CD27 costimulation augments the survival and anti-tumor activity of redirected human T cells in vivo

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Abstract

The costimulatory effects of CD27 on T lymphocyte effector function and memory formation has been confined to evaluations in mouse models, in vitro human cell culture systems and clinical observations. Here, we tested whether CD27 costimulation actively enhances human T cell function, expansion and survival in vitro and in vivo. Human T cells transduced to express an antigen-specific chimeric antigen receptor (CAR-T) containing an intracellular CD3 zeta (CD3ζ) chain signaling module with the CD27 costimulatory motif in tandem exerted increased antigen-stimulated effector functions in vitro, including cytokine secretion and cytotoxicity, compared to CAR-T with CD3ζ alone. After antigen stimulation in vitro, CD27 bearing CAR-T cells also proliferated, upregulated Bcl-XL protein expression, resisted apoptosis and underwent increased numerical expansion. The greatest impact of CD27 was noted in vivo where transferred CAR-T cells with CD27 demonstrated heightened persistence after infusion, facilitating improved regression of human cancer in a xenogeneic allograft model. This tumor regression was similar to that achieved with CD28 or 4-1BB costimulated CARs, and heightened persistence was similar to 4-1BB, but higher than CD28. Thus, CD27 costimulation enhances expansion, effector function and survival of human CAR-T cells in vitro and augments human T cell persistence and anti-tumor activity in vivo.
Introduction

The genetic engineering of T cells to express chimeric antigen receptors (CAR) has emerged as an attractive “off-the-shelf” approach for generating tumor antigen-specific T cells for adoptive immunotherapy. CARs couple the high affinity binding of antibodies with the signaling domains of the T cell receptor (TCR) CD3ζ chain for antigen-specific triggering of T cell activation similar to the endogenous TCR\(^1\). In early phase I/II trials, adoptive transfer of CAR redirected T cells (CAR-T) did not induce tumor regression due to the poor persistence of the gene-modified T cells in vivo\(^2,3\), consistent with the finding that persistence of tumor antigen-specific T cells after adoptive transfer correlates with tumor regression in patients with advanced metastatic cancer\(^4\). The addition of costimulatory domains, including the intracellular domain of CD28 and tumor necrosis factor receptor (TNFR) family members, CD134 (OX-40), inducible costimulator (ICOS) and CD137 (4-1BB), into CARs can significantly augment the ability of these receptors to stimulate cytokine secretion, promote T cell survival and enhance net antitumor efficacy in preclinical animal models of solid tumors and leukemia that lack cognate costimulatory ligands\(^5-12\). Costimulated CAR therapy is now showing promising clinical results in lymphoma\(^13,14\).

CD27 (also known as TNFRSF7) is a member of the TNFR superfamily whose expression is constitutive by human CD8 and CD4 T cells, NKT cells, NK cell subsets and haematopoietic progenitors and induced in FOXP3+ CD4 T cells and B cell subsets. Its ligand, CD70, is transiently expressed by activated T and B lymphocytes and mature dendritic cells. CD27 signaling plays a costimulatory role in T cell activation yet appears to be dispensable for immune development since CD27/- mice are immunocompetent, lack overt autoimmune symptoms and respond to viral infection, however, T cell memory responses in these mice are impaired in formation, kinetics of response and
cell number, which has been attributed to the impact of CD27 on activated T cell survival\textsuperscript{15,16}. Similarly, T cell priming in the absence of CD70 interaction with CD27 results in abortive clonal expansion, dysfunctional antitumor responses, and no CD8+ T-cell memory\textsuperscript{17}. The role of CD27 signaling in human T cell activation, survival and memory formation is less clear. In patients responding to adoptive T cell therapy, the absolute number of CD27+ antigen-specific T cells that persist in the blood after infusion is remarkably stable\textsuperscript{18}, implying that the CD27 subset present in the early aftermath of infusion is the precursor to the emergent memory pool. Response to adoptive T cell therapy is also associated with the transfer of high numbers of T cells expressing CD27 costimulatory receptor\textsuperscript{19}. Whether CD27 costimulatory signaling facilitates improved human T cell survival, memory formation and anti-tumor response \textit{in vivo}, or is a functionally dispensable marker for the evolving human T cell memory pool is not known.

