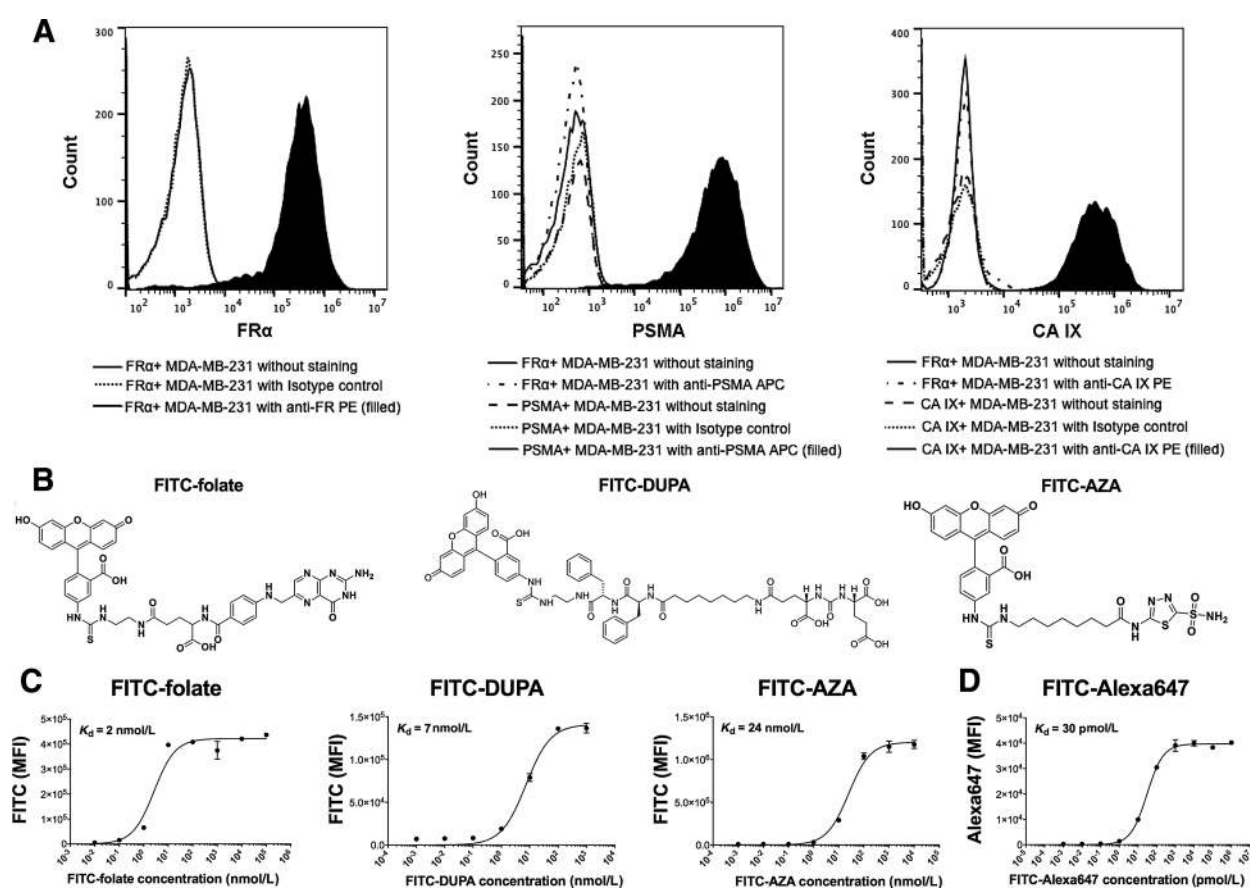


Figure 1.

Characterization of cancer cell clones that express orthogonal antigens and their bispecific CAMs. **A**, Demonstration that MDA-MB-231 breast cancer cells that naturally express folate receptor α (left) can also be transduced to express PSMA (middle) or CA IX (right). In each case, expression of the antigen is evaluated by monoclonal antibody staining, followed by flow cytometry. **B**, Structures of FITC-folate, FITC-DUPA, and FITC-AZA, as labeled. **C**, Evaluation of the binding affinities of the CAMs in **B** to targeted receptors on the tumor cells characterized in **A**. **D**, Evaluation of the binding affinity of FITC-Alexa647 for the anti-FITC CAR used in these studies. Data shown in **C** and **D** are the representative data from two independent experiments.

observed. These data demonstrate that the combination of anti-FITC CAR cell plus its tumor antigen-matched CAM is required for tumor eradication *in vivo*.

