Cancer Immunotherapy with T Cells Carrying Bispecific Receptors That Mimic Antibodies

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Abstract

Tumors are inherently heterogeneous in antigen expression, and escape from immune surveillance due to antigen loss remains one of the limitations of targeted immunotherapy. Despite the clinical use of adoptive therapy with chimeric antigen receptor (CAR)-redirected T cells in lymphoblastic leukemia, treatment failure due to epitope loss occurs. Targeting multiple tumor-associated antigens (TAAs) may thus improve the outcome of CAR-T cell therapies. CARs developed to simultaneously target multiple targets are limited by the large size of each single-chain variable fragment and compromised protein folding when several single chains are linearly assembled. Here, we describe single-domain antibody mimics that function within CAR parameters but form a very compact structure. We show that antibody mimics targeting EGFR and HER2 of the ErbB receptor tyrosine kinase family can be assembled into receptor molecules, which we call antibody mimic receptors (amR). These amR can redirect T cells to recognize two different epitopes of the same antigen or two different TAAs in vitro and in vivo.

Introduction

Chimeric antigen receptors (CARs) are synthetic receptors. Their specificity is determined by the single-chain variable fragment (scFv) obtained from a monoclonal antibody, with their activation controlled by the ζ-chain signaling domain from the T-cell receptor complex and costimulatory endodomains (1, 2). Gene transfer of CARs into T cells redirects T-cell antigen specificity through the scFv. T-cell activation and proliferation is amplified through costimulatory signals (3). The infusion of CAR-T cells in patients with lymphoid malignancies has led to durable complete remission in more than 40% of patients (4, 5). However, up to 20% of patients with acute lymphoblastic leukemia receiving CD19-specific CAR-T cells relapse due to the emergence of leukemic clones that have lost the targeted epitope (4, 6). Furthermore, the heterogeneity of tumor-associated antigen (TAA) expression in solid tumors leads to CAR-T treatment failure when a single TAA is targeted (7).

The generation of multiredirected CAR-T cells, namely, by recognition of two nonoverlapping epitopes of a TAA or two different TAAs, may be necessary to effectively eradicate tumor cells. Several approaches have been proposed to achieve this goal including pooling CAR-T products targeting different TAAs, generating vectors encoding two different CARs that can be expressed simultaneously by each T cell, and engineering cassettes in which two scFv are assembled into a single CAR moiety (2, 8, 9). Although these approaches are feasible, a challenge in using an scFv-based antigen receptor is the need for multiple VH and VL domains to appropriately pair and stabilize in complex structures. In addition to the complexity and costs of manufacturing multiple T-cell products, reduced expression of simultaneously expressed CARs, and compromised protein folding when several scFvs are linearly assembled in one single CAR, remain challenges to current methods of generating multiredirected CAR-T cells.

Achieving multiple tumor-targeting features using scFv-based CAR-T cells is difficult. We hypothesized that extracellular antigen receptors with simple structure, high stability, and small size could address the challenge. Various small protein domains that are not structurally equivalent to the immunoglobulin domains have been developed using various display technologies (10–14). Tumor-homing ligands based on a small protein domain possess advantages over those on a multidomain, complex structure in terms of engineering higher modularity. Ideally, the scaffold should be a monomeric small protein domain with high solubility and stability that is not inclined to aggregation, that tolerates sequence variations, and that is amenable to directed molecular evolution to create antigen-binding ligands with multiple functions (13). Among numerous scaffolds that have been examined, three single-domain antibody mimics are of interest for developing CARs with multiple tumor-targeting features: (i) monobody, based on the type III domain of fibronectin (FN3); (ii) affibody, based on a three-helix bundle Z domain; and (iii) DARPin, based on the designed ankyrin repeat protein (11, 12, 15, 16). These engineered proteins can have high specificity and affinity, despite their simple structures and relatively small size. The FN3 domain...
is a stable protein with a molecular weight (MW) around 10 kDa. Structurally, it has a β-sandwich scaffold similar to that of the immunoglobulin V_{H} domain, with putative ligand-binding sites composed of three solvent accessible surface loops that are structurally analogous to the CDR H1, H2, and H3 of the V_{H} domain [11]. The advantage of the FN3 domain is in its lack of disulfide bonds and posttranslational modifications for biological functions. Affibodies (AFF) are based on the three-helix bundle Z domain derived from Staphylococcal protein A (17, 18). As with the FN3 domain, AFF domains are resistant to proteolysis and heat-induced denaturation and lack disulfide bonds. Finally, DARPin-based targeting ligands that bind to various targets including CD4, EGFR, and HER2 have been generated [19].

Taking into consideration the simplicity, stability, and smaller size of these targeting ligands, as well as their current applications in therapeutics and diagnostics [20], we explored the use of these molecules in generating antigen-specific receptors for T cells. In particular, we investigated if a combination of these single-domain antibody mimics allows the generation of a T-cell-surface antigen receptor that recognizes two different epitopes of the same tumor antigen or two different antigens, aiming to develop T cells with bispecific redirection targeting two epitopes of the same antigen or two different antigens. As proof of principle, we have adapted high-affinity antibody mimics specific for ErbB1 (EGFR) and ErbB2 (HER2), to generate receptor molecules called antibody mimic receptors (amR).

