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# Multispecific targeting with synthetic ankyrin repeat motif chimeric antigen receptors

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## Structured Abstract

**Purpose:** The outgrowth of antigen negative variants is a significant challenge for adoptive therapy with T cells that target a single specificity. Chimeric antigen receptors (CARs) are typically designed with one or two scFvs that impart antigen specificity fused to activation and costimulation domains of T cell signaling molecules. We designed and evaluated the function of CARs with up to three specificities for overcoming tumor escape using Designed Ankyrin Repeat Proteins (DARPins) rather than scFvs for tumor recognition.

**Experimental Design:** A monospecific CAR was designed with a DARPin binder (E01) specific for EGFR and compared to a CAR designed using an anti-EGFR scFv. CAR constructs in which DARPins specific for EGFR, EpCAM and HER2 were linked together in a single CAR were then designed and optimized to achieve multispecific tumor recognition. The efficacy of CAR-T cells bearing a multispecific DARPin CAR for treating tumors with heterogeneous antigen expression was evaluated in vivo.

**Results:** The monospecific anti-EGFR E01 DARPin conferred potent tumor regression against EGFR$^+$ targets that was comparable to an anti-EGFR scFv CAR. Linking three separate DARPins in tandem was feasible and in an optimized format generated a single tumor recognition domain

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S.R.R. is a founder of Juno Therapeutics, a Celgene company and has served as a scientific advisor to Juno Therapeutics, Adaptive Biotechnologies, Nohla and Cell Medica. AP is a cofounder and shareholder of Molecular Partners AG, which commercializes the DARPin technology. The other authors declare that they have no competing interests.

that targeted a mixture of heterogeneous tumor cells, each expressing a single antigen, and displayed synergistic activity when tumor cells expressed more than one target antigen.

**Conclusions:** DARPins can serve as high-affinity recognition motifs for CAR design, and their robust architecture enable linking of multiple binders against different antigens to achieve functional synergy and reduce antigen escape.

**Keywords**

chimeric-antigen-receptor; adoptive T cell therapy; cancer immunotherapy

## Introduction

The adoptive transfer of chimeric antigen receptor modified T cells (CAR-T) targeting single B cell lineage markers CD19 and CD22 has induced durable remissions in some patients with B-cell derived malignancies that have failed all conventional therapies (1-4). However, treatment failure occurs in some patients because of the outgrowth of tumor cells that have lost or express low levels of the target antigen; or, in the case of acute leukemia, that have undergone a lineage switch to a myeloid phenotype (1, 5-8). Solid tumors present an even greater therapeutic challenge because few surface molecules that could serve as CAR targets are solely expressed on tumors and not on normal tissues and because antigen expression is often more heterogeneous on solid tumors than on B cell malignancies (9, 10). Indeed, clinical trials in which CAR-T cells targeting EGFRvIII were administered to treat glioblastoma or targeting c-MET in triple negative breast cancer have resulted in the outgrowth of antigen negative variants (11, 12).

In an attempt to reduce antigen escape, bispecific CAR-T cells with 2 scFv moieties separated by $G_4S$ linkers have been designed to target two antigens simultaneously. Bispecific constructs have been described that target CD19/CD123, CD19/CD22 or CD19/CD20 in B-cell malignancies, or HER2/IL13Rα2 in glioblastoma (1, 13-17). Linking more than 2 CAR encoding transgenes in tandem can also be achieved (18), although vector size becomes a barrier for efficient gene delivery and obtaining the correct stoichiometry to efficiently recognize all target molecules can be challenging. Furthermore, tandem antibody domains are prone to alternative association and aggregations (19).

Advances in protein engineering have led to the development of alternative antigen binding proteins that are smaller in size, more thermodynamically stable and less aggregation prone than antibodies (20). Designed Ankyrin Repeat proteins (DARPins) are one class of such antibody mimetic proteins with such properties and are comprised of a variable number of ankyrin repeat motifs where each ankyrin repeat consists of 33 amino acids, forming 2 α-helices followed by a β-turn (20). DARPin libraries have been generated by consensus design and by randomizing 6 surface interaction residues per repeat without compromising the stability of the hydrophobic core. A library of 2 or 3 internal repeats, flanked by N- and C-terminal hydrophilic capping repeats, has a theoretical diversity of $5.2 \times 10^{15}$ or $3.8 \times 10^{23}$, respectively. Using ribosome display, practical diversities of $10^{11}$ to $10^{12}$ can be routinely reached, and with phage display about one order of magnitude less.

Library screening against many targets has enabled the selection of DARPin binders that have low nanomolar or picomolar affinity, are extremely specific and stable, and have been developed as protein therapeutics for cancer and ocular diseases (21). Previous work has demonstrated that DARPins can function as a single or bispecific recognition motifs in CAR-T cells and confer antigen-specific recognition (22-24). Here, we extend these observations to demonstrate the utility of multiple DARPins for constructing a single highly multispecific CAR that is capable of efficiently recognizing and eliminating heterogeneous tumors expressing disparate antigens.

## Materials and Methods

### Human subjects

Blood samples were obtained from donors who provided written informed consent for research protocols approved by the Institutional Review Board (IRB) at the Fred Hutchinson Cancer Center. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation using Ficoll-Histopaque (Sigma) or Lymphocyte Separation Media (Corning). $CD8^+$ and $CD4^+$ T cells were selected with EasySep™ Direct Human $CD8^+$ and $CD4^+$ T Cell Isolation Kit (Stem Cell Technologies).

### Cell Lines

The tumor cell lines K562 (ATCC® CCL-243™), MDA-MB-231 (ATCC® HTB-26™), NCI-H1975 (ATCC® CRL-5908™) and Raji (ATCC® CCL-86™) were purchased from the American Type Culture Collection, grown in RPMI media with 10% FCS and 100 U/mL penicillin–streptomycin (Gibco), and transduced with firefly luciferase (ffluc). 293T (ATCC® CRL-11268™) were cultured in DMEM with 10% FCS and 100 U/mL penicillin–streptomycin (Gibco). K562 cells were transduced with a lentivirus expressing full-length EGFR. Raji tumor cells were transduced with lentivirus expressing full-length EGFR, EpCAM and/or HER2 alone and in combination to derive cells that express one, two or all 3 of the target antigens (EGFR, EpCAM and HER2). All tumor cell lines were tested routinely for mycoplasma contamination using the MycoAlert Mycoplasma Detection kit (Lonza) as per manufacturer instructions.