This conclusion is further supported by data in Fig. 3B and C, where PSMA- and CA IX-expressing clones of the MDA-MB-231 cells, respectively, were observed to yield complete responses when treated with both CAR T cells plus the antigen-matched CAM, but not when either the CAR T cell or antigen-matched CAM was lacking. Importantly, when the nontransformed (natural) FR α^+ MDA-MB-231 clone was treated with the CAR T-cell preparation plus either the PSMA- or CA IX-targeted CAM, negligible antitumor activity could be detected (Supplementary Fig. S2), suggesting that the killing potency of the anti-FITC CAR T cell requires the presence of the tumor antigen-matched CAM for killing to occur.

Use of a CAM cocktail to prevent cancer recurrence due to selection for antigen-deficient cancer cells

To assess the ability of a cocktail of CAMs to mediate eradication of different solid tumors deriving from antigenically distinct clones of the same cancer, we implanted PSMA- and CA IX-expressing subclones of MDA-MB-231 cells on different locations

of the same mice (neck and flank) and measured their tumor volumes following administration of anti-FITC CAR T cells plus either matched or mismatched CAMs. As shown in Fig. 4A, both cancer cell subclones grew at approximately the same rate in PBS-treated NSG mice. In contrast, similar anti-FITC CAR T-cell-treated mice experienced a complete remission of their PSMA-expressing tumors when treated with the PSMA-targeted CAM, but saw little or no decline in their CA IX-expressing tumors growing on the neck (Fig. 4B). As anticipated, similar CAR T-cell-injected mice underwent complete remission of their CA IX-expressing tumors when treated with CA IX-targeted CAMs, but experienced no decline in their PSMA-expressing cancers growing on the right flank (Fig. 4C). Importantly, complete elimination of both PSMA- and CA IX-expressing tumors was only achieved when a cocktail of FITC-DUPA and FITC-AZA adapter molecules was injected into the tumor-bearing mice (Fig. 4D). These data argue that the same anti-FITC CAR T-cell preparation can be used to eliminate multiple tumors in the same animal when coadministered with the proper antigen-matched set of CAMs. The data also demonstrate that any cytotoxicity arising from allogeneic effects of the polyclonal human T cells used in these studies is minimal if not entirely absent.