To investigate the functional role of CD27 in human T cell memory development and anti-tumor function \textit{in vivo}, we took advantage of the CAR approach and gene transfer technology to develop human T cells bearing a tumor antigen-specific scFv-based CAR fused with an intracellular CD3\(\zeta\) signaling domain alone or in tandem with a CD27 signaling domain. We tested whether addition of CD27 signaling to CAR-T cells promoted antigen-induced proliferation, anti-apoptosis Bcl-X\(L\) protein expression, proinflammatory cytokine production and cytolytic functions \textit{in vitro}, and evaluated whether transfer of CD27-containing CAR-T cells into tumor bearing mice resulted in improved human T cell survival and anti-tumor efficacy in a xenogeneic model of human cancer. We further compared CD27 with CD28 and CD137 (4-1BB) costimulation in CAR-T \textit{in vivo}. Our results support the notion that CD27 promotes primary human T cell survival \textit{in vivo} and serve to rationalize the incorporation of CD27 costimulatory modules in the creation of antigen-specific CAR-T therapy.
Materials and Methods

Chimeric antigen receptor construction. CAR backbone constructs were generated as previously described. The anti-FR scFv sequence was derived from MOv19, a monoclonal antibody directed against FR. MOv19 scFv was amplified using the following primers:

5’-GCAGGATCTCTAGAGCGGCCCAGCCGGCCATGGCCCAGGTG-3’ (Bam-HI underlined)

and 5’-GCGGCTAGCGGCCGCCGTTTTATTTTCTTAACATTTGTCCCCCC-3’ (Nhe-I underlined)

and cloned into the CAR backbone vector. The CD27 (amino acids 213-260) or CD314 (NKG2D, amino acids 1-51) intracellular domain was amplified from cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems) from total RNA isolated from activated T cells by Trizol method and fused to a CD8α hinge-TM and CD3ζ fragment via overlap extension PCR. The primer sequences for FR-27z CAR construction is summarized in Table 1. The insert was then digested with Xba-I and Sal-I and ligated into pELNS, a third generation self-inactivating lentiviral expression vector, based on pRRL-SIN-CMV-eGFP-WPRE, containing the EF-1α promoter. FR-BBz CAR constructs were described previously, as was the -28z CAR backbone into which MOv19 was cloned. The CD19-27z CAR was constructed as above using a CD19 CAR template provided by Michael Milone (University of Pennsylvania). High-titer replication-defective lentiviral vectors were produced and concentrated as previously described.

T Cells. Primary human CD4+ and CD8+ T cells were isolated from healthy volunteer donors following leukapheresis by negative selection, and purchased from the Human Immunology Core at University of Pennsylvania. All specimens were collected under a University Institutional Review Board-approved protocol, and written informed consent was obtained from each donor in accordance with the Declaration of Helsinki. T cells were cultured in complete media (RPMI 1640 supplemented
with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 ug/ml streptomycin sulfate, 10-mM HEPES), and stimulated with anti-CD3 and anti-CD28 mAbs coated beads (Invitrogen) as described \(^27\). 12-24hr after activation, cells were transduced with lentiviral vectors at MOI of ~5-10. CD4+ and CD8+ T cells used for in vivo experiments were mixed at 1:1 ratio, activated, and transduced. Human recombinant interleukin-2 (IL-2; Novartis) was added every other day to 50 IU/ml final concentration and a 0.5-1x10^6 cells/ml cell density was maintained. Once T cells rested down, ~14 days after stimulation and determined by decreased growth kinetics and cell size (~250 femtoliters) using the Multisizer3 Coulter Counter (Beckman Coulter), engineered cells were adjusted to equalize the frequency of transgene expressing cells and level of surface CAR expression (MFI) prior to functional assays.

**Cell lines.** Lentivirus packaging was performed in the immortalized normal fetal renal 293T cell line purchased from ATCC. Mycoplasma-free human cancer cell lines used in immune based assays include the established ovarian cancer cell lines SKOV3, OVCAR8, OVCAR4, A1847, OVCAR3, OVCAR5, C30, PEO-1 and breast cancer cell lines SKBR3, MCF7, MDA-231, MDA-468. For bioluminescence assays, target cancer cell lines were transduced to express firefly luciferase (fLuc) with lentivirus and validated for positive expression by bioluminescence imaging. The mouse malignant mesothelioma cell line, AE17 (provided by Steven Albelda, University of Pennsylvania), was transduced to express FR (AE17.FR) with lentivirus. CD19-expressing K562 (CD19+K562) cells, a human erythroleukemic cell line, were provided by Michael Milone (University of Pennsylvania).

**Cytokine release assays.** Cytokine release assays were performed by coculture of 10^5 T cells with 10^5 target cells per well in triplicate in 96-well round bottom plates in a 200ul volume of complete media. After ~24hr, co-culture supernatants were assayed for presence of IFN-γ using an ELISA Kit.
Values represent the mean of triplicate wells. IL-2, IL-4, IL-10, IFN-γ and TNF-α cytokines were measured by flow cytometry using Cytometric Bead Array (BD Biosciences).