Materials and Methods

Construction of bispecific CAR vectors

To construct bispecific CAR vectors, the codon-optimized (for expression in human cells) coding regions for a monomeric or heterodimeric EGFR- or/and HER2-binding ligand were fused through an optimized flexible linker. The final coding region was cloned into the SFG vector, resulting in a fusion protein that is composed of the signaling peptide from human IgG heavy-chain, EGFR- or HER2-binding domain(s), a FLAG tag, a 45-residue hinge region from the human CD8ε extracellular domain, the transmembrane domain of human CD8ε, the CD28-costimulatory endodomain, and the ζ chain of the TCR/CD3 complex [21]. The CD8ε hinge and transmembrane domains contain native cysteine residues. Single-domain antibody mimics (AFF, DARPin, and FN3) were PCR amplified and cloned into the SFG vector. The scFv derived from the cetuximab mAb was PCR amplified and cloned into the SFG vector. EGFR WT (Addgene plasmid #110110) and pBABE-puro-ErbB2 (Addgene plasmid #40978) were gifts from Matthew Meyerson Dana-Farber Cancer Institute. Full-length EGFR and HER2 were amplified by PCR and cloned into the SFG retroviral vector. A truncated form of HER2 lacking an intracellular domain was amplified by PCR and also cloned into the SFG retroviral vector. All retroviral supernatants were prepared as previously described [22].

Expression and purification of recombinant EGFR and HER2 binding protein domains

Coding sequences codon-optimized for expression in E. coli with a C-terminal His tag were cloned into the pET28b vector. To express the ligands, vectors were transformed into E. coli BL21 (DE3) Rosetta cells, and positive clones were selected on lysogeny broth (LB) plates containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. Single colonies were picked and grown overnight at 37°C. Overnight cell cultures were added to 1 L of LB media and grown at 37°C. When the OD 600 was between 0.6 and 0.8, 1 mmol/L IPTG was added to induce expression for 4 hours at 37°C. To purify the binding ligands, the cell pellet was resuspended in buffer A (25 mmol/L HEPES pH 7.4 and 300 mmol/L NaCl) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and sonicated on ice for 10 minutes on a Sonifier 450 sonicator (Branson). After cell lysis, the soluble fraction was recovered by centrifugation at 4°C. The resulting soluble fraction was loaded onto an IMAC Ni-charged affinity column (Bio-Rad) preequilibrated with buffer A. The column was washed with buffer A containing 20 mmol/L imidazole (buffer B) and then 50 mmol/L imidazole (buffer C) and the proteins were eluted with buffer D (buffer A and 200 mmol/L imidazole). Following dialysis against 1 × PBS, the quality of the purified proteins was verified by SDS-PAGE.

Characterization of target-binding features

Bio-layer interferometry (BLI) analyses of the monomeric and heterodimeric EGFR and HER2-binding domains were performed on a Octet QK system (FortBio LLC.) at 30°C. The ErbB binding ligands and corresponding receptors diluted to required concentrations with an assay buffer (1 × PBS, 1% BSA, 0.05% Tween 20, pH 7.4) as well as the buffer are assigned to black 96 well plates (Greiner Bio-One). Streptavidin (SA) biosensors (Fort_eBio) were used to immobilize biotinylated ErbB binding ligands and ErbB with Fc fusions [AcroBiosystems] at three different concentrations against antigen-binding SA sensors. Assays run in triplicate were acquired and analyzed on the ForteBio Data Acquisition 6.4 software. Savitzky–Golay filtering was applied to the averaged reference biosensors and then globally fitted at a 1:1 model.

Cell lines

Tumor cell lines Panc-1, BxPC-3, HPAP-II, and AsPC-1 (pancreatic cancer), MCF-7 (breast cancer), and BV173 (B cell lymphoma) were purchased from ATCC. BxPC-3 and BV173 were cultured in RPMI-1640 (Gibco). AsPC-1 was cultured in RPMI-1640 supplemented with 1 mmol/L sodium pyruvate (Gibco). HPAP-II and MCF-7 were cultured in MEM (Gibco). Panc-1 was cultured in DMEM (Gibco). All media were supplemented with 10% FBS (Sigma), 2 mmol/L Glutamax (Gibco) and penicillin (100 units/mL) and streptomycin (100 mg/mL, Gibco). All cells were maintained at 37°C with 5% CO2. All cell lines are regularly tested for Mycoplasma, and the identity of the cell lines was validated by flow cytometry for relevant cell-surface markers and were also monitored for morphological drift in culture. Cell lines were maintained in culture no longer than 30 days and then replaced with cells from stored vials. The number of previous passages of these cell lines was unknown. Panc-1 cells were transduced with a retroviral vector encoding the eGFP-Flirey-Luciferase (eGFP-FFluc) gene [21]. BV173 cells were transduced with a retroviral construct encoding full-length human EGFR to generate BV173-EGFR cells or a truncated form of HER2 to generate BV173-HER2 cells. Panc-1 cells were transduced with a retroviral vector encoding HER2 to make Panc-1-HER2.