### Lentiviral vector construction, transduction and generation of CAR-T cells

Monospecific CARs were designed using codon-optimized nucleotide sequences for the cetuximab scFv and DARPins E01 (EGFR), Ec1 (EpCAM) and G3 (HER2) linked to sequences encoding a modified IgG4 hinge with or without a $(G_4S)_2$ linker between the DARPin and hinge, followed by the CD28 transmembrane domain and the 4-1BB costimulatory and intracellular CD3ζ domains (25-28). Multispecific DARPins were linked in tandem each separated by a $(G_4S)_2$ or $(G_4S)_4$ linker from the neighboring DARPin followed by a $(G_4S)_2$ linker, the IgG4 hinge and transmembrane and signaling domains as described above. CAR sequences were cloned into an epHIV7 lentiviral vector downstream of a GM-CSF receptor leader sequence and separated by a T2A sequence from a truncated CD19 tag (tCD19) used for detection and enrichment of transduced T cells (29, 30) . A MYC tag (amino acid sequence EQKLISEEDL) was fused to the N-terminus of monospecific and multispecific DARPins CARs and these constructs were used solely to assess surface expression of the CARs. Lentivirus was generated by transient transfection of psPAX2 and pMD2G packaging plasmids in HEK293-T cells. Primary CD8$^+$ and CD4$^+$ T cells were separately activated by anti-CD3/CD28 beads (Life Technologies), transduced one day after activation by centrifugation at 800g for 90 minutes at 32°C with lentiviral supernatant and 1μg/mL polybrene (Millipore). Transduced cells were then sorted for expression of tCD19, restimulated using the rapid expansion protocol as described (31), and tested in functional assays on day 11 or 12 after restimulation.

## In vitro functional assays

T cell cytotoxicity was analyzed in a 4-hour chromium release assay, cytokine secretion was detected by ELISA of supernatants collected after 24 hours, and proliferation of T cells was measured by dilution of carboxyfluorescein succinimidyl ester (CFSE) or Cell Trace Violet labelled cells by flow cytometry as described previously (32). Activation-induced cell death (AICD) of T cells was detected by Annexin V and propidium iodide staining after incubation with target cells for 24 hours (BD Biosciences# 556547).

## Flow cytometry and immunoblot

T cells were stained with mAbs against human CD8 (SK1-BD Biosciences), CD4 (RPA-T4-BD Biosciences), human PD-1 (J105-eBioscience) and corresponding isotype antibody. Flow analysis was performed on a FACSCantoII or FACSARIA II and data were analyzed with FlowJo (Treestar). Transduced T cells were detected by staining with anti-human CD19 mAbs (BD Biosciences #555415) to detect tCD19. A MYC tag fused to the N-terminus of DARPin-based CARs was used to determine CAR surface expression on the T cells by staining with the anti-MYC antibody (Cell Signaling Technology #2233S) (33). Flow analysis of target expression on tumor cells was performed using mAbs against EGFR, EpCAM and HER2 (Biolegend #352910, Biolegend #324208, Biolegend #324420). Immunoblot analysis was performed against anti-human CD247 pTyr142 (K25–407.69, BD Biosciences) and pan CD247 to detect CAR expression as previously described (25).

## NOD/SCID/$\gamma c^{-/-}$ (NSG) mouse model

The Institutional Animal Care and Use Committee approved all mouse experiments. 6-8 week old NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ [NOD/SCID/γc−/− (NSG)] female mice were procured from Jackson Laboratory or bred in-house. A mixture of CD8$^+$ and CD4$^+$ T cells (1:1 ratio), unless otherwise specified, were transferred via tail vein injection one week after subcutaneous implantation of MDA-MB-231 or after intravenous implantation of Raji. Tumor growth was monitored by bioluminescence imaging as previously described (32). Transferred T cells were detected in blood or tumors harvested at specified intervals after therapy using mAbs specific for CD8 and CD4. Analysis of EGFR, EpCAM and HER2 antigen expression on tumor cells harvested at euthanasia was performed by flow cytometry.

## MHC binding prediction

Approximate binding affinities of 9-mer peptides derived from the DARPins and scFvs to MHC were predicted using NetMHC (34).

### Statistics

Log-transformation were conducted for the data with large value before analysis. To compare two paired groups, two-sample paired t-test was used. The 2-way ANOVA was used if there were two factors in group comparisons. Kaplan Meier curves were generated for survival analysis and the curves compared by Log-rank test. Results with a p value of <0.05 were considered significant. To compare multiple groups with a single control, Dunnett's test was used for multiplicity adjustment. For survival curve comparison, an un-adjusted p-value was reported.

### Study approval

Normal donors were enrolled for peripheral blood collection and provided written informed consent for research protocols approved by the Institutional Review Board of the FHCRC. Blood sample collection was conducted in accordance with the Declaration of Helsinki.The in vivo mouse experiments were approved by the FHCRC Institutional Animal Care and Use Committee.

## Results

### DARPins function as monospecific recognition motifs in chimeric antigen receptors

The E01 DARPin has been described previously and recognizes a similar region on domain III of the EGFR protein as cetuximab (35). We designed a second-generation 4-1BB/CD3ζ CAR with monospecific E01 DARPin as the recognition motif and compared its function in T cells to a similarly designed CAR in which the DARPin was replaced with the cetuximab scFv (Figure 1A). Separate aliquots of CD8$^+$ T cells were transduced with lentivirus encoding the E01 DARPin or the cetuximab scFv CARs and transduced tCD19$^+$ T cells were sorted to high purity (Figure S1A). The E01 DARPin and cetuximab scFv CAR-T cells were tested for recognition of a panel of target cells expressing EGFR including K562 cells that were transduced with full-length human EGFR (K562/EGFR), as well as MDA-MB-231 breast cancer and NCI-H1975 lung cancer cell lines that endogenously expressed EGFR (Figure S1B). K562/EGFR cells but not the EGFR$^-$ K562 parental line, and endogenous EGFR$^+$ tumor cell lines were efficiently lysed by both the E01 DARPin CAR and cetuximab CAR (Figure 1B, C).

DARPins are small (15-18 kDa) and we hypothesized that the addition of a flexible linker between the DARPin and the hinge and/or an increase in spacer length might optimize the geometry of the T cell/tumor cell interaction and improve CAR function. We constructed the E01 DARPin CAR with and without the addition of a $(G_4S)_2$ linker between the DARPin and the hinge in a short (IgG4 hinge) and modified long (hinge-CH2-CH3) spacer format (Figure S1C) (36). The expression of these different DARPin CARs was evaluated by Western blotting using an antibody against the CD3ζ chain, with the endogenous CD3ζ chain (16 kDa) serving as a loading control. Both the short and long spacer E01 CARs were expressed in T cells at the expected size of 42 kDa and 67 kDa respectively and appeared to be at comparable levels to the cetuximab scFv CAR (Figure 1D). Each DARPin CAR sample had doublet bands at the predicted size, which is likely due to incomplete unfolding of the DARPin in SDS-PAGE gels due to their high thermodynamic stability (37).