**Cytotoxicity Assays.** For the cell based bioluminescence assay, 5x10⁴ firefly Luciferase expressing (fLuc+) tumor cells were cultured with complete media in the presence of different ratios of transduced T cells using a 96-well Microplate (BD Biosciences). After incubation for ~20hrs at 37°C, each well was filled with 50ul DPBS resuspended with 1ul D-luciferin (0.015 g/ml) and imaged using a Xenogen IVIS Spectrum. Percent tumor cell viability was calculated as the mean luminescence of the experimental sample minus background divided by the mean luminescence of the input number of target cells used in the assay minus background times 100. All data are represented as a mean of triplicate wells. For extended-duration killing assays, tumor cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFDASE, CFSE; Molecular Probes, Inc., Eugene, OR) and then cultured with T cells as above. FACS analysis was performed using forward and side scatter gating to determine viable cells, while CFSE and CD3 staining was used to distinguish T cells from unlabeled tumor cells.

**Flow cytometric analysis.** The following mAbs were used for phenotypic analysis: APC-Cy7 Mouse Anti-Human CD3; FITC-anti-human CD4; APC-anti-human CD8; PE-anti-human CD45; FITC-anti-human CD45RO; PE-anti-human CD62L; PE-anti-human CD27; FITC-anti-human CD28 (BD Biosciences). In transfer experiments, blood was obtained via retro-orbital bleeding and stained for the presence of human CD45, CD4, and CD8 T cells. Human CD45+ gated, CD4+ and CD8+ subsets were quantified using TruCount tubes (BD Biosciences) per manufacturer’s instructions. Spleens pooled from each group were homogenized in RPMI cell culture medium. RBCs were lysed by adding 1xFACS Lysing Solution (BD Biosciences). Cells were washed in FACS buffer (1X PBS, 2% FCS). 1x10⁶ cells per sample were analyzed for transferred T cells phenotype.
Tumor cell surface expression of FR was detected by Mov18/ZEL antibody (Enzo Life Sciences). CD70 (CD27 ligand) expression on SKOV3 cells was detected by FITC-anti-human CD70 antibody (BD Biosciences). CAR expression was detected by biotin-labeled Protein L (GeneScript, cat. number M00097) at 100ng per 10^6 cells followed by incubation with APC-conjugated streptavidin or PE-conjugated goat anti-mouse IgG F(ab’)2 (Jackson ImmunoResearch). PE-conjugated anti-Bcl-XL antibody was purchased from Southern Biotech. Isotype matched control Abs were used in all analyses. Flow cytometric data were analyzed by FlowJo software.

**T cell proliferation, expansion and apoptosis.** For cell division, 2x10^7 T cells in PBS were labeled with 2.5 μM CFSE for 5 minutes at room temperature. Labeled cells were co-cultured with tumor cells at 2:1 ratio in the absence of exogenous IL-2. After 5 days, cells were stained with CD3, and analyzed for CFSE dilution by FACS analysis. For expansion, viable T cells were co-cultured with tumor cells at 1:1 ratio in the absence of IL-2, and T cell counts were determined by the Multisizer 3 Coulter Counter (Beckman Coulter) and Trypan blue exclusion on day 5 and 10. For apoptosis assays, T cells were co-cultured with tumor cells at 2:1 ratio in the absence of IL-2. After 3 days, T cells were detected by simultaneous staining with anti-CD3 antibody, Annexin V-FITC(AV) and 7-AAD-PerCP-Cy5.5, according to the manufacturer’s instructions (BD Pharmingen).

**Xenograft model of ovarian cancer.** Animals were obtained from the Stem Cell and Xenograft Core of the Abramson Cancer Center, University of Pennsylvania. Eight to 12-week-old NOD/SCID/γ-chain-/- (NSG) mice were bred, treated and maintained under pathogen-free conditions in-house under University of Pennsylvania IACUC approved protocols. Female NSG mice were inoculated s.c. with 3x10^6 SKOV3 fluc+ cells on the flank on day 0. Five mice were randomized per group prior to treatment. After tumors became palpable, human primary T cells (CD4+ and CD8+ cells
mixed at 1:1 ratio) were activated, and transduced as described above. After 2 weeks expansion, when tumor burden was ~250-300 mm³, mice were injected with 10⁷ CAR-T cells (~2×10⁷ at 50% CAR+) intratumorally or intravenously and again 5-7 days later. Tumor dimensions were measured with calipers, and tumor volumes calculated using the formula \( V = \frac{1}{2}(\text{length} \times \text{width}^2) \), where length is greatest longitudinal diameter and width is greatest transverse diameter. Animals were imaged prior to T cell transfer and about every two weeks thereafter. Tumors were resected immediately after euthanasia 25 days after first T cell dose for size measurement.