Generation of redirected T cells

T cells expressing CAR and amRs were generated in accordance with standard operating procedures currently used
Flow cytometry

We used mAbs specific for human CD3 (APC-H7; SK7; 560176), CD45 (BV510; H130; 563204), CD4 (BV711; SK3; 563028), CD8 (APC; SK1; 340584), CD19 (FITC; SJ25C1; 340409), CD45RA (PE; H1100; 555489), CD45RO (BV786; UCHL1; 564290), CD69 (FITC; L78; 347823), and HER2 (PE; Neu24.7; 340879) from BD Biosciences, CCR7 (FITC; 150503; FAB197F-100) from R&D Systems, and EGFGR (PE; AY13; 352904) from BioLegend. We detected the expression of the EGFRCAR or anti-mRs using anti-FLAG mAb (APC; L5; 637308). An anti-idiotypic mAb was used to detect the expression of the CD19 CAR as previously described (21). All samples were acquired on a BD LSRFortessa, and a minimum of 10,000 events were analyzed on FlowJo 9 (FlowJo LLC).

Western blot analysis

T-cell lysates were resuspended in 2× Laemelli buffer (Bio-Rad) in reducing or nonreducing conditions. To assess signaling through the CAR or anti-mR, T cells on ice were incubated with 1 µg of anti-FLAG Ab (clone M2) for 15 minutes and then 1 µg of goat anti-mouse secondary Ab (BD Biosciences) for an additional 15 minutes. Cells were then transferred to a 37°C water bath for the indicated time points and lysed with 4× Laemelli buffer. All lysates were separated in 4% to 15% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (all Bio-Rad). Blots were probed for human CD3ζ (Santa Cruz Bio-technology), p-Y142 CD3ζ (Abcam), pan-ERK (BD Biosciences), and pan-Akt, p-S473 Akt, and p-T202/Y204 MAPK (all Cell Signaling Technology) diluted 1:1,000 in TBS-Tween/5% skim milk. Membranes were then incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (both Santa Cruz) at a dilution of 1:3,000 and imaged using the ECL Substrate Kit on a ChemiDoc MP System (both Bio-Rad) according to the manufacturer’s instructions.

In vitro activation

Biostinylated recombinant EGFR and EGFRVIII protein (Acro-Biosystems) were added to 96-well plates coated with 1 µg of avidin (Thermo Fisher Scientific) at a 3:1 ratio. Recombinant EGFR-Fc and HER2-Fc (R&D Systems) were coated on 96-well plates overnight at a concentration of 1 µg/well. T cells were seeded in duplicate or triplicate for 6 hours, and supernatant was collected for IFNγ and T cells were assessed for CD69 by flow cytometry.

Proliferation assay

T cells were labeled with 1.5 mmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) and plated with irradiated AsPC-1 at a 4:1 effector-to-target (E:T) ratio in the absence of exogenous cytokines. CFSE dilution of CAR-T cells or amR-T cells was analyzed on day 5 using flow cytometry (21). The proliferation index was quantified using FlowJo 9.

Long-term in vitro cytotoxicity

Tumor cells were seeded at 2.5–5 × 10^5 per well in 24-well plates. Donor-matched T cells normalized for transduction efficiency were added at 1:5 E:T ratio. On day 3–5 of coculture, cells were collected, and the frequency of T cells and residual tumors cells was measured by flow cytometry. Tumor cells were identified as CD19^+ for BV173 tumor cells or CD4^+ CD8^+ in the case of all adherent tumor cell lines (27).

Repetitive coculture assay

For multiple rounds of coculture, tumor cells were seeded at 5 × 10^5 per well in 24-well plates. Donor-matched T cells normalized for transduction efficiency were added at 1:2 E:T ratio. On day 3 of coculture, a fraction of the cells was collected, and the frequency of T cells and residual tumors cells was measured by flow cytometry. Between cocultures, T cells were washed and resuspended in fresh medium, without the addition of exogenous cytokines, and left to rest for 3 days (27).

Cytokine analysis

Supernatant was collected from 0.5–1 × 10^5 CAR-T cells or amR-T cells plated in in vitro cytotoxicity assays at 1:5 E:T ratio after 24 hours or from 1.25 × 10^5 CAR-T cells or amR-T cells plated for 6 hours. IFNγ and IL2 were measured by ELISA per the manufacturer’s instructions (R&D Systems) in duplicate.