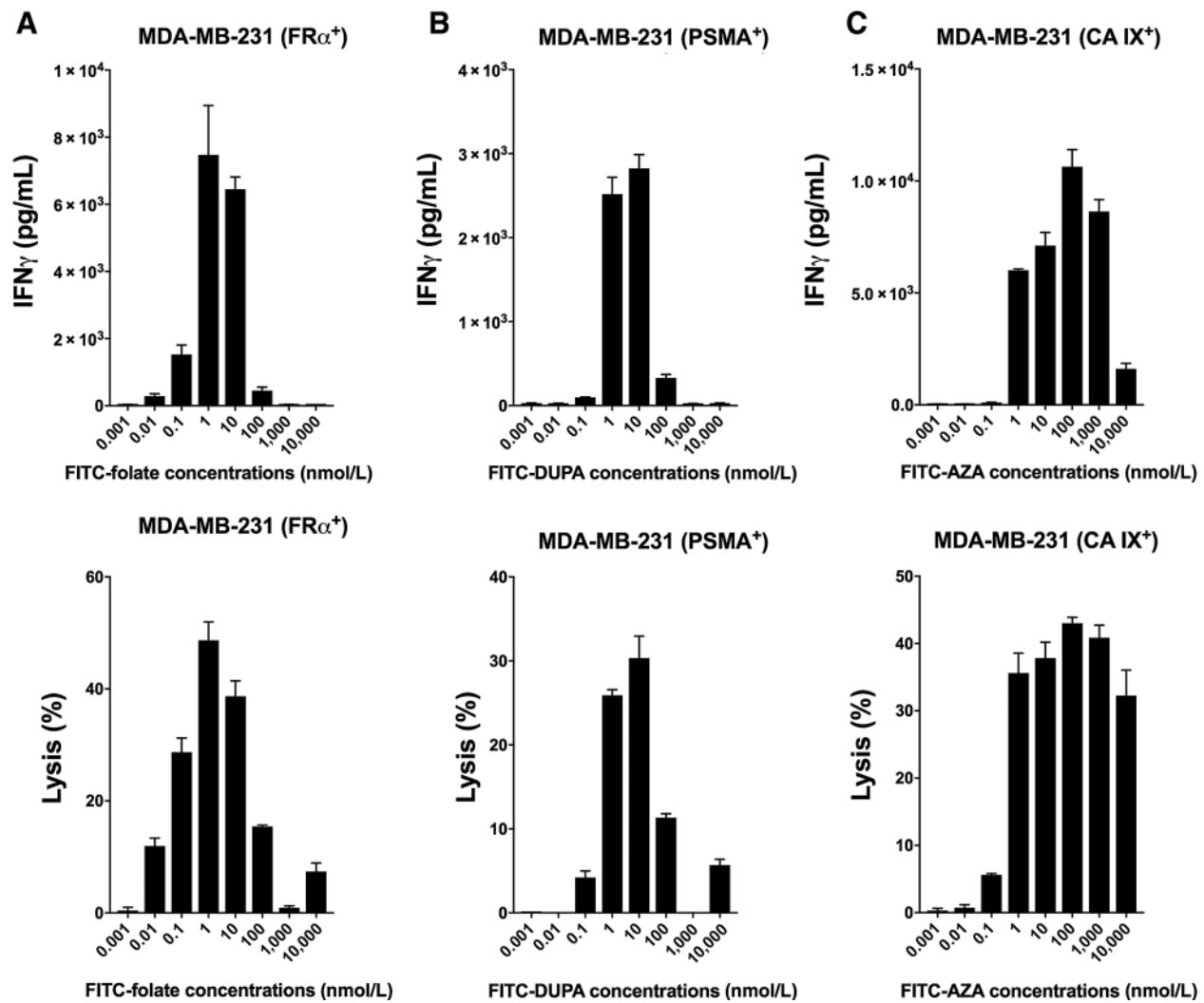


Figure 2. Effect of CAM concentration on cytokine release and cancer cell lysis by anti-FITC CAR T cells *in vitro*. Naturally occurring FR α -expressing MDA-MB-231 cells (A), or MDA-MB-231 cells transduced to express PSMA (B) or CA IX (C) were incubated in the presence of anti-FITC CAR T cells plus different concentrations of FITC-folate (A), FITC-DUPA (B), or FITC-AZA (C) prior to analysis of IFN γ release and tumor cell lysis as described in Materials and Methods. Effector:tumor cell ratio = 5:1. Bar graphs represent mean \pm SD. *n* = 3. Data shown are the represent data from two independent experiments.

Next, in order to mimic the more common situation where multiple heterogeneous clones of the same parent cancer cell are simultaneously present in the same tumor mass, approximately equal numbers of PSMA- and CA IX-expressing subclones of MDA-MB-231 cells were mixed and allowed to proliferate in NSG mice until approximately 100 mm³ tumors comprised of similar numbers of the two subclones were formed. As shown in Fig. 5A, mice treated with CAR T cells plus only PBS showed rapid and uninterrupted growth of all heterogeneous tumors, whereas similar animals injected solely with FITC-DUPA or FITC-AZA experienced delayed but persistent tumor enlargement. To evaluate the compositions of the residual tumor masses, these tumor nodules were excised and analyzed by flow cytometry for expression of either PSMA or CA IX; i.e., diagnostic of the clones that were surviving the CART-cell therapy. As shown in Fig. 5B, mice treated with only PBS grew tumors with roughly the same clonal composition as the implanted mixture. In contrast, mice treated with

the PSMA-targeted CAM proliferated tumors by day 30 that were approximately 5% PSMA positive and 95% CA IX positive. Analogously, residual tumors in mice treated with the CA IX-targeted CAM displayed almost the opposite clonal composition (i.e., 7% CA IX positive and 93% PSMA positive). Most significantly, mice injected with a combination of PSMA- and CA IX-targeted CAMs underwent complete remission of their tumors, suggesting again that the correct mixture of adapter molecules can enable anti-FITC CAR T cell killing of all tumor clones.

Assessment of anti-FITC CAR T-cell efficacy in an unrelated tumor model

Finally, to evaluate the potency of the anti-FITC CAR T-cell therapy in a different tumor model, HEK293 cells were transformed with FR α , PSMA, CA IX, or the neurokinin 1 receptor (NK1R), that is often overexpressed on neuroendocrine cancers (13). Following selection of subclones that expressed significant