**Bioluminescence imaging.** Imaging of tumor was performed using Xenogen IVIS imaging system and the photons emitted from fLuc+ cells within the animal body were quantified using Living Image software (Xenogen). Briefly, mice bearing SKOV3 fLuc+ tumor cells were injected intraperitoneally with D-luciferin (150 mg/kg stock, 100 µL of D-luciferin per 10 grams of mouse body weight) suspended in PBS and imaged under isoflurane anesthesia after ~10 minutes. Pseudocolor images representing light intensity (blue, least intense; red, most intense) were generated using Living Image. Imaging findings were confirmed at necropsy.

**Statistical analysis.** The data are reported as means and standard deviations (SD). Statistical analysis was performed using two-way repeated measures ANOVA for the tumor burden (tumor volume, photon counts). Student’s \( t \) test was used to evaluate differences in absolute numbers of transferred T cells, cytokine secretion and specific cytolysis. GraphPad Prism 4.0 (GraphPad Software) was used for the statistical calculations. \( P < 0.05 \) was considered significant.
Results

**CAR construction and T cell transduction.** FR-specific CARs were constructed containing the mouse anti-human scFv MoV19, which has high affinity for FR $\left(10^{-8}$-$10^{-9}$ M$^{-1}\right)$. Folate receptor-alpha (FR) is a glycosylphosphatidylinositol-anchored protein that is overexpressed on the surface of cancer cells in a wide array of epithelial malignancies, including ovarian, lung, breast, colorectal, renal, and other solid cancers but limited in normal tissues. FR CAR constructs were comprised of the MoV19 scFv linked to a CD8α hinge and transmembrane region, followed by a CD3ζ signaling moiety alone (FR-z) or in tandem with the CD27 intracellular signaling motif (FR-27z; Figure 1A). A CD19-specific CAR containing CD3ζ and CD27 signaling motifs in tandem (CD19-27z) was constructed to control for antigen specificity. CAR constructs were subcloned into the pELNS lentiviral vector where transgene expression is driven off the EF-1α promoter. Using gene transfer technology established for clinical application, lentiviral vectors efficiently transduced primary human T cells to express anti-FR or CD19 CARs (Figure 1B). Transduced cells showed a CD45RO+ CD62L+ CD28+ CD27+ central memory-like phenotype (Supplemental Figure S1A). For *in vitro* assays, whole CD3+ cells or enriched CD8+ or CD4+ T cells were transduced independently with CAR. Reproducibly, the efficiency of cell transduction with CAR was high (>70%), as assessed by flow cytometry, with transduction efficiency with FR-z CAR being identical or slightly higher than with FR-27z CAR. The frequency of CAR-expressing T cells was equalized prior to all functional assays.

**CAR T cells with CD27 signaling exert enhanced antigen specific reactivity in vitro.** Ovarian and breast cancers frequently express FR. A panel of established human ovarian and breast cancer cell lines that express surface FR at varying levels was selected for assays (Figure 2A). Flow cytometry results confirmed that ovarian cancer lines SKOV3, A1847, OVCAR8, OVCAR4, OVCAR3 and
OVCAR5 and breast cancer cell lines MCF7, MDA231 and MDA468 all expressed surface FR protein, as did control lines AE17.FR (a mouse mesothelioma cell line engineered to express human FR) and CD19+ K562 (a human erythromyeloblastoid leukemia cell line engineered to express human CD19). Two ovarian cancer lines, C30 and PEO-1, were negative for FR, as were the parental AE17 line and the 624 melanoma line.

To evaluate the impact of CD27 costimulation on antigen-specific antitumor function of CAR-T cells in vitro, engineered human T cells and cancer cells were co-cultured and T cell reactivity measured by proinflammatory cytokine secretion. FR-z or FR-27z CAR-T cells recognized FR-pos ovarian and breast cancer lines and secreted high levels of IFN-γ, which was associated with the level of FR expression by tumor cells, but not when stimulated with FR-negative cell lines (Figure 2B). Invariably, increased quantities of secreted IFN-γ were detected when FR-27z CAR-T cells where stimulated with the various FR-pos target cells, relative to FR-z CAR-T cells. FR-27z and FR-z CARs functioned in both primary human CD4+ and CD8+ T cells, with CD8+ T cells producing more Th-1 type cytokines than CD4+ T cells (Supplementary Figure S2). CD8+ FR-27z CAR-T cells also secreted higher levels of TNF-α and IL-2 than FR-z CAR-T cells when stimulated with FR-pos cancer cells (Supplementary Figure S2). CD4+ and CD8+ CAR-T cells produced low levels of IL-4 and IL-10, which were uniformly lower in FR-27z CAR-T cells compared to FR-z, indicating that CD27 signaling reinforces the Th-1 response. When the CD27 domain was replaced by the CD314 endodomain, which is similar in length but has no intrinsic signaling capacity, FR-314z CAR-T cells failed to increase IFN-γ secretion after co-culture with FR+ SKOV3 cells, compared to FR-z (Supplemental Figure S3), confirming that the costimulatory CAR effects are not due to simple extension of the intracellular domain. CD19-27z CAR-T cells did not produce IFN-γ, except when
co-incubated with CD19+ K562 cells, and untransduced T cells did not secrete cytokine when
stimulated with FR\textsuperscript{pos} cancer cells, illustrating the requirement for antigen specificity for CAR-T cell
activity.

