Chimeric Antigen Receptor T Cells with Dissociated Signaling Domains Exhibit Focused Antitumor Activity with Reduced Potential for Toxicity In Vivo

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Abstract
Adoptive immunotherapy using T lymphocytes genetically modified to express a chimeric antigen receptor (CAR-T) holds considerable promise for the treatment of cancer. However, CAR-based therapies may involve off-target toxicity against normal tissues expressing low amounts of the targeted tumor-associated antigen (TAA). To specify T cells for robust effector function that is selective for tumor but not normal tissue, we developed a trans-signaling CAR strategy, whereby T-cell activation signal 1 (CD3ζ) is physically dissociated from costimulatory signal 2 (CD28) in two CARs of differing antigen specificity: mesothelin and α-folate receptor (FRα). Human T cells were genetically modified to coexpress signal 1 (anti-Meso scFv-CD3ζ) and signal 2 (anti-FRa scFv-CD28) CARs in trans. Trans-signaling CAR-T cells showed weak cytokine secretion against target cells expressing only one TAA in vitro, similar to first-generation CAR-T cells bearing CD3ζ only, but showed enhanced cytokine secretion upon encountering natural or engineered tumor cells coexpressing both antigens, equivalent to that of second-generation CAR-T cells with dual signaling in cis. CAR-T cells with dual specificity also showed potent anticancer activity and persistence in vivo, which was superior to first-generation CAR-T cells and equivalent to second-generation CARs. Importantly, second-generation CAR-T cells exhibited potent activity against cells expressing mesothelin alone, recapitulating normal tissue, whereas trans-signaling CAR-T cells did not. Thus, a dual specificity, trans-signaling CAR approach can potentiate the therapeutic efficacy of CAR-T cells against cancer while minimizing parallel reactivity against normal tissues bearing single antigen. Cancer Immunol Res; 1(1): 43–53. © 2013 AACR.

Introduction
Genetic redirection of T cells with chimeric antigen receptors (CAR) that link an antigen-specific single-chain antibody fragment (scFv) to intracellular signaling domains is at the forefront of cancer immunotherapy (1, 2). CARs functionally redirect T cells with high specificity to various surface antigens on tumor cells independent of MHC restriction and antigen processing, and therefore bypass major mechanisms by which tumors escape immune recognition. T cells bearing a first-generation CAR having only the T-cell CD3ζ intracellular signaling domain either fail to persist or become anergic, as tumor cells frequently lack requisite ligands for costimulation (3). This incomplete activation of CAR-T cells in vivo seems to limit their persistence, and has thus hampered their efficacy in clinical trials for lymphoma (4), neuroblastoma (5), ovarian cancer (6), or renal cell cancer (7). To overcome these limitations, second-generation CAR-T cells were developed that incorporate the intracellular domain of various costimulatory molecules such as CD28, 4-1BB, OX-40, and CD27 leading to improved expansion, persistence, and activity of the CAR-T cells in preclinical mouse models (8, 9) and in clinical studies (2, 10, 11). Still, the enhanced potency of these CARs can be associated with autoimmunity due to on-target toxicities against normal tissues expressing lower levels of the tumor-associated antigen (TAA). For instance, administration of high numbers of T cells bearing an anti-ErbB2 CAR comprising the CD28 and 4-1BB costimulatory domains to a lymphodepleted patient with metastatic colon cancer resulted in rapid onset of pulmonary toxicity with lung infiltrates and a “cytokine storm” followed by cardiac arrest and death (12). Clearly, the development of strategies limiting potential early- or late-phase toxicity is of importance. We have previously generated a fully human anti-mesothelin CAR capable of conferring potent in vitro and in vivo effector functions to primary T cells against mesothelin-expressing tumors (13). Mesothelin-directed CAR-T cells also hold the potential to inflict damage against normal mesothelial cells lining the pleura, peritoneum as well as epithelial cells of the trachea, tonsils, fallopian tube, and the
rete testis, which express low levels of mesothelin (14, 15). To limit “on-target” toxicity and improve tumor-focused targeting and attack, we have developed and tested the concept of a trans-signaling CAR strategy where the T-cell activation signal 1 (CD3ζ module) is physically dissociated from the costimulatory signal 2 (CD28 module). Because mesothelin and a-folate receptor (Fra) are TAAs coexpressed in the majority of epithelial ovarian cancers, but expressed differentially and at low levels in normal tissues (14, 16–19), 2 independent CARs of distinct specificity were used: a signal 1 CAR (meso-CD3ζ only), and a signal 2 CAR (Fra-CD28 only) using prevalidated scFvs (13, 20). In this fashion, T cells transduced to coexpress both CARs exhibit potent in vitro and in vivo effector functions that are driven by tumor encounter and coupled with diminished damage to normal tissues.