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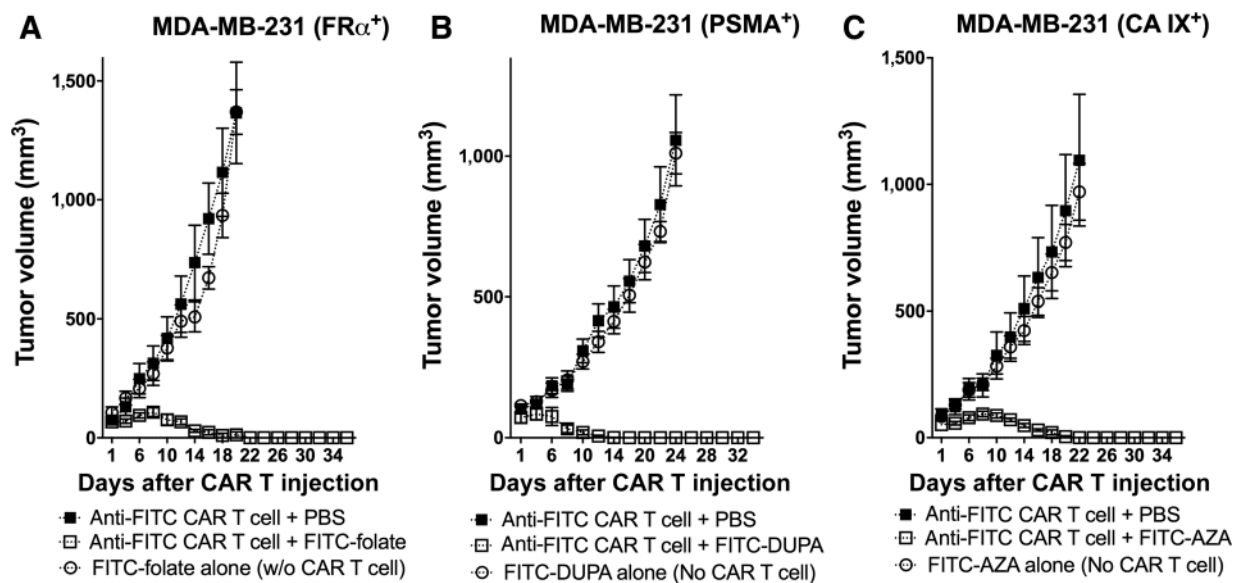


Figure 3. Effect of anti-FITC CAR T-cell infusion on tumor volume upon administration of the correct antigen-specific CAM. NSG mice were implanted with two million of either naturally occurring MDA-MB-231 cells (FR α^+ ; **A**) or MDA-MB-231 cells transduced with PSMA (**B**) or CA IX (**C**) and infused with anti-FITC CAR T cells (10^7 cells) when tumor volumes reached approximately 100 mm^3 (day 1). Mice were then intravenously injected every other day with either PBS or increasing amounts of the CAM that was designed to bind the tumor antigen that was overexpressed on the MDA-MB-231 tumor (i.e., 5 nmol/kg on days 1 and 2, 50 nmol/kg on days 4 and 6, 100 nmol/kg on days 8 and 10, and 500 nmol/kg from day 12 onward). To determine whether CAM addition might have any direct effect on tumor growth, mice were also treated with each of the CAMs in the absence of anti-FITC CAR T cells. $n = 5$ mice per group. All data represent mean \pm SEM. Data shown are the represent data from two independent experiments.