We next evaluated the antigen-specific cytolytic potential of FR-specific CD8+ CAR-T cells \textit{in vitro} using overnight luminescence and 5 day co-culture assays. CAR-T cells were co-cultured with
FR\textsuperscript{pos} SKOV3 and A1847 cancer cells, or FR\textsuperscript{neg} C30 and AE17 cells expressing firefly luciferase
(fLuc+) and assessed for bioluminescence after overnight culture. Representative luminescence results
shown in Figure 3A reveal that both FR-z and FR-27z CAR-T cells specifically eliminated FR\textsuperscript{pos}
A1847 ovarian cancer cells but not FR\textsuperscript{neg} C30. Untransduced or CD19-27z CAR-T cells did not lyse
ovarian cancer cells, but the latter did efficiently lyse CD19+ K562 cells (Supplemental Figure S4). FR-z and FR-27z CAR-T cells directly and efficiently lysed FR\textsuperscript{pos} human ovarian cancer cell lines
SKOV3 and A1847, but not FR\textsuperscript{neg} C30 or AE17 cell lines (Figure 3B). Similar to cytokine production
results, FR-27z CAR-T cells demonstrated enhanced cytotoxicity, particularly at high E/T ratios,
compared to FR-z CAR-T cells, but not at lower ratios (p=0.024). To assess the cytotoxic function of
CAR-T cells in extended culture, CAR-T cells were co-cultured with CFSE-labeled tumor cells for 5
days. Representative dot plots show that CFSE+ CD3-negative SKOV3 cancer cells, but not C30 cells,
were eliminated when cultured with FR-specific CAR-T cells but control T cells (Figure 3C). Co-cultures containing FR-specific CAR-T cells were dominated by CFSE-negative CD3+ human T
cells that had eliminated all FR\textsuperscript{pos} cancer cells tested (SKOV3, A1847, OVCAR4, AE17.FR) in
extended culture, but not FR\textsuperscript{neg} lines (Figure 3D).

\textbf{CD27 signaling improves the survival of antigen-stimulated CAR-T cells \textit{in vitro}.} To determine
the impact of CD27 costimulation on antigen-stimulated proliferation of redirected T cells,
CFSE-labeled CAR-T cells were co-cultured with FR<sup>pos</sup> SKOV3 or FR<sup>neg</sup> C30 cell lines, respectively. CFSE-labeled control CD3<sup>+</sup> T cells did not dilute CFSE or eliminate CFSE<sup>neg</sup> CD3<sup>neg</sup> tumor cells after 72hrs incubation (Figure 4A). However, FR-z and FR-27z CAR-T cells both proliferated, and to a similar extent, in response to FR<sup>pos</sup> tumor stimulation as measured by CFSE dilution and eliminated the tumor cell fraction, in agreement with above results (Figure 3D). Since T cell division does not adequately measure the extent of overall cell expansion, which encompasses both cell proliferation and apoptosis, viable cell counting was performed. In response to FR<sup>pos</sup> tumor stimulation, FR-27z CAR-T cells underwent increased numerical expansion after ten days, compared to FR-z CAR-T cells, suggesting a survival advantage for cells receiving CD27 costimulation (Figure 4B). Non-specific stimulation with FR<sup>neg</sup> tumor cells did not induce substantial cell expansion. CD27 signaling can induce expression of Bcl-X<sub>L</sub><sup>40,41</sup>, an anti-apoptotic protein of the Bcl-2 family. When stimulated with FR<sup>pos</sup> tumor cells, more FR-27z CAR-T cells (~20% of CAR+) expressed Bcl-X<sub>L</sub> compared to FR-z (~2.4%) and CD19-27z CAR-T cells (~0.9%) (Figure 4C). Bcl-X<sub>L</sub> upregulation was restricted to the CAR+ T cell population, confirming dependence on antigen-specific stimulation. Consistent with these findings, more viable (7-AAD<sup>neg</sup> AnnexinV<sup>neg</sup>) and fewer early apoptotic (7-AAD<sup>neg</sup> AnnexinV<sup>pos</sup>) and late apoptotic (7-AAD<sup>pos</sup> AnnexinV<sup>pos</sup>) T cells were detected in co-cultures of FR-27z CAR-T cells and FR<sup>pos</sup> SKOV3 tumor cells, compared to FR-z CAR-T cell cultures where apoptosis was more prevalent (Figure 4D).