We evaluated T cells expressing these modified E01 DARPin CARs in functional assays. $CD8^+$ T cells expressing E01 DARPin CARs in the long and short spacer format with the $(G_4S)_2$ linker lysed $EGFR^+$ MDA-MB-231 comparable to $CD8^+$ T cells expressing the cetuximab CAR. In contrast, the short spacer without the $(G_4S)_2$ linker exhibited a minor reduction in cytolysis not observed with the long spacer (Figure 1E). Interestingly, $CD8^+$ T cells expressing the short spacer E01 DARPin CAR without the $(G_4S)_2$ linker showed markedly lower production of IFN-$\gamma$ and IL2, and decreased proliferation after co-culture with MDA-MB-231 target cells compared to all other EGFR CAR-T cells tested (Figure 1F,G). $CD8^+$ T cells expressing the $(G_4S)_2$ linker containing short and long spacer E01 CARs proliferated similarly to T cells expressing the cetuximab CAR (Figure 1G), and produced IFN-$\gamma$ and IL2, although the level of cytokines produced by these E01 DARPin CAR-T cells was less than that produced by the cetuximab CAR-T cells (Figure 1F). A similar hierarchy in cytokine production was observed when $CD4^+$ T cells were transduced with the E01 DARPin and cetuximab scFv CARs. $CD4^+$ T cells expressing the short spacer E01 CAR lacking $(G_4S)_2$ produced significantly less IFN-$\gamma$ and IL-2 and proliferated poorly after stimulation with MDA-MB-231, and the addition of the $(G_4S)_2$ linker or the CH2-CH3 sequences improved function of the short spacer construct (Figure S2A,B). The long spacer formats of the DARPin CAR showed equivalent proliferation and slightly less cytokine production than the cetuximab CAR in $CD4^+$ T cells. Because of the high affinity of E01 and cetuximab for EGFR, we also measured whether engagement of tumor cells might induce activation-induced cell death (AICD) of CAR-T cells. We found that the cetuximab CARs showed slightly higher AICD (Annexin $V^+$, $PI^+$, $CD8^+$ cells) *in vitro* than the E01 DARPins CARs after 24 hours of incubation with MDA-MB-231 (Figure S2C).

We next examined whether the monospecific DARPin CAR-T cells were effective in treating NSG mice engrafted with MDA-MB-231. Tumor cells were inoculated subcutaneously and 1 week later mice received a 1:1 ratio of $CD8^+$ and $CD4^+$ CAR-T cells (38). T cells expressing all of the E01 DARPin CARs with various spacer lengths with or without the $(G_4S)_2$ linker exhibited superior tumor reduction compared to the non-transduced T cells, however the effects of spacer design on CAR-T cell function were more evident in vivo than suggested by the in vitro functional assays. E01 DARPin CAR-T cells with the short spacer CAR lacking the $(G_4S)_2$ linker induced only modest tumor regression and improvement in survival compared to control T cells (Figure 1H-J). This reduced tumor control by E01 short spacer specific cells lacking $(G_4S)_2$ was predicted based on in vitro functional assessments, but it was unexpected that the E01 long spacer formats $(+/-(G_4S)_2)$ were less effective in tumor control compared with the $(G_4S)_2$ linker containing short E01 CAR, despite equivalent *in vitro* function (Figure 1 H-I). Only E01 DARPin specific cells with the short spacer and $(G_4S)_2$ linker and the cetuximab scFv specific cells completely eradicated tumor and cured all mice (Figure 1H-J). CAR-T cells were present in similar levels in the blood of mice with MDA-MB-231 tumors in all groups early after T cell transfer (Figure S2D). However the frequency of E01 $(+(G_4S)_2)$ short spacer CAR-T cells was higher than the long spacer $(+(G_4S)_2)$ at the tumor site but not in the spleen, suggesting enhanced local survival or proliferation of T cells expressing the E01 $(+(G_4S)_2)$ short spacer CAR (Figure S2E). Together, these results indicate that DARPin binders can function efficiently as recognition motifs in CARs in vitro and in vivo and show that spacer and linker modifications can be decisive in achieving optimal efficacy.

## DARPins can function in tandem to generate multispecific CARs

Ankyrin repeats are more stable and aggregation-resistant than scFvs and it is feasible to link DARPins in tandem repeats in one molecule (27). To evaluate DARPins in a multispecific CAR format, we first linked EGFR-specific (E01) and EpCAM-specific (Ec1) DARPins in tandem either separated from each other by a $(G_4S)_2$ or $(G_4S)_4$ linker, followed by a $(G_4S)_2$ linker, the hinge, and the 4-1BB and CD3ζ signaling domain (26, 27). We designed the CARs to have either E01 or Ec1 as the membrane-proximal DARPin, expressed them in T cells, and tested recognition of Raji tumor cells transfected to express EGFR or EpCAM alone, or both EGFR and EpCAM (Figure 2A; Figure S3A,B). T cells expressing each of the bispecific constructs recognized single EGFR or EpCAM positive target cells but there was a hierarchy in cytolytic activity and cytokine production. CAR-T cells expressing the Ec1-$(G_4S)_4$-E01 bispecific DARPin exhibited slightly higher cytotoxicity and cytokine secretion against the single positive targets compared to the other bispecific CAR formats (Figure 2B), and showed the highest cytotoxicity and cytokine secretion against Raji target cells expressing both EGFR and EpCAM (Figure 2B,C).

We then examined if DARPins could be used for designing targeting structures of even higher multispecificity by fusing a third DARPin (G3) that recognizes HER2 either upstream or downstream of the optimal bispecific DARPin CAR (Ec1-$(G_4S)_4$-E01) (Figure 2D) (28, 39). Evaluation of T cells that expressed these constructs against single EpCAM, EGFR, or HER2 positive Raji targets (Supplemental Figure 3A,B), demonstrated that the trispecific DARPin CAR produced IFN-γ and mediated cytotoxicity in response to $HER2^+$ cells only when G3 was membrane-distal (G3-Ec1-E01), and not when in a membrane-proximal (Ec1-E01-G3) position (Figure 2D,E). This was in line with published data that G3 DARPin binds to a membrane-proximal epitope on HER2, and therefore might require a membrane-distal location in the trispecific CAR format for effective T cell synapse formation (39). T cells expressing the trispecific CAR with the E01 DARPin in a membrane-proximal position also exhibited better IFN-γ production against single EGFR expressing target cells than when E01 was placed between the G3 and Ec1 DARPins (Figure 2D). Cytokine production by the Ec1 DARPin against single EpCAM expressing target cells was equivalent either in a membrane-distal position or when placed between the G3 and E01 DARPin (Figure 2D). Based on these results, we chose the G3-Ec1-E01 construct as a x.