Xenograft murine models

Six- to 8-week-old male or female nonobese diabetic severe combined immunodeficiency/γ,−/− (NSG) mice were injected intravenously (i.v.) with tail vein with the Panc-1 tumor cell line (1 × 10^6 cells/mouse) transduced with the GFP-FFLuc reporter i.v. by tail vein injection. In other experiments, HPAF-II GFP-FFLuc tagged cells were suspended in Matrigel and inoculated intraperitoneally (i.p.): 1 × 10^6 cells/mouse. In rechallenge experiments, Panc-1 GFP-FFLuc cells (1 × 10^6 cells/mouse) were injected 10 days prior to T-cell injection. Upon clearance of the Panc-1 tumor cells, mice were then infused with BV173-HER2 cells (2 × 10^6 cells/mouse). Mice were matched based on the bioluminescence intensity and injected with 5 × 10^5 or 1 × 10^5 cells i.v. 12 to 14 days after tumor cell engraftment. The IVIS-Kinetic Optical System (PerkinElmer) was used to monitor tumor burden. Mice were monitored and euthanized according to UNC-IACUC Standards.

Statistical analysis

Data are reported as the mean and standard deviation, unless otherwise reported. To compare significant differences between two samples, a two-tailed Student t test was applied. An ANOVA with Tukey post hoc analysis was applied when a comparison between multiple groups was required. A P value of less than 0.05 was considered statistically significant. All figures were generated using GraphPad Prism (GraphPad Software).
A soluble single-domain antibody targets EGFR in a bispecific manner

To develop a single-domain–based tumor antigen–binding moiety that targets two different epitopes of EGFR, we assembled the previously identified Z domain–based EGFR-binding affibody ZEGRF:1907 and an FN3-based EGFR-binding monobody (17). The length and flexibility of the linker between the two EGFR-binding domains were optimized to retain the target-binding affinity, specificity, and independent folding of each EGFR-binding domain. We observed that when the linker is too short, both domains failed to bind targets. An overly long linker resulted in the loss of the bivalent effect as well as instability of the ligands. Because the linker length appeared to be antigen and binder dependent, we only used the linker length that worked well in this proof-of-concept work. In this study, the two antigen-binding moieties were separated by a 25-residue flexible linker. The resulting bispecific affinity molecule, from now on referred to as Bi-EGFR, binds to the extracellular domain of human EGFR at two nonoverlapping epitopes with an affinity (Kd) around 0.38 ± 0.07 nmol/L (Fig. 1A), which is approximately 10 times higher than that of the monomeric EGFR-binding FN3 domain (FN3.EGFR: 3.1 ± 0.9 nmol/L; Fig. 1B) or Z domain (AFF.EGFR: 3.2 ± 0.3 nmol/L; Fig. 1C).

Bi-EGFR.amR T cells resemble those expressing conventional EGFR-specific CAR

To construct the CAR vector, the sequence of the biochemically optimized Bi-EGFR was fused to the CD28 and CD3ζ-chain signaling domains via the CD8ζ hinge and transmembrane domains (21). To detect the expression of the Bi-EGFR.amR in T cells (Bi-EGFR.amR-T cells) following transduction, we included a FLAG tag into the Bi-EGFR.amR cassette (Supplementary Fig. S1). A conventional EGFR-specific CAR (EGFR.CAR) generated using the scFv derived from the monoclonal antibody cetuximab (Supplementary Fig. S1), and the CD3ζ-specific CAR (CD19.CAR: ref. 21) were used as controls. AmRs composed of either the EGFR-A binding moiety (FN3.EGFR.amR) or the EGFR-B binding moiety (AFF.EGFR.amR) alone were also constructed (Supplementary Fig. S3A). Upon retroviral gene transfer, T cells stably expressed the Bi-EGFR.amR (Fig. 1D and E), expanded in vitro in response to exogenous cytokines (Fig. 1F), and maintained T-cell composition comparable with EGFR.CAR-T cells and CD19.CAR-T cells (Fig. 1G). Western blot analysis of lysates from Bi-EGFR.amR-T cells detecting the CD3ζ chain under reducing conditions showed the native ζ-chain (17 kDa) and a band at the expected size of 55 kDa, indicating the integrity of the assembled Bi-EGFR.amR (Fig. 1H). We then analyzed proximal and distal signaling in EGFR.CAR-T cells and Bi-EGFR.amR-T cells upon receptor cross-linking. As shown in Fig. 1I, receptor cross-linking in both Bi-EGFR.amR-T cells and EGFR.CAR-T cells triggered similar phosphorylation of proximal (CD3ζ) and distal (Akt and ERK) signaling molecules.