**CD27 promotes CAR-T cell survival and anti-tumor function in vivo.** Addition of CD28 or CD137(4-1BB) endodomains to CARs can improve CAR-T cell persistence and anti-tumor function in vivo<sup>5,25,42</sup>. To test and compare CD27 to these domains, we generated MOv19-based FR-28z and FR-BBz CARs<sup>25</sup>. Transduced human CD4<sup>+</sup> and CD8<sup>+</sup> T cells efficiently expressed the various
FR-specific CAR constructs (Figure 5A). CAR-T cells bearing -z, -27z, -28z or -BBz endodomains directly and efficiently lysed FR<sup>pos</sup> SKOV3 cells in vitro, but not FR<sup>neg</sup> C30 (Figure 5B). Next, immunodeficient NOD/SCID/IL-2R<sub>γc</sub><sup>γcnull</sup> (NSG) mice were inoculated subcutaneously with fLuc+ FR<sup>pos</sup> SKOV3 human ovarian cancer cells on the hind flank and received intravenous injections of 10<sup>7</sup> CAR+ T cells on days 40 and 45 post-tumor inoculation (p.i.), when tumors were ~250 mm<sup>3</sup> in size and evident by bioluminescence imaging. Transfer of FR-27z CAR-T cells by intravenous or intratumoral administration resulted in rapid regression of established human tumor (Figure 5C,D and Supplemental Figure S5A; <i>P</i>&lt;0.0001), which was superior to first generation FR-z CAR therapy. FR-27z CAR–mediated regression was similar to that achieved using FR-28z and FR-BBz CAR-T cells.

Although SKOV3 cells do express low levels of CD70, the CD27 ligand, FR-z CAR-T cells that expressed endogenous cell surface CD27 at the time of cell infusion had only a modest impact on tumor outgrowth, compared to animals receiving untransduced T cells (<i>P</i>=0.015), and did not express surface CD27 three weeks after transfer (Supplemental Figure 1A). While these observations do not define the role for endogenous CD27 in this system, it does demonstrate that enforced expression of CD27 in CAR-T cells augments their anti-tumor function in vivo.

Significantly higher numbers of circulating human CD4<sup>+</sup> and CD8<sup>+</sup> T cells were observed in tumor-treated mice receiving either FR-27z or FR-BBz CAR-T cells three weeks after the first T cell dose, compared to FR-z and FR-28z CAR groups (Figure 5D; <i>p</i>&lt;0.05). Improved engraftment of FR-27z CAR-T cells required both antigen-specific stimulation and CD27 costimulation. Persistence was not a product of constitutive CD27 activity since CD19-27z CAR-T cells did not engraft or treat tumor (Supplemental Figures S6,7). Further, cognate antigen recognition reinforces the persistence of CAR-T cells in vivo, since mice that received FR-z CAR-T cells had increased CAR+ T cell counts
compared to mice in CD19-27z CAR-T (P=0.017) or gfp T cell groups, which had little to no detectable persistence of CAR+ T cells, respectively (Supplemental Figure 6).

Notably, surface CAR expression was better maintained in the FR-28z group (Figure 5F), compared to other groups (p<0.01), consistent with prior findings. As such, the absolute number of circulating CAR+ T cells in tumor-bearing mice administered FR-27z, FR-28z and FR-BBz were not statistically different (p>0.05), but higher than that observed in mice receiving first generation FR-z CAR-T cells (Supplemental Figures S5B).
Discussion

In this study, we elucidate a role for CD27 costimulatory function in promoting the survival of adoptively transferred human T cells in vivo and enhancing the anti-tumor efficacy of chimeric antigen receptor (CAR) therapy. Primary human T cells expressing a FR-specific CAR, outfitted with intracellular TCR CD3ζ and a CD27 costimulatory signaling domain in tandem, recognized and killed FR-expressing cancer cell lines in vitro and demonstrated improved antigen-dependent memory formation and anti-tumor potency in vivo in a mouse xenograft model of advanced cancer.