The function of trispecific G3-Ec1-E01 CAR against Raji cells expressing either EGFR or EpCAM or HER2 was then compared to the respective monospecific CAR constructs to determine whether tandem linking might reduce function against single antigen positive target cells. We first assessed the level of expression of each CAR on T cells by fusing an N-terminal MYC tag to the monospecific and trispecific CARs to allow direct measurement of CAR surface expression by flow cytometry. We observed lower surface expression of the trispecific G3-Ec1-E01 construct compared to each monospecific CAR (Figure 3A). Consistent with the reduction in expression, trispecific CAR-T cells exhibited reduced cytotoxic activity against Raji tumor cells expressing EGFR, EpCAM or HER2 alone, and produced lower levels of IL-2 against single positive targets compared with the corresponding monospecific CAR-T cells (Figure 3B,C). T cells expressing the trispecific CAR proliferated after incubation with Raji tumor cells expressing only EGFR, EpCAM or HER2 at levels similar to or slightly less than T cells expressing the corresponding monospecific CARs (Figure 3D). We did not observe basal CAR phosphorylation in the monospecific,bispecific or trispecific DARPin CAR T cells as observed in previously published tonic signaling CARs such as the ROR1- CD28/CD3ζ CAR T cells (Figure S4A) (40). The monospecific, bispecific or trispecific DARPin CAR T cells also did not demonstrate cell surface expression of PD-1 prior to activation with target cells, and only upregulate PD-1 with activation as shown for the E01 CAR stimulated with MDA-MB-231 tumor cells (Figure S4B) (41). Comparison of function between the trispecific CAR (G3-Ec1-E01) and the optimal bispecific CAR construct (Ec1-$(G_4S)_4$-E01) demonstrated equivalent IFN-γ production against Raji cells expressing EGFR or EpCAM or both EGFR and EpCAM (Figure S4C). Further, the bispecific and trispecific CAR recognized endogenously expressed antigens on the MDA-MB-231 breast tumor line (Figure S4D).

## Trispecific targeting against a homogeneous versus heterogeneous tumor

Heterogeneity of antigen expression in tumors is a barrier to effective immunotherapy and might be overcome by multispecific targeting, either by infusing T cells expressing different monospecific CARs or expressing a single multispecific CAR. We evaluated the efficacy of $CD4^+$ and $CD8^+$ T cells expressing the trispecific G3-Ec1-E01 DARPin CAR, each monospecific CAR, or a mixture of T cells expressing each monospecific CAR for treating a heterogeneous tumor in NSG mice. Mice were injected with 0.5 million Raji tumor cells expressing either EGFR, EpCAM or HER2 in a 1:1:1 mix and treated 7 days later with CAR-T cells against one of the targets, a mix of monospecific CAR-T cells or trispecific (G3-Ec1-E01) CAR-T cells at the same total cell dose (Figure 4A). The monospecific CAR-T cells did not exert measurable tumor control compared with mock T cells, however a mixture of T cells expressing each monospecific CAR and T cells expressing the trispecific CAR exhibited a delay in tumor growth and improved survival (Figure 4B-D). $CD8^+$ and $CD4^+$ expressing the trispecific and each monospecific DARPin CAR were detectable in peripheral blood for over 3 weeks after T cell transfer (Figure 4E). The $CD8^+$ and $CD4^+$ trispecific CAR T cells demonstrated an early peak expansion higher than the mix of monovalent CARs followed by a decline at later time points that correlated with decreased tumor burden. On examining tumors from mice that met the euthanasia criteria, we observed that in the heterogeneous tumor setting, monospecific specific cells eliminated tumor cells that expressed their cognate antigen and outgrowth represented tumor cells that expressed only the non-targeted antigens or were antigen null (Figure 4F). In mice treated with trispecific CAR-T cells or a mix of monospecific CAR-T cells, most of the tumor cells were antigen null indicating outgrowth of tumor cells that had either lost cognate antigen or rare tumor cells that were not transfected (Figure 4F). We observed Raji tumor cells that were antigen null in all groups but the percentage was low in mice treated with mock T cells or with the E01 or G3 monospecific DARPin CARs where the harvested Raji tumors were predominantly $EpCAM^+$, possibly due to more aggressive growth of tumor cells transfected with EpCAM (Figure 4F).

## Synergistic activity of the trispecific DARPin CAR

The goal of multispecific targeting is to eliminate tumor cells that express any constellation of target antigens. Therefore, we examined potential differences in in vivo anti-tumor efficacy of the trispecific construct against Raji tumor cells expressing just a single target, a combination of 2 targets or all 3 targets. Expression of the target antigens (EpCAM and HER2) was similar in the single, double or triple transfectants but expression of EGFR in the triple transfected Raji cells was 0.6 fold of the level in single or double EGFR$^+$ targets (Figure S3B). We observed that a dose of 9 million trispecific CD4$^+$ and CD8$^+$ CAR-T cells was insufficient to clear Raji tumors that expressed only HER2 or EpCAM but was curative against Raji tumor cells expressing EGFR suggesting that the potency of the trispecific CAR is different against tumor cells expressing different single target antigens (Figure 5A). However, at the same CAR-T cell dose, we observed more rapid tumor clearance and more prolonged tumor control in mice engrafted with Raji tumor cells co-expressing both EpCAM and HER2 compared with mice engrafted with single antigen (Raji/EpCAM and Raji/HER2) tumors, although the survival difference was not significant (Figure 5A,B). The superior antitumor activity did not reflect differences in CAR-T cell expansion suggesting that efficacy was due to superior recognition by the trispecific CAR of tumor cells expressing these two antigens (Supplemental Figure 5A).