Bi-EGFR.amR-T cells demonstrate activity against tumor cells expressing EGFR

To demonstrate that Bi-EGFR.amR-T cells specifically target EGFR, we used the EGFR+ tumor cell line BV173 (BV173-WT) and transduced it to express EGFR (BV173-EGFR) with a retroviral vector encoding the full-length human EGFR (Supplementary Fig. S2A). We then cocultured BV173-WT or BV173-EGFR cells with control nontransduced T cells (NTs), CD19.CAR-T cells, EGFR.CAR-T cells, and Bi-EGFR.amR-T cells. NTs did not eliminate either BV173-WT or BV173-EGFR cells, CD19.CAR-T cells eliminated both cell types (Supplementary Fig. S2B and S2C), and Bi-EGFR.amR-T cells and EGFR.CAR-T cells only eliminated BV173-EGFR cells (Supplementary Fig. S2B and S2C), indicating the antigen specificity of the redirected T cells. Antitumor activity was then tested against tumor cell lines that physiologically express EGFR (Supplementary Fig. S2D). In coculture experiments, Bi-EGFR.amR-T cells and EGFR.CAR-T cells demonstrated similar antitumor activity in vitro (Fig. 2A) and released comparable amounts of IFNγ (Fig. 2B) and IL2 (Fig. 2C). Using a CFSE dilution assay, we demonstrated the proliferation of Bi-EGFR.amR-T cells in response to EGFR-expressing targets (Fig. 2D and E). Finally, we evaluated the antitumor activity of Bi-EGFR.amR-T cells in a metastatic model of EGFR-expressing pancreatic cancer in NGS mice (Fig. 2F). Bi-EGFR.amR-T cells and EGFR.CAR-T cells equally controlled human Panc-1 tumor cell growth as assessed by measurement of tumor bioluminescence intensity (Fig. 2G and H).

Bi-EGFR.amR-T cells recognize two nonoverlapping epitopes of EGFR

AmRs composed of either the FN3.EGFR.amR or the AFF.EGFR.amR alone were transduced in T cells (Supplementary Fig. S3A). Both AFF.EGFR.amR-T cells and FN3.EGFR.amR-T cells showed comparable activity in vitro against tumor cells expressing the full-length EGFR (Supplementary Fig. S3B–S3F) and proliferated in response to EGFR-expressing CAR vectors (Supplementary Figs. S3G and S3H). To demonstrate the bispecific feature of the Bi-EGFR, we analyzed the binding of Bi-EGFR and each of its monomeric domains using the recombinant extracellular domain of the wild-type EGFR (wtEGFR) and the EGFRvIII (EGFRvIIIInv) mutant, a constitutively active and ligand-independent variant of EGFR with deletions in exons 2 to 7. We found that Bi-EGFR and Z domain–based AFF.EGFR bound both wtEGFR and EGFRvIII. However, FN3 domain–based FN3.EGFR bound to wtEGFR but not EGFRvIII (Supplementary Fig. S4A), suggesting that it recognizes an antigen encoded in the 267 AA that are absent in EGFRvIII. To assess recognition of the two different EGFR epitopes, we used the extracellular domain of wtEGFR and EGFRvIII mutant recombinant proteins. Control, AFF.EGFR.amR-T cells, FN3.EGFR.amR-T cells, and Bi-EGFR.amR-T cells were seeded in tissue culture plates coated with either wtEGFR or EGFRvIII, and the CD69 expression and IFNγ release by T cells were measured. Both wtEGFR and EGFRvIII recombinant proteins activated AFF. EGFR.amR-T cells and BiEGFR.amR-T cells, whereas only the wtEGFR protein activated FN3.EGFR.amR-T cells (Fig. 3A–C), indicating that the epitope recognized by the FN3.EGFR binding moiety is either located in the 267 AA region deleted in EGFRvIII, or the mutation has altered the epitope accessibility due to the deletion-induced conformational changes. In contrast, the AFF. EGFR binding moiety recognizes an epitope that is conserved between EGFR and EGFRvIII (Fig. 3A–C). To ensure that both AFF. EGFR and FN3.EGFR binding moieties can induce the activation of T cells when assembled into the Bi-EGFR.amR, we generated an AFF. EGFR.amR mutant–binding moiety (mAFF. EGFR.amR) in which critical residues at the EGFR-binding alpha helices were mutated to alanine to reduce binding to EGFR. As shown in Supplementary Fig. S4B, mAFF. EGFR.amR was expressed in T cells, but mAFF. EGFR.amR-T cells did not eliminate
Figure 1.
Bi-EGFR.amR-T cells and conventional EGFR.CAR-T cells show comparable activity. A–C, Biolayer interferometry (BLI) was used to measure the affinity of the monomeric or heterodimeric single-domain antibody mimics binding to recombinant Fc-EGFR on the ForteBio Octet system. Biotinylated antibody mimics (100 nmol/L) were immobilized onto a streptavidin biosensor. Binding kinetics were measured against various concentrations of EGFR (0, 25, 50, and 100 nmol/L). The $K_d$ was calculated based on kinetic fitting. D, Representative expression of the Bi-EGFR.amR, EGFR.CAR, or CD19.CAR in T cells as assessed by flow cytometry. Activated T cells were transduced with retroviral vectors encoding the Bi-EGFR.amR, EGFR.CAR, or CD19.CAR. With the exception of the CD19.CAR, all constructs were detected using an anti-FLAG Ab. The CD19.CAR was detected using an anti-idiotype Ab. Shaded and unshaded histograms indicate nontransduced and specific mAb, respectively. E, Summary of amR or CAR expression ($n = 4$). F, Expansion kinetics of Bi-EGFR.amR-T cells, EGFR.CAR-T cells, or CD19.CAR-T cells NT ($n = 4$); error bars denote SD. G, Phenotypic composition of Bi-EGFR.amR-T cells, EGFR.CAR-T cells or CD19.CAR-T cells 10 days after transduction ($n = 4$); error bars denote SD. H, Reducing immunoblots of Bi-EGFR.amR-T cell and EGFR.CAR-T cell lysates. Immunoblots were probed with anti-CD3ζ. Top and bottom represent detection of the receptors and endogenous ζ-chain, respectively. I, EGFR.CAR-T cells and Bi-EGFR.amR-T cells were incubated at the indicated time points with the anti-FLAG Ab and cross-linked with a secondary Ab to induce the aggregation of the receptors. Cell lysates were immunoblotted to detect proximal (CD3ζ p-Y142) and distal (Akt p-S473 and ERK p-T202/204) phosphorylation events following receptor cross-linking. Total CAR.CD3ζ or amR.CD3ζ and endogenous CD3ζ were used as loading controls. Data are representative of 4 experiments.
BV173-EGFR cells (Supplementary Fig. S4C and S4D), indicating that the mutations had abrogated binding to EGFR. However, T cells expressing a Bi-EGFR.amR constructed with the FN3.EGFR and mAFF.EGFR binding moieties (mBi-EGFR.amR) upregulated CD69 and released IFN-γ when seeded in wells coated with wtEGFR recombinant protein, but not in response to EGFRvIII protein, indicating that the FN3.EGFR binding moiety alone can induce T-cell activation when the function of the other EGFR-binding moiety (AFF.EGFR) is abolished in the mBi-EGFR.amR (Fig. 3D–F).