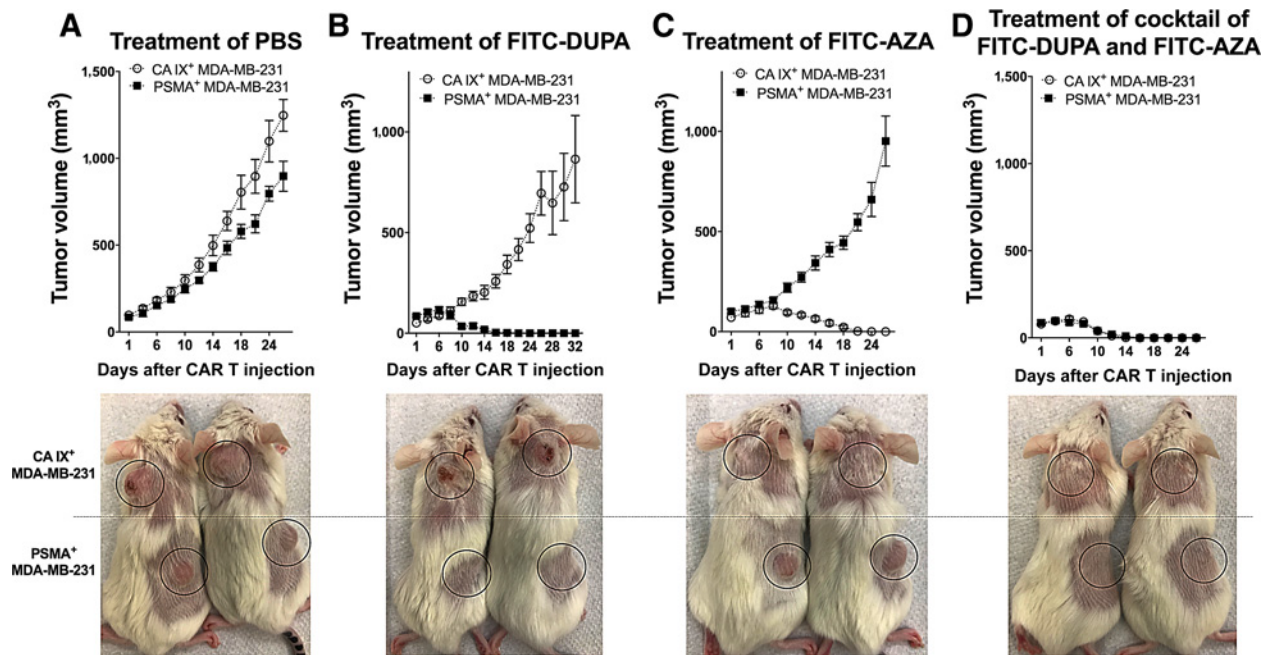


Figure 4. Demonstration that antigenically different tumor clones can be simultaneously eradicated by a single anti-FITC CAR T-cell preparation in combination with a cocktail of antigen-matched CAMs. Anti-FITC CAR T cells (10^7 cells) were infused into NSG mice bearing MDA-MB-231 (PSMA $^+$) solid tumors on their right flanks and MDA-MB-231 (CA IX $^+$) solid tumors on their necks after tumors reached 100 mm^3 . Mice were then treated with either PBS (**A**), FITC-DUPA only (**B**), FITC-AZA only (**C**), or a mixture of FITC-DUPA and FITC-AZA (**D**) using the dose-escalation schedule described in Fig. 3. $n = 5$ mice per group. All data represent mean \pm SEM. Data shown represent data from two independent experiments.

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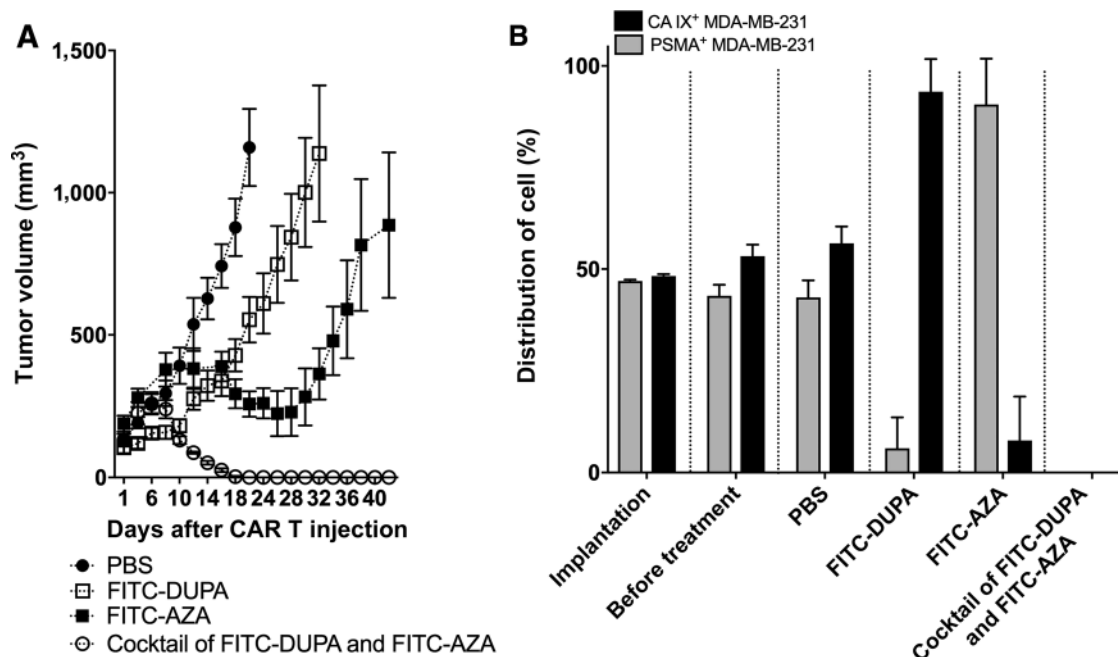


Figure 5.