Materials and Methods

CAR constructs

The F-28 CAR was constructed by using lentiviral vector backbone constructs previously described (20). CAR construction and lentiviral production are detailed in Supplementary Materials and Methods.

Recombinant lentivirus production

High-titer replication-defective lentiviral vectors were produced and concentrated as previously described (13).

Human T-cell transduction

Primary human T cells, purchased from the Human Immunology Core at University of Pennsylvania (Philadelphia, PA), were isolated from healthy volunteer donors following leukapheresis by negative selection. All specimens were collected under a University Institutional Review Board–approved protocol, and written informed consent was obtained from each donor. T-cell activation and lentiviral transduction was conducted as previously described (13).

Functional assays

Cytokine release assays were carried out using an IFN-γ ELISA Kit (Biolegend). 51Cr-release and CD107 degranulation assays of cytolysis were conducted as previously described (13, 21). The Cytometric Bead Array and apoptosis assay were conducted according to manufacturer’s instructions (BD Biosciences). Functional assays are further detailed in the Supplementary Materials and Methods.

Xenograft model of ovarian cancer

Mouse studies were carried out as detailed in the Supplementary Materials and Methods.

Immunohistochemistry

Fresh frozen tumor samples were sectioned for immunohistochemical analysis as described in the Supplementary Materials and Methods.

Statistical analysis

Statistical evaluation was conducted using two-tailed Student t test. GraphPad Prism 4.0 (GraphPad Software) was used for the statistical calculations. P values less than 0.05 were considered significant.

Results

CAR construction

Anti-mesothelin CAR constructs comprised the P4 scFv linked to a CD8α hinge and transmembrane region, followed by a CD3ζ signaling moiety alone (referred to as M-ζ) or in tandem with the CD28 intracellular signaling motif (M-28ζ), which were previously shown to confer specific mesothelin-directed activity in vitro and in vivo (ref. 13; Fig. 1A and B). The costimulatory anti-FRA CAR (F-28) construct comprised the MOv-19 scFv linked to a CD8α hinge and the CD28 transmembrane region and intracellular signaling motif; the signaling-deficient F-Dζ construct lacks a functional signaling domain. F-28 CAR expression was monitored through the GFP transgene, which is included in the CAR lentiviral construct with its expression driven from the same EF1α promoter, which regulates CAR expression, GFP signal was highly correlated with protein-l binding to the mouse scFv of the CAR highlighting the reliability of this reporter gene expression (Supplementary Fig. S1). Primary human T cells were efficiently transduced with the 2 CAR-encoding lentiviral vectors with more than 40% dual-transduced T cells reproducibly expressing both CARs (Fig. 1C). CAR-T cell populations were adjusted to equivalent frequencies of anti-mesothelin CAR-T cells (60%–70%) by adding untransduced T cells for all functional assays.

Trans-signaling CAR-T cells exert superior antigen-specific cytokine secretion in vitro

To evaluate the in vitro effector functions of CAR-T cells in response to cells that express mesothelin alone versus tumor cells that coexpress mesothelin and FRa, TAA-negative C30 cancer cell lines, which lack endogenous CD80/86 costimulatory ligands were engineered to overexpress either one or both of these antigens (Fig. 2A). T cells engineered to express the M-ζ CAR recognized and secreted similar levels of IFN-γ when cocultured with C30 cells expressing either mesothelin (C30M) or mesothelin and FRa (C30M/F). In comparison, second-generation M-28ζ CAR-T cells exerted superior IFN-γ secretion against all C30 cell variants expressing mesothelin. In contrast, trans-signaling M-ζ/F-28 CAR-T cells produced low levels of IFN-γ against C30M, similar to M-ζ CAR-T cells, but exerted enhanced IFN-γ production when exposed to C30M/F tumor cells (Fig. 2B). M-ζ/F-28 CAR activity against C30M/F cells surpassed that of M-ζ CAR-T cells showing that expression of both antigens by tumor cells promotes the costimulation of M-ζ/F-28 CAR-T cells through the F-28 CAR (Fig. 2B). Consistent with this notion, coexpression of the M-ζ and the signaling-deficient F-Dζ CAR in T cells did not enhance their response against C30M/F cells. M-28ζ CAR-T cells produced more IFN-γ than M-ζ/F-28 CAR-T cells using this artificial target cell system where cells are engineered to express antigens at supraphysiologic levels. T cells expressing the F-28 CAR alone did not produce any IFN-γ, consistent with a lack of CD3-ζ.