Bispecific amR against HER2 demonstrates antitumor activity

We also constructed an amR receptor against another member of the ErbB family of receptor tyrosine kinase, HER2, to demonstrate the applicability of this technology to other TAAs. Using a similar approach to the construction of Bi-EGFR, a tumor antigen–binding moiety that targets two different epitopes of HER2 (Bi-HER2) was generated, which showed a HER2-binding affinity ($K_d$) around 0.25 ± 0.04 nmol/L (Fig. 4A), compared with 1.8 ± 0.6 nmol/L (Fig. 4B) of a HER2-binding DARPin (15) and 1.3 ± 0.4 nmol/L of a HER2-binding Z domain.
Based on this HER2-binding ligand, a bispecific Bi-HER2.amR was constructed (Supplementary Fig. S5A). T cells stably expressed the Bi-HER2.amR (Bi-HER2.amR-T cells) upon retroviral transfer, expanded in vitro in response to exogenous cytokines (Supplementary Fig. S5B) and maintained T-cell composition comparable to control T cells (Supplementary Fig. S5C). Bi-HER2.amR-T cells demonstrated targeting of HER2-expressing cells (Supplementary Fig. S5D–S5F). Furthermore, Bi-HER2.amR-T cells exhibited antitumor activity (Fig. 4D) and released IFNγ (Fig. 4E) and IL2 (Fig. 4F) in a HER2-dependent manner when cocultured with tumor cell lines expressing HER2, but not with HER2-negative cell lines (Supplementary Fig. S5G). We also evaluated the efficacy of Bi-HER2.amR-T cells in a metastatic tumor model of HER2-expressing human HPAF-II pancreatic cancer in NSG mice (Fig. 4G). Bi-HER2.amR-T cells and EGFR.CAR-T cells equally controlled tumor cell growth as assessed by measurement of tumor bioluminescence intensity (Fig. 4H and I).

Bispecific EGFR-HER2.amR-T cells target tumor cells expressing EGFR and HER2

To generate amRs targeting two different TAAs, we integrated the EGFR-specific FN3 binding moiety and the HER2-specific DARPin binding moiety and created the EGFR-HER2.amR with a 27-residue flexible linker between the two antigen-binding moieties (Supplementary Fig. S6A). T cells stably expressed the