To assess synergy of trispecific CAR T cells against tumor cells expressing EGFR and additional antigens, we lowered the dose of T cells since the higher dose was curative against Raji tumors expressing EGFR. At a T cell dose of 5 million CAR-T cells, we saw equivalent anti-tumor activity from trispecific CAR-T cells in mice engrafted with Raji tumors expressing only EGFR and those engrafted with Raji expressing EGFR and EpCAM (Figure 5C,D). However, the trispecific CAR cleared tumor and improved the survival in mice with Raji tumors expressing all 3 antigens compared with tumors expressing only one or two antigens (Figure 5C, D). T cell expansion was also observed in blood in all treatment groups (Figure S5B). We did not observe significant differences in short term cytotoxicity, cytokine secretion or proliferation assays of the trispecific DARPin CAR against target cells expressing single, dual or triple antigens (Figure S5C-E) (14).The trispecific CAR also did not show an increase in activation-induced cell death (AICD) on encountering single versus multiple antigens (Figure S5F). These results illustrate the ability of trispecific CARs to target heterogeneous tumors and exhibit synergistic activity in vivo against tumor cells that express multiple antigens.

## Prediction of Immunogenicity

A barrier that could limit the use of antibody mimetic proteins in CARs is the potential for immunogenicity of non-scFv based motifs. MHC restricted CD8$^+$ and CD4$^+$ T cell responses to transgene encoded epitopes have been observed in some patients treated with T cells expressing a foreign protein, including CAR-T cells designed from murine scFvs (30, 42). Since mouse models are poor predictors of immunogenicity in humans due to differences in MHC proteins, we used an *in silico* approach to identify candidate epitopes for human class I MHC. We focused on the immunogenicity of the core monospecific and trispecific DARPins and compared them to the cetuximab scFv (25). We identified 9-mer binding sequences with a predicted affinity of less than 100 nM to the human MHC I supertype alleles using the NetMHC prediction algorithm (Table S1). We found that both monospecific DARPins and the cetuximab scFv have between 1-2 peptides, predicted to bind to the MHC I supertype. The trispecific DARPin has an increase in the number of peptides (n=5) predicted to bind MHC I alleles over the monospecific construct (Table S1). However, on screening the predicted immunogenic peptides against the human proteome, we found that most of the DARPin but not mouse cetuximab scFv hits matched highly similar sequences in the human proteome. These results indicate that DARPin sequences could potentially be even less susceptible to T cell mediated immunogenicity than murine scFvs because of the abundance of natural ankyrin repeat proteins in the human proteome.

## Discussion

Antigen loss as a mechanism of escape after T cell therapy for cancer can limit the efficacy of this approach (1, 5-7, 11, 12). Strategies to target multiple antigens are likely to be necessary for optimal efficacy and multiple groups are working to develop multispecific CARs using scFv based technologies (1, 13-17). In this study, we show that single domain antibody mimetic proteins such as DARPins can serve as multispecific recognition motifs and may offer advantages over scFvs due to their stable nature and aggregation resistance (20).

We tested multispecific recognition using three previously characterized DARPins that target EGFR (E01), EpCAM (Ec1) and HER2 (G3) (26-28). We acknowledge that these antigens are expressed on normal tissues in addition to high expression on tumors, however they serve as model antigens for proof-of-principle experiments to evaluate multispecific targeting. In the design of monospecific E01 DARPin CARs, we found that the addition of a $(G_4S)_2$ linker between the DARPin and the hinge improved CAR function most likely due to a more optimal spacer length or to the added flexibility that improved target binding (35). The design of multispecific DARPin CARs, including the trispecific CAR was empirically determined. Evaluation of the functionality of each DARPin in all 3 positions of the trispecific CAR identified G3-Ec1-E01 construct as the optimal design which was in agreement with the published crystal structures of the G3 DARPin recognizing a membrane-proximal epitope of HER2 and E01 recognizing a membrane-distal epitope of EGFR (35, 39). Although a published crystal structure of the Ec1 DARPin and its target is not available, Ec1 functioned in all locations in the trispecific design, thus no inferences can be made about the optimal location.

Functional testing of the trispecific DARPin CAR-T cell in both in vitro and mouse studies revealed a hierarchy in anti-tumor efficacy against the different targets. A CAR-T cell dose that was ineffective in clearing tumors expressing only EpCAM or HER2 was sufficient to clear an EGFR expressing tumor. The divergence may be partly explained by differences in growth kinetics between the transfected Raji targets or the efficiency of engagement of individual DARPins with their target on tumor cells. However, the potency of the trispecific CAR was lower than the corresponding monospecific CARs against tumor cells expressing a single target, revealing a potential limitation of multispecific designs, possibly requiring refinement in the geometric presentation of the binders to various epitopes. The optimal level of CAR expression on T cells is not known, however the trispecific CAR was expressed at lower levels than the monospecific CARs, which might explain the decreased activity of the trispecific CAR against single antigen targets. Alternatively, positioning of the G3 and Ec1 DARPin may not be optimal due to the additional length of the extra-cellular domain of the trispecific CAR.

Importantly, in the context of a mixture of heterogeneous antigen positive tumor cells, the trispecific CAR-T cells were as effective as a mix of monospecific CARs. The trispecific CAR also exhibited increased in vivo antitumor activity at suboptimal T cell doses against tumors that expressed multiple (2 or 3) antigens compared to activity against tumor cells expressing only a single antigen. The synergistic increase in trispecific CAR activity could be due to enhanced T cell activation due to binding of multiple antigens simultaneously, which may potentiate signaling at the immune synapse (14). This could also possibly generate a safety window for targeting a combination of antigens on tumors when any of the individual antigens is expressed on normal tissue. These data suggest that for treatment of a heterogeneous solid tumor a single T cell product expressing a multispecific CAR is an alternative that avoids the complexity of engineering multiple monospecific vectors and multiple T cell products.

Our data also reaffirm the obstacle of antigen escape. We consistently observed immune selection when a heterogeneous mixture of tumor cells expressing the three target antigens were engrafted into mice and then treated with monospecific or trispecific CAR-T cells. Tumors that grew out in mice treated with the trispecific CAR or with a mixture of monospecific CAR-T cells were mostly comprised of antigen null variants. In our system the target molecules were transfected into Raji cells and were not essential for the malignant phenotype. Furthermore, the studies were performed in immunodeficient mice where engagement of an endogenous host response cannot occur. For clinical translation, multispecific receptors should target molecules that are involved in tumor cell maintenance or possibly be combined with checkpoint inhibitors to engage endogenous immune response at the tumor site (43-45). An additional challenge with multispecific CARs is the potential for enhanced immunogenicity of complex binding domains.