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signaling. Moreover, control T cells transduced to express GFP or an anti-CD19 CAR containing CD3ζ with CD28 signaling motifs in tandem (CD19-28z; ref. 22) did not produce cytokines after stimulation with CD19-negative C30 cells, illustrating the need for antigen-specificity (Fig. 2B).

We next tested trans-signaling CAR-T cell activity against an ovarian cancer cell line that naturally expresses both mesothelin and FRA, A1847 (Fig. 2C). M-z/F-28 CAR-T cells secreted IFN-γ in response to A1847 that was significantly higher than that secreted by M-z CAR-T cells but similar to cis-signaling M-28z CAR-T cells, showing that the natural expression of both antigens on tumor cells is capable of inducing costimulatory effects in trans-signaling CAR-T cells (Fig. 2D). Interleukin (IL)-2 secretion from the dual transduced M-z/F-28 CAR-T cells was also similar to second-generation M-28z CAR-T cells and higher than that of M-z CAR-T cells indicating that integrated delivery of signal 1 and 2 in trans or cis can enhance production of IL-2 cytokine (Fig. 2E).

**Trans-signaling CAR-T cells show in vitro cytolytic potency**

Degranulation is a quantitative indicator of lytic function by T cells (21). M-z and M-z/F-28 CAR-T cell degranulation was accompanied by upregulation of surface coexpression of mobilized CD107 (lysosomal-associated membrane protein 1) and the activation-associated marker CD69 in response to C30M but not when stimulated with either C30 or C30F cells (Fig. 3A). Consistent with active costimulation, M-28z CAR-T cells displayed a superior cytolytic phenotype against C30M compared with M-z and M-z/F-28 CAR-T cells. However, exposure to A1847 (M⁺/F⁺) tumor cells with both antigens led to enhanced degranulation by both M-28z and M-z/F-28 CAR-T cells, relative to M-z CAR-T cells (Fig. 3A). Anti-CD19 CAR-T cells did not degranulate in response to C30 or A1847 cells (Supplementary Fig. S2). In short-term chromium release assays, all of the various mesothelin-directed CAR-T cell populations (M-28z, M-z, and M-z/F-28) showed cytolytic activity against single
antigen-expressing C30-M target cells with M-28z CAR-T cells exhibiting greater cytolytic activity than M-z/F-28 and M-z cells at all ratios tested. A1847 target cells were also lysed by all anti-mesothelin CAR-T groups, yet M-28z and M-z CAR-T showed statistically comparable and higher lysis than M-z/F-28 CAR-T cells against C30-M/F cancer cells. C, coexpression of mesothelin and FRα by A1847 ovarian cancer cell line. D, trans-signaling CAR-T cells secrete higher levels of IFN-γ in response to A1847. Mean IFN-γ concentration ± SEM (pg/mL) is shown. *, P < 0.05 comparing M-z and M-z/F-28 CAR-T cells against A1847 cancer cells. E, trans-signaling CAR-T cells secrete more IL-2 (pg/mL) in response to A1847 than M-z CAR-T. Values represent the mean ± SEM concentration of triplicate wells.