Demonstration that an antigenically heterogeneous tumor comprised of different tumor clones can be eradicated by a single anti-FITC CAR T cell preparation in combination with a cocktail of antigen-matched CAMs. Anti-FITC CAR T cells (10^7 cells) were infused into NSG mice bearing single 100 mm^3 tumors comprised of the aforementioned MDA-MB-231 clones (PSMA⁺ and CA IX⁺) and then intravenously treated with either PBS only, FITC-DUPA only, FITC-AZA only, or a mixture of FITC-DUPA and FITC-AZA. **A**, Measurement of tumor volume. **B**, Analysis of the change in clonal composition of the tumor masses following treatment. The dosing schedule was the same as described in Fig. 3. $n = 5$ mice per group. All data represent mean \pm SEM. Data shown represent data from two independent experiments.

amounts of the desired receptor (Fig. 6A), the cultured cells were coincubated with anti-FITC CAR T cells in the presence of antigen-matched and mismatched CAMs. For use in bridging between an NK1R-expressing HEK293 cell and the anti-FITC CAR T cell, the NK1R-specific CAM (termed FITC-NKRL) shown in Fig. 6B was synthesized ($K_d = 4 \text{ nmol/L}$, Fig. 6C). As shown in Fig. 6D and E, HEK293 cell killing and production of IFN γ were both strongly dependent on the presence of the correct antigen-matched CAM, confirming the CAM bridging mechanism of CAR T-cell engagement and killing. Moreover, eradication of HEK293 tumors *in vivo* expressing the NK1R antigen also required coadministration of the NK1R-targeted bispecific CAM (Fig. 6F).

Discussion

In response to the antigenic heterogeneity that is believed to arise in almost all solid tumors, many laboratories have explored strategies to create a universal CAR T cell capable of recognizing different cancer cell clones that express unrelated tumor antigens (29–32). Bispecific adapter approaches similar to ours have consequently been examined for their abilities to mediate CAR T-cell eradication of heterogeneous tumors, only in most of these cases antibodies or antibody fragments have been used as tumor-specific ligands in the bispecific adapter constructs (33–36). Although each of the above strategies has shown considerable promise, we believe that use of low-molecular-weight adapters/CAMs may have advantages over their macromolecular counterparts. First, low-molecular-weight CAMs such as FITC-folate ($M_R \sim 873$) have demonstrated an ability to reach all cancer cells

in a solid tumor in human cancer patients (16), whereas the much larger antibodies ($M_R \sim 150,000$) have not (28). Thus, surgical resection of solid tumors from patients receiving FITC-folate shortly before surgery has established that virtually all folate receptor-expressing cancer cells accumulate FITC-folate (16). In contrast, similar studies of patients treated with antibody-drug conjugates have revealed that significant areas of tumors can remain devoid of derivatized antibodies, largely due to poor penetration of the antibodies through a tumor's dense extracellular matrix (28). Second, low-molecular-weight CAMs penetrate solid tumors rapidly and clear from receptor-negative tissues with half-lives of the order of approximately 90 minutes (20). Systemic residence times of antibodies, in contrast, commonly have half-lives on the order of days (37). Thus, one would anticipate that when a reduction in the number of CAR T cells engaged with cancer cells is urgently needed in order to mitigate a life-threatening cytokine release syndrome or destruction of healthy tissues, modulation of the numbers of CAR T-cell-cancer cell bridges might be achieved more rapidly with a low-molecular-weight CAMs (Y.G. Lee; submitted for publication). Finally, when the length of the adapter between the CAR T cell and cancer cell is so long that it prevents intercellular engagement of the coreceptors required for CAR T-cell activation, cancer cell killing can be compromised. Such steric obstructions, however, can be easily remedied by shortening the synthetic spacer between FITC and its cancer-specific ligand in the CAM, but might require re-engineering of the antibody in a macromolecular-bispecific adapter. Taken together, the flexibility offered by use of low-molecular-weight CAMs for promoting and regulating a CAR