Notwithstanding the aforementioned challenges, our results demonstrate that in principle DARPin motifs can serve as effective monospecific and multispecific tumor recognition domains for CARs and function effectively in T cells to reduce antigen escape. Refinements such as improved cell surface expression, computational modelling of ligand binding, and evaluation of immunogenicity can further contribute to the use of antibody mimetic proteins in CAR design and facilitate clinical translation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations list:

| | |
|---|---|
| **scFv** | (single-chain variable fragment) |
| **DARPin** | (Designed Ankyrin Repeat proteins) |
| **CAR** | (chimeric antigen receptor) |
| **EGFR** | (epidermal growth factor receptor) |
| **EpCAM** | (Epithelial cell adhesion molecule) |
| **HER2** | (human epidermal growth factor receptor 2) |
| **AICD** | (activation-induced cell death) |
| **tCD19** | (truncated CD19) |
| **PBMC** | (Peripheral blood mononuclear cells) |
| **kDa** | (kilodalton) |
| **$G_4S$** | ((Glycine)$_4$-Serine) |
| **IFN-γ** | (Interferon gamma) |
| **IL2** | (interleukin 2) |
| **MHC** | (major histocompatibility complex) |
| **nM** | (nanomolar) |

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## Statement of translational relevance:

The simultaneous targeting of multiple antigens with engineered immune cells could overcome the barrier posed by the heterogeneity of antigen expression in tumor cells, which is frequent in many cancer types and enables the escape and subsequent outgrowth of antigen loss variants. We show that stable antigen binding proteins comprised of designed ankyrin repeat proteins (DARPins) can be linked in tandem as targeting domains for chimeric antigen receptors (CARs). These higher-order receptors retain specificity and function against multiple target antigens thereby overcoming the challenge of target heterogeneity and antigen escape that can occur after adoptive therapy with engineered immune cells.

**Statement of significance**: Multispecific chimeric antigen receptor (CAR) modified T cells generated by linking DARPins in tandem demonstrate potent anti-tumor function against heterogeneous tumors and synergistic function against tumor cells that express multiple antigens.

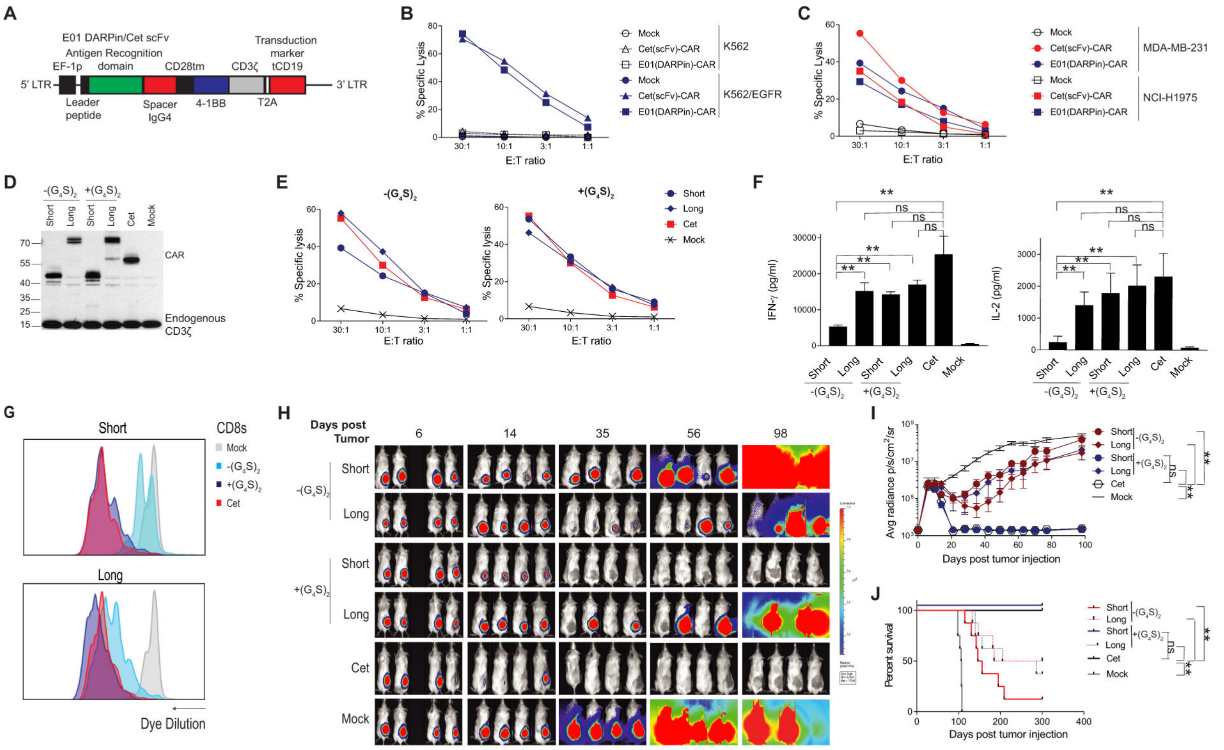

**FIGURE 1:**

EGFR recognition by E01 DARPin CARs. (A) Schematic of the structure of EGFR-specific CARs consisting of the EF-1 promoter (EF-1p), GM-CSF receptor leader, E01 DARPin or Cet scFv, spacer sequences, CD28 transmembrane, 4-1BB costimulation and CD3ζ activation domain with a truncated CD19 transduction marker after the T2A element. (B) Cytotoxicity of E01 DARPin and cetuximab scFv specific cells against K562 cells and K562 cells transduced to express full-length EGFR (n=3). (C) Cytotoxicity of E01 DARPin and cetuximab scFv specific cells against tumor lines expressing EGFR (n=3). (D) Immunoblot with anti CD3ζ measuring CAR and endogenous CD3ζ on sort-purified specific cells (E) Cytotoxicity of CD8$^+$ E01 DARPin specific cells against MDA-MB-231 tumor cells with short and long spacer (+/−$(G_4S)_2$ linker) (n=3). (F,G) Cytokine secretion and proliferation of CD8$^+$ E01 DARPin specific cells with short and long spacer (+/−$(G_4S)_2$ ) against MDA-MB-231 target cells (n=3). (H) Bioluminescent imaging of MDA-MB-231 breast cancer cells transduced with firefly luciferase in NSG mice treated with 5 million CAR-T cells (CD8:CD4:1:1). (I,J) Average tumor radiance and survival of E01 DARPin CAR with short and long spacer (+/− $(G_4S)_2$ linker) compared to the cetuximab scFv CAR (n=8 mice from 2 donors). (**-p<0.05, ns-not significant)