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**Figure 3.** Bi-EGFR.amR-T cells are activated by recognition of two nonoverlapping epitopes on EGFR. Control (NTs), AFF.EGFR.amR-T cells, FN3.EGFR.amR-T cells, and Bi-EGFR.amR-T cells were seeded in tissue culture plates coated with either recombinant human EGFR WT protein (EGFR) or the truncated mutant EGFRvIII recombinant protein (EGFRvIII). A, Representative expression of CD69 on NTs, AFF.EGFR.amR-T cells, FN3.EGFR.amR-T cells, and Bi-EGFR.amR-T cells 6 hours after stimulation with plate-bound EGFR or EGFRvIII protein as assessed by flow cytometry. Shaded and dashed histograms indicate media and PMA/Iono controls, respectively. Solid and dashed lines indicate EGFR and EGFRvIII protein stimulation, respectively. B, Summary of CD69 expression on total live T cells (n = 4), P < 0.01 when comparing FN3.EGFR.amR-T cells seeded in EGFR and EGFRvIII-coated wells; two-way ANOVA with Tukey correction. C, IFNγ released in the supernatant by AFF.EGFR.amR-T cells, FN3.EGFR.amR-T cells, and Bi-EGFR.amR-T cells as assessed by ELISA (n = 3), P < 0.01 when comparing FN3.EGFR.amR-T cells seeded in EGFR and EGFRvIII-coated wells; two-way ANOVA with Tukey correction. D, mAFF.EGFR.amR-T cells or mBi-EGFR.amR-T cells were cocultured with BV173-WT or BV173-EGFR cells at 1:5 E:T for 3 days. CD19.CAR-T cells were used as a positive control. Cells were collected, and T cells (CD3+) and tumor cells (CD19+) were quantified by flow cytometry. Representative flow plots are illustrated. E, Quantification of BV173-WT or BV173-EGFR cells remaining after 3 days of coculture. F, IFNγ released in the supernatant by NT, CD19.CAR-T, mAFF.EGFR.amR-T, and mBi-EGFR.amR-T cells as assessed by ELISA (n = 2–4), P < 0.01 when comparing the percentage of residual BV173-WT and BV173-EGFR cells remaining in the mBi-EGFR.amR-T wells; two-way ANOVA with Tukey correction.
EGFR-HER2.amR (Supplementary Fig. S6B and S6C), expanded in vitro in response to exogenous cytokines (Supplementary Fig. S6D) and maintained T-cell composition comparable to monospecific FN3.EGFR.amR-T cells and DARPin.HER2.amR-T cells (Supplementary Fig. S6E). To establish the bispecificity of EGFR-HER2.amR-T cells, we seeded control, FN3.EGFR.amR-T cells, DARPin.HER2.amR-T cells, and EGFR-HER2.amR-T cells in tissue cultures plates coated with either the extracellular domain of rhEGFR or rhHER2 recombinant proteins and assessed CD69 expression and IFNγ release by T cells. EGFR-HER2.amR-T cells expressed CD69 (Supplementary Fig. S6F) and secreted IFNγ (Supplementary Fig. S6G) when seeded in wells coated with either rhEGFR or rhHER2 proteins, whereas FN3.EGFR.amR-T cells and DARPin.HER2.amR-T cells were only activated by

Figure 4.

Bi-HER2.amR-T cells have antitumor activity against HER2-expressing primary cells in vitro and in vivo. A–C, Biolayer interferometry (BLI) was used to measure the affinity of the monomeric or heterodimeric single-domain antibody mimics binding to recombinant Fc-HER2 using the FortéBio Octet system. Biotinylated antibody mimics (100 nmol/L) were immobilized onto a streptavidin biosensor. Binding kinetics was measured against various concentrations of HER2 (0, 25, 50, 100 nmol/L). The Kd was calculated based on kinetic fitting. D, Control T (NT), Bi-HER2.amR-T cells, and EGFR.CAR-T cells were cocultured with Panc-1 cells (HER negative) or HER2-expressing pancreatic adenocarcinoma cell lines (AsPC-1 and HPAF-II) at 1:5 E:T. Cells were collected and quantified by flow cytometry on day 5. The frequency of residual tumor cells was identified as CD4+ CD8+ live cells (n = 3–6). P < 0.01 when comparing Bi-HER2.amR-T cells or EGFR.CAR-T cells with NTs; two-way ANOVA with Tukey correction. E, IFNy and (F) IL2 released in the coculture supernatant by NTs, Bi-HER2.amR-T cells or EGFR.CAR-T cells after 24 hours of coculture with tumor cells as assessed by ELISA (n = 3–6). P < 0.01 when comparing Bi-HER2.amR-T cells or EGFR.CAR-T cells with NTs; two-way ANOVA with Tukey correction. G, Schematic representation of a metastatic pancreatic cancer model in NSG mice using the FFLuc-labeled human HPAF-II cell line. Representative images of tumor bioluminescence (BLI) (H) and kinetics (I) of tumor growth as assessed by BLI measurements. Data are representative of two independent experiments with 5 mice per group; P < 0.01 when Bi-HER2.amR-T cells or EGFR.CAR-T cells are compared with NTs; two-way ANOVA with Tukey correction.
Figure 5.