Figure 2. Trans-signaling CAR-T cells exert superior antigen-specific cytokine secretion in vitro compared with first-generation CAR-T cells. A, detection of surface mesothelin and/or FRα protein expression in genetically modified C30 tumor cells by flow cytometry. B, primary human T cells transduced with M-z and F-28 CARs exert superior IFN-γ secretion compared with M-z CAR-T cells. Mean IFN-γ concentration ± SEM (pg/mL) is shown. **, P < 0.01 comparing M-z and M-z/F-28 CAR-T cells against C30-M/F cancer cells. C, coexpression of mesothelin and FRα by A1847 ovarian cancer cell line. D, trans-signaling CAR-T cells secrete higher levels of IFN-γ in response to A1847. Mean IFN-γ concentration ± SEM (pg/mL) is shown. *, P < 0.05 comparing M-z and M-z/F-28 CAR-T cells against A1847 cancer cells. E, trans-signaling CAR-T cells secrete more IL-2 (pg/mL) in response to A1847 than M-z CAR-T. Values represent the mean ± SEM concentration of triplicate wells.

Trans-signaling CAR-T cells resist AICD

Incorporation of costimulatory domains into CARs can increase resistance of CAR-T cells to apoptosis upon activation by tumors (23). To investigate if provision of CD28 costimulation in trans protects T cells from antigen-induced cell death (AICD), single or dual CAR-bearing T cells were measured for their rate of apoptosis following coculture with A1847 (M⁺/F⁺) tumor cells. Apoptosis [7-aminoactinomycin D (7-AAD)+ Annexin V⁺] was elevated in M-z T cells exposed to A1847 (34%) for 3 days but reduced in trans-signaling M-z/F-28 CAR-T cells (0.5%–1%; Fig. 4A). Consistent with past studies (24), cis-signaling M-28z CAR-T cells were also resistant to AICD. Control F-28 or CD19-28z CAR-T cells displayed no AICD in the absence of specific antigenic stimulation (Fig. 4B).

Dual-specific CAR-T cells possess enhanced antitumor potency and persistence in vivo

The capability of trans-signaling CAR-T cells to inhibit human tumor outgrowth was evaluated in vivo in immunodeficient NOD/SCID/IL-2R-γcnull (NSG) mice inoculated subcutaneously with 1 × 10⁶ firefly luciferase-expressing A1847 cells. Mice with established tumors (150–200 mm³) received intravenous injections of CAR-T cells. Four weeks after the first T-cell dose, tumor growth was inhibited modestly in mice receiving M-z CAR-T cells (P = 0.045), compared with saline,
CD19-28z CAR-T cells, F-28 CAR-T cells, or GFP-T cell control groups (Fig. 5A). Compared with M-z CAR-T cells, transfer of M-28z or M-z/F-28 CAR-T cells mediated even more potent inhibition of tumor outgrowth (*P* = 0.028) indicating that incorporation of CD28 signaling domain in cis or in trans enhances antitumor activity in vivo against tumors coexpressing both TAAs (Fig. 5A). Bioluminescence imaging results from treated mice confirmed the lower tumor burden in those treated with the trans-signaling CAR-T cells compared with first-generation CAR-T cells. Tumors exhibited similar low levels of bioluminescence regardless of whether the mice were treated with trans- or cis-signaling CAR-T cells (Fig. 5B). Two weeks after first T-cell dose, peripheral blood CD8+ T and CD4+ T cell counts from mice injected with M-28z or M-z/F-28 CAR-T cells were similar and higher than in the M-z group (*P* < 0.05; Fig. 5C). No substantial human T-cell persistence was observed in mice treated with CD19-28z CAR-T, F-28 CAR-T, or GFP-T cells.

Trans-, but not cis-, signaling CAR-T cells exhibit more limited activity against cells bearing single antigen in vivo

FRα-deficient A1847 cells (A1847M+/-/F-) were generated via transduction with lentiviral particles encoding a FRα-specific short hairpin RNA (shRNA), as a surrogate for normal human mesothelial cells expressing only mesothelin for use in modeling in vivo. Fluorescence-activated cell sorting resulted in an enriched cancer cell population (~98%), which lacked surface FRα expression (Supplementary Fig. S3A). FRα expression was unaltered after engineering cells with control shRNA (A1847M+/-/F-). Besides the lack of FRα expression, the 2 lines seemed identical by all measures. No difference in in vitro growth kinetic or viability of A1847M+/-/F- and A1847M+/F- cells was observed (Supplementary Fig. S4) and control anti-FRα (F-28z) CAR-T cells produced minimal IFN-γ in response to stimulation with A1847 cells after FRα silencing; similar to levels seen in response to PEO-1 ovarian cancer cells, which have undetectable surface FRα expression via flow cytometry (ref. 9; Supplementary Fig. S5A and S5B). In coculture assays, IFN-γ secretion by trans-M-z/F-28 CAR-T cells was significantly reduced in response to A1847M+/-/F- compared with A1847M+/F-, a confirmation of potent effector function only upon engagement of both antigens (Supplementary Fig. S6). In addition, M-z/F-28 and M-z CAR-T cells reacted equivalently against A1847M+/F- cells. As expected, cis-M-28z CAR-T cells secreted significantly higher amounts of IFN-γ against A1847M+/F-, similar to the IFN-γ levels achieved against A1847M+/F-.