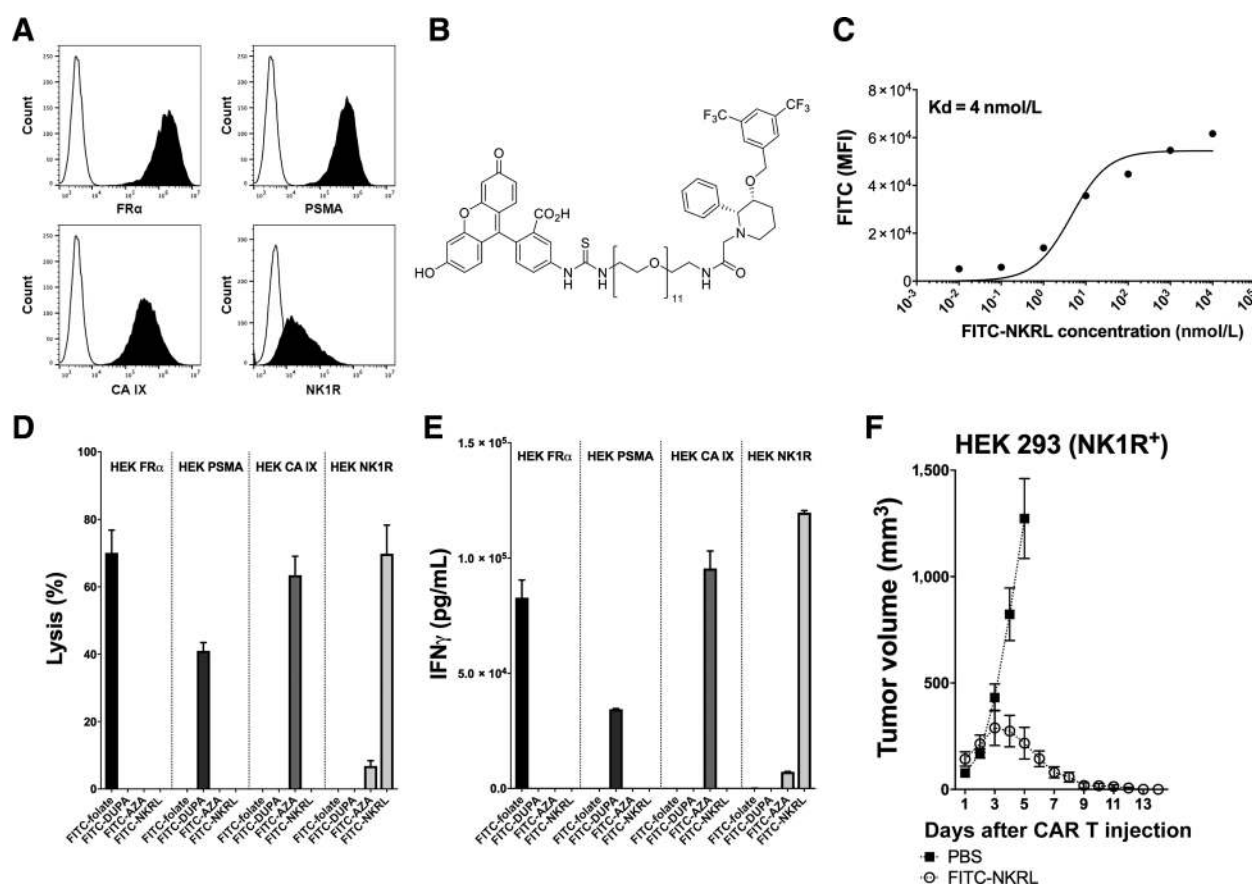


Figure 6.

Demonstration that the same anti-FITC CAR T cells plus the correct tumor antigen-matched CAMs can also eliminate an orthogonal tumor type. **A**, Demonstration that HEK293 cells can be transduced to express FR α , PSMA, CA IX, or NK1R. In each case, expression of the antigen is evaluated by staining with the correct FITC-labeled CAM, followed by flow cytometry. **B**, Structure of the FITC-NKRL CAM used in this study. Structures of all other CAMs are shown in Fig. 1. **C**, Evaluation of the binding affinity of NKRL for the neurokinin receptor 1 transduced into HEK293 cells ($K_d = 4$ nmol/L). **D**, Demonstration that a single anti-FITC CAR T cell can lyse multiple HEK293 cells expressing orthogonal antigens upon addition of the antigen-matched CAM but not upon addition of a mismatched CAM. **E**, Demonstration that the same anti-FITC CAR T-cell preparation can be stimulated to release IFN γ in the presence of antigenically different HEK293 cells upon addition of the correct antigen-matched CAM but not upon addition of a mismatched CAM. **F**, Demonstration that anti-FITC CAR T cells can eradicate HEK 293 (NK1R $^+$) tumors upon addition of FITC-NKRL, but not PBS. All conditions are as described in Fig. 3 except 5 million HEK293 expressing NK1R were implanted, and FITC-NKRL was used as the CAM. $n = 5$ mice per group. Data shown in **D**, **E**, and **F** are the representative data from two independent experiments.

T-cell–cancer cell immunologic synapse argues that further exploration of low-molecular-weight adaptors warrants continued effort.