In our study, the role of CD27 costimulatory signaling in T cell effector proliferation, activity and survival was evaluated via incorporation of the CD27 receptor endodomain into the intracellular signaling portion of a FR-specific CAR-z construct. Addition of the CD27 signaling domain to CAR supported significant improvement in tumor antigen-specific T cell reactivity in vitro, relative to first generation FR-z CAR. Consistently, the production of proinflammatory cytokines such as IFN-γ by FR-27z CART cells was substantially increased after co-culture with all FRpos ovarian and breast cancer cell lines, compared to FR-z T cells. Interestingly, the levels of cytokine secretion measured were associated with the expression of surface FR expression by target cancer cells for both FR-27z and FR-z CART cells. In overnight killing assays, provision of CD27 costimulation to CAR resulted in marginally increased cytolytic function, however, with extended co-culture both FR-27z and FR-z CARs induced complete tumor killing with no viable FR-expressing tumor cells detected. Mechanisms accounting for increased effector function by CD27 costimulated CART cells in vitro is not known but appears linked in part to their ability to resist antigen-induced cell death, and suggested that CAR-T cells with CD27 costimulatory capacity might demonstrate improved function and therapeutic efficacy in vivo.
Using *in vitro* assay systems, CD27 has been established as a costimulator of human T cell responses \(^4^3\) however its importance for immune responses and memory formation *in vivo* have been primarily restricted to studies using mouse model systems. While CD27-/- mice are immunocompetent, intranasal infection with influenza virus revealed that CD27 costimulation regulates the survival and accumulation of virus specific T cells at the site of infection independent and complementary to CD28 \(^1^5;^1^6\). Moreover, T cell memory responses are reduced in CD27-/- mice upon secondary infection \(^1^5;^1^6\). Similar results have been observed in a model of CD8+ T cell-mediated cardiac allograft rejection where disruption of CD27-CD70 interactions contributes to reduced CD28-independent effector T cell accumulation, function and memory response \(^4^4\). Alternatively, constitutive CD27 costimulatory signaling in CD70 transgenic mice promotes virus- and cancer-specific CD8+ memory T cell formation by initially reducing effector phase contraction and supporting the maintenance of high numbers of antigen-specific T cells with increased effector function in the memory pool \(^4^5\). Notably, superior cancer-specific CD8+ memory formation induced by constitutive CD27 ligation can protect CD70 transgenic mice against lethal tumor dose challenge in an IFN-γ dependent manner \(^4^5\).

The intracytoplasmic domain of CD27 used in our study and triggered naturally by CD70 ligation mediates pro-survival effects through interaction with TRAF2 and activation of Jun kinase and NF-κB, which is required for optimal effector/memory CD8+ T cell generation \(^4^6\). The mechanism by which CD27 prevents activation-induced cell death and promotes T cell survival includes the upregulation of anti-apoptotic Bcl-X\(_L\) protein expression \(^4^1\) and resistance to Fas mediated apoptosis \(^4^7\). After stimulation *in vitro*, increased Bcl-X\(_L\) protein expression has been observed in T cells engineered to express a CD70-specific, chimeric CD27-CD3z CAR consisting of the both the extracellular and intracellular CD27 domains \(^4^0\). Consistent with this finding, we observed increased expression of Bcl-X\(_L\) protein in
FR-27z CAR-T cells following antigen stimulation, compared to FR-z alone. Moreover, incorporation of the CD27 intracellular signaling domain to CAR also reduced T cell apoptosis following antigen-induced activation and consequently increased numerical expansion of transduced T cells in vitro. These results suggested that provision of CD27 costimulatory signaling through CAR engagement with cognate antigen in vivo might improve T cell survival and numerical expansion, thereby significantly augmenting their potential for anti-tumor activity after adoptive transfer.

The role of CD27 on human T cell activity and memory formation in vivo has not been formally evaluated to date. We first reported that following adoptive transfer of heterogeneous tumor infiltrating lymphocytes (TILs) to patients with metastatic melanoma in vivo, a subpopulation of CD27+ tumor antigen-specific T cells was temporally selected for in vivo, suggesting that these cells possessed a function advantage in long-term survival and that CD27+ cells detectable in the blood in the early aftermath of adoptive transfer represented the precursors to the emerging memory pool18. These results were later reproduced at the clonal level for HIV-specific CD8+ T cells persisting after adoptive transfer48. Similarly, a population of CD27+ HIV-specific CD8+ T cells with memory characteristics and direct effector capabilities has been observed in vivo when viral replication is inhibited with antiretroviral therapy49. Consistent with these results, retrospective analysis of TILs administered to patients with melanoma revealed a significant association between the absolute number of administered CD27+ CD8+ TILs and the ability of those TILs to persist in vivo and mediate objective clinical response after transfer19. As T cell persistence after infusion is a major factor limiting adoptive cell therapy1, the elucidation of approaches to promote T cell survival has the potential to significantly improve response rates in adoptive immunotherapy.