To evaluate the in vivo potency of trans- or cis-signaling T cells against A1847 cells coexpressing or lacking FRα, A1847M+/F- and A1847M+/F- cells were inoculated subcutaneously separately in the same NSG mice on opposite hind flanks. A1847M+/F- and A1847M+/F- tumors were established comparably and grew similarly in vivo (Fig. 1A and B). Mice bearing 2 established A1847 (~330 mm3) tumors received tail vein injections of CAR-T cells and tumor
outgrowth was monitored. The efficiency of A1847M⁺/F⁺ tumor outgrowth inhibition was identical between trans-M-z/F-28 CAR-T and cis-M-28z CAR-T cell groups (Fig. 6A). However, inhibition of A1847M⁺/F⁺/C₀ outgrowth was significantly attenuated in the trans-M-z/F-28 CAR-T cell group compared with the cis-M-28z group (P = 0.0045; Fig. 6B). Notably, trans-signaling CAR-T cells were less effective in inhibiting the outgrowth of A1847M⁺/F⁺/C₀, compared with their activity against the A1847M⁺/F⁺ tumor in the same mice (P = 0.0001; Fig. 6C). Bioluminescence imaging of the tumors confirmed these results (Fig. 6D).

**Discussion**

Adoptive immunotherapy involving genetic modification of T cells with antigen-specific, chimeric, single-chain receptors is a promising approach for the treatment of cancer (25). However, the therapeutic value of adoptively transferred, gene-engineered T cells may be compromised by extensive autoimmune damage to normal tissues expressing the target antigen (26). This adverse side effect has been encountered in clinical trials using T cells genetically modified to express a tumor antigen-specific CAR (27). Mild to severe autoimmune toxicity has been reported following transfer of tumor-reactive T cells including liver toxicity upon adoptive transfer of anti-CAIX CAR-T cells (28) due to CAIX expression on bile duct epithelium. Serious adverse events (SAE) involving death of a patient has been observed in 2 distinct clinical trials following adoptive transfer of CAR-T cells redirected against ErbB2 (12) or CD19 (29), though the role for modified T cells in the latter SAE remains unclear. Collectively, studies in preclinical mouse models and in patients have indicated that the number of T cells administered, the expression levels and localization of antigen in normal tissues, the type of signaling domains incorporated into chimeric receptors and the level of immune preconditioning used are parameters that must be carefully considered in the design of safe engineered T-cell therapeutic strategies (26, 30–35).

CARs with one or more costimulatory signals confer a new potential to respond to target antigen with sustained proliferation and cytotoxicity, and resistance to AICD and regulatory T-cell suppression (20, 36). However, powerful costimulation raises a potential danger for cross-reactivity against normal...
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Figure 5. Trans-signaling CAR-T cells exert superior antitumor effector functions in vivo compared with first-generation CAR-T cells. A, in vivo inhibition of large preestablished tumors by M-z/F-28 CAR-T cells: effect of the CD28 costimulatory signaling domain in trans. NSG mice bearing established subcutaneous tumors were treated with 2 intravenous injections of 7.5 × 10^6 M-z, M-z/F-28, or M-28z CAR-T cells or control anti-CD19-28z and GFP T cells or saline on days 55 and 59 posttumor inoculation (arrows). Tumor growth was assessed by caliper measurement. Mean tumor volume (mm^3 ± SEM) is shown with n = 5 for all groups. **P < 0.05 comparing M-z CAR-T–treated mice with M-z/F-28 or M-28z CAR-T–treated mice. B, A1847 fluc + bioluminescence signal is similar in M-z/F-28 and M-28z CAR-T–treated mice and less than in M-z–treated mice 4 weeks after first T-cell dose. Control groups show no decrement in the bioluminescence signal. C, stable persistence of CD28 cis- or trans-costimulated CAR-T cells in vivo. Peripheral blood was collected 2 weeks after the first T-cell infusion and quantified for the absolute number of human CD4^+ and CD8^+ T cells/μL of blood. Mean cell count ± SEM is shown with n = 5 for all groups. **P < 0.05 comparing M-z CAR-T–treated mice with M-z/F-28 CAR-T–treated mice.