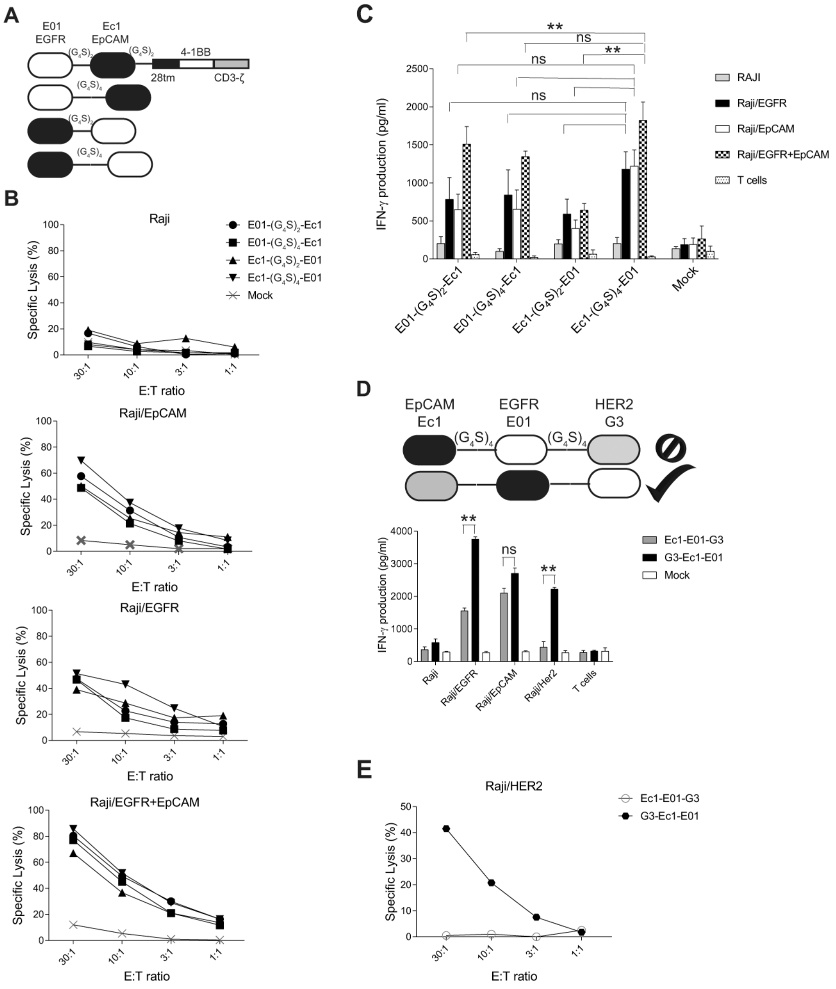

**FIGURE 2:**

Design of multispecific DARPin CARs. (A) Bispecific DARPin CARs constructed with either the Ec1 DARPin or E01 DARPin as the membrane-proximal DARPin separated by $(G_4S)_2$ or $(G_4S)_4$ linkers. (B,C) Cytotoxicity and cytokine production of each bispecific DARPin CAR against single positive or double positive target cells (n=3). (D) Trispecific CAR designs and recognition of single positive tumor targets measured by IFN-γ production by specific cells (n=4) (E) Cytotoxicity of trispecific specific cells against Raji/HER2. (**-$p<0.05$, ns-not significant)

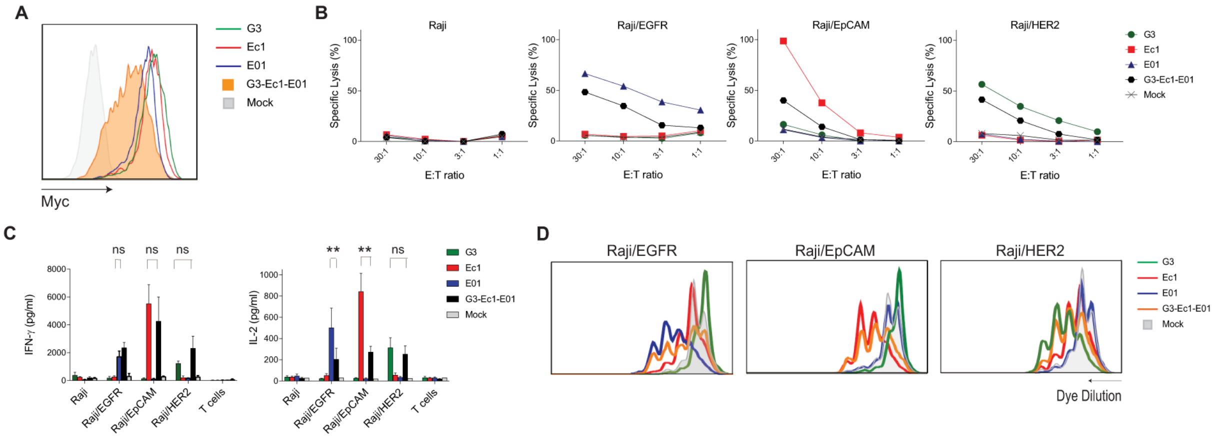

**FIGURE 3:**

Trispecific CAR function against target cells expressing single antigens. (A) Surface expression of monospecific and trispecific DARPins CARs measured by staining of N-terminal MYC tag. (B,C) Cytotoxicity and cytokine production of monospecific versus trispecific DARPin specific cells against Raji cells expressing EGFR or EpCAM or HER2 (n=3). (D) Proliferation of the trispecific G3-Ec1-E01 specific cells against Raji cells expressing single targets. (**-p<0.05, ns-not significant).