Given the documented antigenic heterogeneity found in almost all solid tumors (12, 38), the question naturally arises whether sufficient CAMs can be designed to enable universal CAR T-cell killing of most human cancers. Although considerable work remains to achieve this goal, the increasing availability of low-molecular-weight tumor targeting ligands argues that a pan-cancer cocktail of CAMs can eventually be achieved. Thus, considerable data on FR α expression demonstrates that approximately 40% of all human tumors overexpress this isoform of FR (27). Considered with the fact that the same FITC-folate CAM should mediate eradication of FR β -expressing tumor-associated macrophages and myeloid-derived suppressor cells (both of which can facilitate tumor proliferation and survival; ref. 39), the impact of an FITC-folate CAM on tumor growth could extend beyond the 40% of cancers that express FR α . Similarly, although PSMA is only overexpressed on approximately 90% of prostate cancer cells, PSMA is also upregulated on the neovasculature of almost all solid

tumors (26), suggesting that a CAR T cell directed to PSMA could augment CAR T-cell potency in many solid tumors. Recent analyses of CA IX expression in a large number of human tissues have also revealed that this hypoxia-induced antigen is upregulated on almost all solid tumors (25), suggesting that a CA IX–targeted CAM might also belong in a pan-cancer cocktail of CAMs. And although *in vivo* documentation of the efficacy of CAMs containing still other tumor-targeting ligands may still be lacking, the growing availability of low-molecular-weight tumor-specific ligands suggests (40, 41) that the raw material for production of a library of tumor-specific CAMs may soon exist. Taken together, these data argue that if the CAM technology can prove effective in treating human cancers, the availability of CAMs to target CAR T cells to heterogeneous human tumors should not be limiting.

Although availability of a desired tumor-specific CAM should not be problematic, a concern should still exist that expression of the tumor-specific antigen on healthy tissues would cause unacceptable damage to healthy cells. For this reason, selection of any tumor-associated antigen for CAR T-cell therapies must involve a

thorough multimethodological examination of the proposed antigen's expression pattern followed by selection of only those tumor antigens whose expression on healthy cells is very limited. Importantly, this vetting process has already been performed on two of the three tumor-specific antigens examined above, and both were found to satisfy most of the expected criteria. Thus, mRNA and IHC analysis of folate receptor expression in both healthy and malignant tissues along with folate receptor–targeted imaging data on several hundred cancer patients has shown that folate receptors are predominantly expressed on malignant and inflamed cells (42), with little expression on normal cells. Moreover, a folate-targeted conjugate of the highly cytotoxic drug, tubulysin B, has been shown in clinical trials of lung and ovarian cancer patients to display little off-target toxicity (43), suggesting that any normal cell that expresses a folate receptor is either not readily accessed or not damaged by the folate-linked drug. Because a folate–NIR dye conjugate currently in late-stage clinical trials for fluorescence-guided surgery of lung and ovarian cancer has demonstrated 98% sensitivity and 96% positive-predictive value with little if any toxicity (44), the folate receptor would seem at this juncture to qualify for possible further evaluation as a tumor antigen for CAR T-cell therapies. Similarly, many mRNA and IHC expression studies (26) together with toxicity data on PSMA-targeted radiotherapeutic agents in clinical trials for metastatic castration-resistant prostate cancer (23) suggest that PSMA may also constitute an attractive candidate for CAR T-cell applications. Although a previous clinical trial in which a classical CAR T-cell therapy was directed against CA IX had to be terminated due to toxicity (45), it is possible that this toxicity might have been mitigated if a CAM had been used whose induction of CAR T-cell–cancer cell engagement could be controlled by adjusting the affinity, concentration, or dosing schedule of the CAM. Thus, with further vetting studies, it is not inconceivable that one or more of the tumor-associated receptors used in these studies could be used in clinical applications of a CAR T-cell therapy.

Finally, with recent advances in methods for deimmunogenizing a donor's T cells (46–48), the possibility of producing a single "off-the-shelf" CAR T-cell clone for use in the general population seems increasingly achievable. Although preparation of a library

of deimmunogenized CAR T-cell clones containing multiple scFv constructs that recognize most tumor antigens would indeed be possible, attainment of an "off-the-shelf" CAR T-cell goal might be more easily achieved if a single deimmunogenized CAR T-cell clone could be combined with a universal cocktail of low-molecular-weight tumor-specific CAMs. Such a combination might eventually allow treatment of additional devastating cancers with this powerful immunotherapy.

Disclosure of Potential Conflicts of Interest

Y.G. Lee has ownership interest (including stock, patents, etc.) in patents. H. Chu has ownership interest (including stock, patents, etc.) in Endocyte, Inc. P.S. Low is Chief Science Officer at, reports receiving commercial research grant from, and has ownership interest (including stock, patents, etc.) in Endocyte Inc. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.G. Lee, I. Marks, H. Chu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.G. Lee, I. Marks, H. Chu, P.S. Low
Writing, review, and/or revision of the manuscript: Y.G. Lee, P.S. Low
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.K. Kanduluru
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