One approach to forestall this problem involves the physical separation of the signal 1 module (CD3ζ) from the signal 2 module (costimulation) through their incorporation into 2 distinct CARs specific for 2 different antigens, to recapitulate natural T-cell biology and function. In this way, dual CAR-T cells may selectively traffic, survive, and exert sustained proliferation within the tumor microenvironment, as synergistic signals would be delivered to T-cells preferentially at that location. Hence, the potential for “on-target” toxicity should be reduced commensurately. Indeed, CARs can be engineered to provide costimulation alone (37). For example, Jurkat cells engineered to coexpress hapten-specific CD3ζ- and CD28-based CARs, triggered complementary signaling leading to IL-2 production (38). So far, early attempts to preferentially redirect CAR-T cells against tumors expressing multiple antigens to limit potential toxicity were not successful enough to hold promise for clinical application. In one study, CAR-T were outfitted with 2 first-generation CARs specific for ErbB2 and FRα to increase specificity for tumor. These dual-transduced CAR-T cells expressed similar amounts of total surface CARs as monotransduced T cells, that is each individual CAR was expressed at a lower level (39). Thus, the dual-transduced T cells cross-reacted considerably less avidly with target cells expressing only a single antigen than with tumor cells expressing both antigens. Because these CAR-T cells exhibited no costimulation activity due to the lack of any costimulatory domains in either of the CARs, they were unlikely to survive, persist, and clear tumors in vivo. Another recent study proposed dual targeting of ErbB2 and MUC1 in breast cancer by generating dual CAR-T cells transduced with both a CD28 containing MUC1 CAR and a CD3ζ containing ErbB2 CAR (40). These dual CAR-T cells were even less efficient than first-generation CAR-T cells and failed to secrete IL-2 in response to trans-signaled costimulation upon encounter with a second antigen, thus rendering the system unsuitable for further preclinical investigation.

Here, we used 2 distinct CARs, redirected against mesothelin and FRα, to test the concept of combinatorial CAR signaling for highly selective antitumor activity. The anti-mesothelin CAR contained the CD3ζ signaling motif alone, whereas the anti-FRα CAR included only the intracellular domain of the CD28 costimulatory molecule. Proper costimulation of these engineered T cells relies upon the coexpression of both FRα and mesothelin on the tumor cell surface. In comparisons between trans-signaling CAR-T cells with conventional first- and second-generation anti-mesothelin CAR-T cells, trans-signaling CAR-T cells showed similar in vitro potency to cis-signaling CAR-T cells and were capable of producing increased levels of T-helper cell (Th1) cytokines
compared with first-generation CAR-T cells. Unlike the findings of Wilkie and colleagues (40), tumor-induced costimulation through the anti-FRa-28 CAR triggered enhanced secretion of IL-2, a cytokine known to promote T-cell proliferation and persistence in vivo (41, 42). Furthermore, costimulation delivered in trans was sufficient to protect those CAR-T cells from AICD, similar to the effects seen in cis-signaling CAR-T cells (9, 24). The cytolytic potential of trans-signaling CAR-T cells in vitro was similar to first and second-generation CAR-T cells. This is consistent with previous studies showing no statistically significant difference in specific tumor lysis by CAR-T cells that include or lack incorporated costimulatory domains (11, 20, 43).