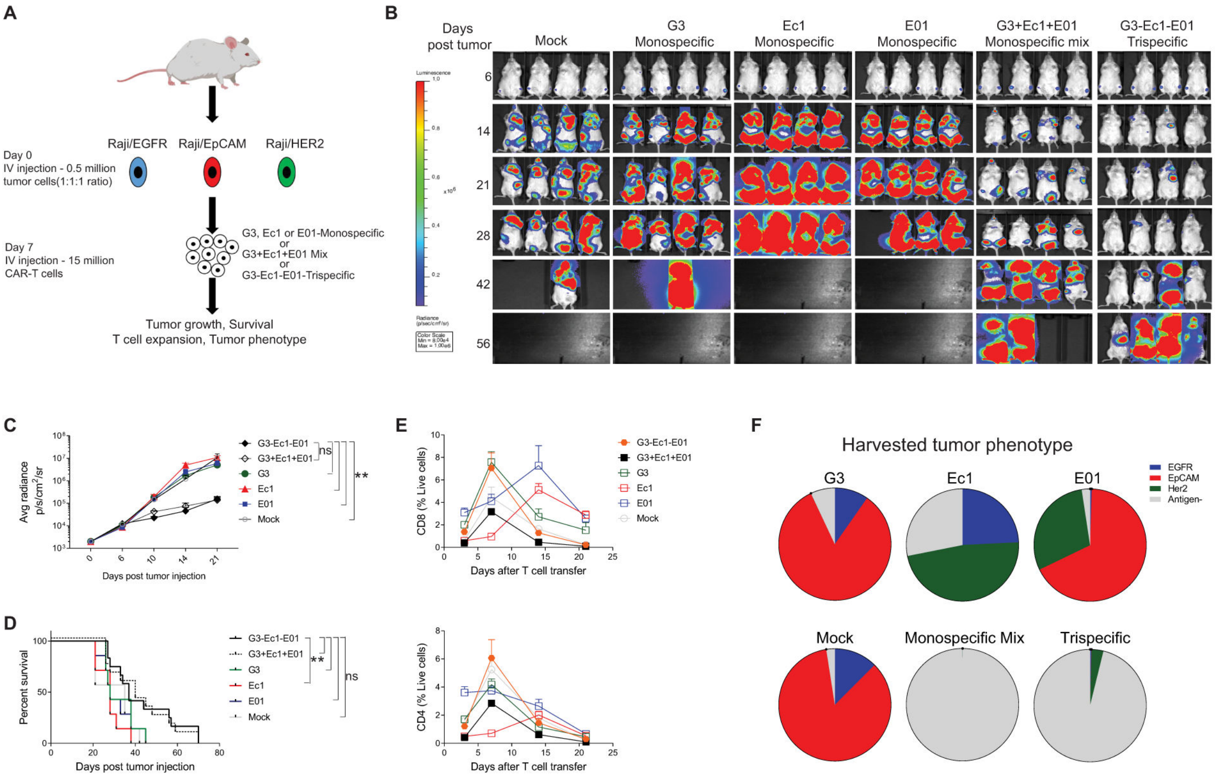

**FIGURE 4:**

In vivo anti-tumor function of trispecific specific cells against heterogeneous tumor cells. (A) NSG mice were inoculated with a 1:1:1 mix (0.5 million) of Raji/EGFR, Raji/EpCAM, and Raji/HER2 intravenously (IV) followed by IV injection of CAR-T cells (15 million CD8:CD4 1:1) 1 week after tumor cell injection. (B) Bioluminescence imaging of mice treated with trispecific CAR-T cells, monospecific CAR-T cells, or a mix of monospecific CARs at the same total T cell dose. (C,D) Average tumor radiance and survival of mice in each treatment cohort. (n=7-12 from 2 donors). (E) CAR-T cell frequencies in blood for monospecific versus trispecific CAR (n=7). (F) Antigen expression on tumor cells harvested on euthanasia in the heterogeneous tumor model in each treatment cohort (**- p<0.05, ns-non significant)

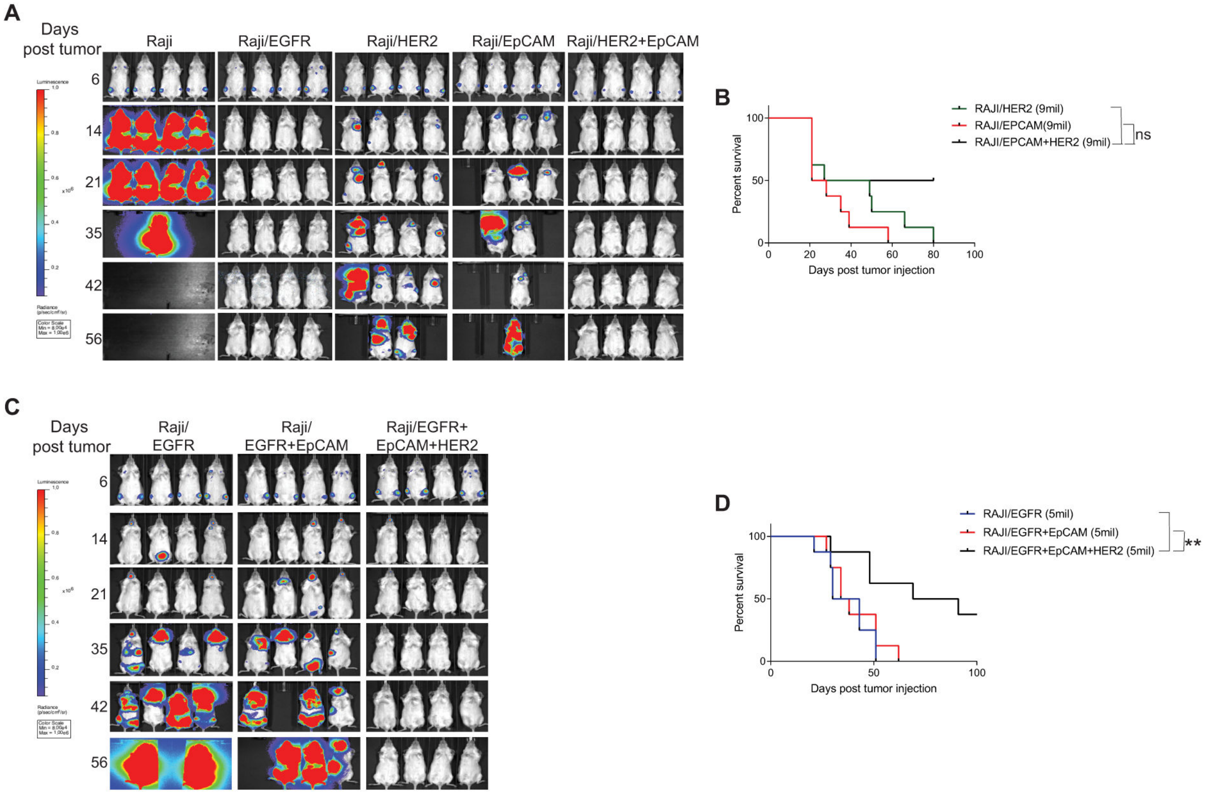

**FIGURE 5:**

Trispecific CAR G3-Ec1-E01 T cells demonstrate synergy against tumor cells expressing multiple antigens. (A) Bioluminescence imaging of tumors expressing single (EGFR, EpCAM or HER2) antigens or both HER2 and EpCAM in mice treated with 9 million trispecific CAR-T cells. (B) Survival curves of cohorts of mice after treatment with 9 million trispecific CAR-T cells. (C) Bioluminescence imaging of tumors expressing single, double or all 3 antigens (EGFR, HER2 and EpCAM) in mice treated with 5 million trispecific CAR-T cells. (D) Survival curves of cohorts of mice after treatment with 5 million trispecific CAR-T cells. (**-p<0.05, ns-not significant)