EGFR-HER2.amR-T cells show dual specificity. Coculture experiment in which T cells were plated with its respective BV173 target cells, and 3 days later T cells were collected and replated with the same BV173 tumor cell line or BV173 cells expressing the nonspecific target. A, Schema of the repetitive coculture experiments with control (CD19.CAR-T cells), monospecific FN3.EGFR.amR-T cells, DARPin.HER2.amR-T cells, and bispecific EGFR-HER2.amR-T cells all plated at 1:2 E:T ratio. B, Representative flow plots of the repetitive coculture experiments on the second round. Cells were collected and quantified by flow cytometry on day 3. The frequency of residual tumor cells was identified as CD3−CD19+ live cells. C, Quantification of tumor cells remaining after the second coculture (n = 2–3), *P < 0.01 when comparing FN3.EGFR.amR-T cells and DARPin.HER2.amR-T cells and their respective targets against WT tumor cells; two-way ANOVA with Tukey correction. D, IFNγ released in the coculture supernatant by CD19.CAR-T cells, FN3.EGFR.amR-T cells, DARPin.HER2.amR-T cells, or EGFR-HER2.CAR-T cells after 24 hours of coculture with tumor cells as assessed by ELISA (n = 2–3), *P < 0.01 when comparing FN3.EGFR.amR-T cells and DARPin.HER2.amR-T cells and their respective targets against WT tumor cells; two-way ANOVA with Tukey correction. E, Schematic representation of the rechallenge tumor model in NSG mice using the EGFR+/HER2+/human Panc-1 cell line labeled with the FF-Luc and the EGFR+/HER2 human BV173-HER2 cell line. Representative images of the Pan-1 tumor BLI (F) and BLI kinetics (G) of tumor growth of one experiment 3–7 mice per group. H, Kaplan-Meier survival curve of NSG mice after rechallenge with the BV173-HER2 cell line; *P < 0.01 when mice treated with EGFR.amR-T cells are compared with mice treated with EGFR-HER2.amR-T cells.
rhEGFR and rhHER2, respectively. Next, we evaluated the functionality of EGFR-HER2.amR-T cells against BV173-WT, BV173-EGFR, and BV173-HER2 cells (Supplementary Fig. S6H). We performed a series of cocultures in which T cells were plated with respective BV173 target cells, and 3 days later, T cells were collected and replated with the same BV173 tumor cell line or BV173 cells expressing a nonspecific target (Fig. 5A). As shown in Fig. 5B–D, FN3.EGFR.amR-T cells and DARPin.HER2.amR continued to eliminate only BV173-EGFR and BV173-HER2, respectively, whereas EGFR-HER2.amR-T cells eliminated both BV173-EGFR and BV173-HER2 targets in both the primary and secondary cocultures. In order to demonstrate the bispecificity of the EGFR-HER2.amR-T cells in vivo, mice were engrafted with the EGFR+HER2+ human Panc-1 cell line labeled with FF-Luc, and infused with either EGFR.amR-T cells or EGFR-HER2.amR-T cells. Tumor growth was equally controlled by both EGFR.amR-T cells and EGFR-HER2.amR-T cells (Fig. 5E–G). However, when these mice were rechallenged with the EGFR+HER2+ BV173 tumor cell line, EGFR.amR-T-treated mice developed limb paresis due to growth of the BV173 tumor within the spinal cord, whereas EGFR-HER2.amR-T cell–treated mice remained healthy (Fig. 5H).

Discussion

The generation of multiredirected CAR-T cells may be necessary to effectively eradicate tumors in which TAAs of interest can be lost or are heterogeneously expressed in tumor cells. Here, we demonstrated that modular single-domain antibody mimics are a practical alternative to the conventional scFvs to generate T cells with engineered multiple antigen-targeting features. We constructed antibody mimics that can be assembled with signaling molecules of the T-cell receptor and costimulatory endodomains. We further demonstrated the functionality in vitro and in vivo of T cells expressing antibody mimics showing their ability to recognize simultaneously two epitopes of the same antigens or two distinct antigens.

In this proof-of-principle study, we have assessed whether single-domain antibody mimics can be used to redirect the specificity of human T lymphocytes (30, 31). We integrated single-domain antibody mimics binding to the nonoverlapping regions of EGFR to create an EGFR-binding ligand with bispecificity. We showed that T cells expressing the single-domain AFF.EGFR.amR or FN3.EGFR.amR have comparable activity in vitro and in vivo to the conventional scFv-based cetuximab EGFR CAR. To further demonstrate the applicability of redirecting T-cell specificity by using antibody mimics, we also validated the approach by targeting HER2 expressing tumors using a Bi-HER2 targeting ligand.

Targeting two epitopes of the same molecule may help prevent tumor escape when the targeted antigen can be expressed by alternative mRNA splicing, which may cause loss of a targeted epitope (6). When the AFF-EGFR and FN3.EGFR were combined in one single amR cassette, we demonstrated that two epitopes of EGFR can be targeted without causing detrimental effects in T cells. Targeting two distinct antigens expressed by tumor cells remains challenging. We demonstrated that single-domain antibody mimics can be assembled in one single cassette to efficiently target two different antigens. As a proof of principle, we showed that both EGFR and HER2 can be effectively targeted by dual-specific amRs neither with impairing targeting of each single antigen nor with detrimental effects of engineered T cells.

In summary, we provided proof of concept that antibody mimics can be used to generate combinatorial targeting of engineered T cells. Taking in consideration that antibody mimics are generated synthetically, our proposed approach can be adapted to a systematic screening of combinatorial antigens to be tested in human malignancies.

Disclosure of Potential Conflicts of Interest

R. Liu is the founder of, has ownership interest in, and is a consultant/advisory board for Panacase Bio Inc. No potential conflicts of interest were disclosed by the other authors.

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