The significance of a trans-signaling CAR approach is best tested in preclinical models where the targeting of both human tumor cells and representative normal tissue cells by CAR-T cells can be evaluated simultaneously. Ovarian tumors generally overexpress both FRα (90%) and mesothelin (70%), rendering ovarian cancer an attractive study paradigm (17, 44). Moreover, the pattern of mesothelin and FRα expression on normal tissues is largely nonoverlapping. Mesothelin is expressed by mesothelial cells lining the pleura and peritoneum and at low levels by epithelial cells of the trachea, tonsils, fallopian tube, and the testis (14, 15). FRα expression is limited to the apical surface of the kidney proximal tubules, choroid plexus, lung epithelium, thyroid, and intestinal brush border epithelial cells (45, 46). In our study, in vivo antitumor activity of trans-signaling CAR-T cells was initially tested by adoptively transferring the different CAR-T cell populations into immunodeficient mice with established ovarian cancer, using the A1847 human ovarian cancer cell line expressing both mesothelin and FRα. Trans-signaling CAR-T cells exhibited potent antitumor activity against ovarian cancer in vivo, equivalent to that achieved using conventional cis-signaling CAR-T cells. More importantly, the finding that trans-signaling CAR-T cells are more selective, potent, and localized within cancers furnished with both antigens, but spare normal cells bearing a single antigen, may have important translational ramifications. Indeed, tumor localization seems to be a key factor for...
were counted in 10 randomly selected intratumoral
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Wilkie and colleagues (40) and were rationalized by the
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eral regression of tumors was observed in our model using
CAR-T cells which receive combined CD3
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CAR-T cells into the tumor sites where both antigens were
ticantly higher in
z/F-28 CAR-T cells. This was further supported by the statis-
the control of tumor bearing 2 antigens versus the more rapid
preclinical model where there was a signi-
tible to AICD or drive them into anergy (24, 36, 48) and
antigen contact. This may render the designer T cells suscep-
lin alone, trans-signaling CAR-T cells receive only signal 1 upon
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cking to the normal tissues expressing mesothe-

findings extend beyond the
in vitro
study of
Wilkie and colleagues (40) and were rationalized by the finding
that our dual CAR-T cells are more favorable effector cells for
adoptive cell transfer than first-generation CAR-T cells due to
their resistance to AICD and ability to secrete elevated levels of
T_h2 cytokine including IL-2 in response to trans-signalized
costimulation upon encounter with a second antigen. Since
the submission of our article, an additional study has now
confirmed these enhanced effector properties of dual signaling
CAR-T cells upon secondary antigen encounter in
vivo
(49).
Using CARs specific for prostate-specific membrane antigen
and prostate stem cell antigen, this report con-
fi-
ted to better de-

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Figure 7. Trans-signaling CAR-T cells preferentially localize to dual antigen-expressing tumors in vivo. A, NSG mice with subcutaneously established A1847 tumors expressing or not FRα in opposite flanks received intravenous injections of 7.5 × 10⁶ CAR-T cells expressing CD19-28z (top), M-z/F-28 (middle), or M-28z (bottom) on days 45 and 49 posttumor inoculation. Mice were euthanized after 4 weeks, and tumors were collected and stained for human CD3 expression (brown) or isotype control. Representative sections for M
M-z/F-28 (middle), or M-28z (bottom) on days 45 and 49 posttumor inoculation. Mice were euthanized after 4 weeks, and tumors were collected and

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of the potential side effects of nontumor cell recognition by CAR-T cells can be overcome by the coexpression of conditional suicide genes such as incorporation of HSV-TK. The cytoplasmic domain of Fas, or an inducible caspase into gene-engineered T cells to abort aberrant T-cell responses (30–33). Indeed, the iCas9 cell-suicide system has been shown to increase the safety of cellular therapies in patients that received T cells depleted of alloreactive progenitor cells (34). Alternatively, electroporation of T cells with optimized RNAs for transient CAR expression has been effective in preclinical mouse models and might bypass the associated safety concerns of integrating gene vectors (50). These approaches toward cell product safety are not mutually exclusive and future application of dual CAR-T cells engineered in combination with suicide switches as described earlier may better permit preferential CAR-T cell accumulation and activity within the tumor microenvironment and in a safe manner.

Disclosure of Potential Conflicts of Interest
C.H. June has ownership interests (including patents) in Novartis, which has licensed inventions related to this research, and he may receive royalties from inventions licensed to Novartis. No potential conflicts of interest were disclosed by the other authors.

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domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. Mol Ther 2009;17:1453–64.


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