The impact of CD27 costimulation on human T cell survival and anti-tumor activity was most
evident in vivo where CD27 bearing CAR-T cells facilitated superior regression of established tumors in a human ovarian cancer xenograft model, compared to the marginal inhibition achieved with conventional, non-costimulated CAR-T cells. Consistent with clinical observations\textsuperscript{4,50}, tumor regression in our model was associated with enhanced T cell persistence in vivo. Tumor regression and T cell persistence were antigen-specific as transfer of CD19-27z T cells had no impact on tumor progression and resulted in poor persistence after intratumoral infusion. Moreover, tumor regression and T cell persistence was increased in mice that received first generation FR-z CART cells, compared to costimulated CD19-27z CAR, demonstrating that provision of a CD27 costimulatory domain in the absence of antigen-specific ligation of the CAR does not substantially bolster T cell survival and effector function in vivo. In this line, the greatest number of CAR+ T cells persisting in the blood one month after the first T cell dose was observed in those animals administered FR-27z CAR-T cells followed by FR-z and then CD19-27z, indicating that simultaneous TCR CD3\textsubscript{ζ} signaling and CD27 costimulation triggered by CAR ligation with antigen improves upon TCR signaling alone, implicating a role for CD27 costimulation in memory T cell formation in vivo.

Our results support the notion that human T cells that are enabled to receive CD27 costimulatory signals in conjunction with CD3\textsubscript{ζ} signaling receive amplified anti-apoptotic signals that facilitate enhanced effector activity and better survival in vivo, assuring the generation of stable human T cell memory. The addition of costimulatory domains, particularly the intracellular domains of CD28, and the tumor necrosis factor receptor family members, CD134 and 4-1BB (CD137), can also significantly augment the ability of these receptors to stimulate cytokine secretion, support T cell survival and enhance antitumor efficacy in preclinical animal models using both solid tumors and leukemia that lack the expression of their cognate costimulatory ligands\textsuperscript{5,12}. Results of comparative in vivo studies of
CARs containing these various costimulatory domains demonstrated that CD137 (4-1BB), like CD27, another member of the TNFR superfamily enhances T cell antitumor activity and persistence. In contrast, CD28, which can support enhanced antitumor activity, does not support robust numerical T cell persistence. Rather, CD28 supports stable surface CAR expression, which may be attributed in part to the inclusion of its transmembrane region in the CAR construct, as suggested previously. Notably, each of these costimulatory molecules can dramatically improve the activity and overcomes the limitations of first generation CAR approaches by improving the persistence of transferred T cells in vivo. These results rationalize the clinical investigation of CD27 costimulated CAR-T cell therapy in combination with lymphodepleting pre-conditioning for the treatment of cancer. Our data further suggest that the combination of CD27 or CD137 endodomains with the CD28 signaling domain may be more effective than CD27, CD137 or CD28 alone.
Acknowledgements

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Authorship Contributions

DS, QY, MP and GH performed research. MF provided vital new reagent. DS collected data, and performed statistical analysis. DJP designed research and interpreted data. DS and DJP wrote the manuscript. The authors of this manuscript declare no conflict of interest.
References


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Note: TM: Transmembrane; ICD: intracellular domain; F: Forward; R: Reverse.
Restriction enzyme recognition sites are underlined.
**Figure Legends**

**Figure 1. CAR constructs and expression of CD27 containing FR CAR or CD19 CAR on primary human CD4 and CD8 T cells.** (A) Schematic representation of anti-FR MOv19 scFv based CAR constructs containing the CD3 zeta cytosolic domain alone (FR-z CAR) or in combination with CD27 intracellular domain (FR-27z CAR). CD19-27z CAR was also constructed and used as an antigen specific control. VL, variable light chain; L, Linker; VH, variable heavy chain; TM, transmembrane region. (B) Both primary human CD4 and CD8 T cells can efficiently express either FR-specific CARs or CD19-specific CAR as measured by flow cytometry. A viable CD3+ lymphocyte gating strategy was used.

**Figure 2. Enhanced immune recognition of tumor cells by CD27 bearing CAR T cells.** (A) FR surface expression on human ovarian cancer and breast cancer cell lines by flow cytometry. FR-specific mAb Mov18 was used to measure FR expression on tumor cell lines (open empty histogram), compared to a matched isotype Ab control (filled gray histogram). (B) Antigen-specific cytokine production by FR CAR-transduced T cells. IFN-γ secretion of FR-z and 27z CAR-transduced T cells following 20h coculture with the indicated tumor lines at a 1:1 ratio. Untransduced T cells (UNT) and CD19-27z CAR T cells were used as negative controls. Anti-CD3/28 beads stimulation served as positive control. CD19+K562 cell line was used as positive target control for CD19 CAR. Results are expressed as a mean and SD of triplicate wells from 1 of at least 3 separate experiments.

**Figure 3. Specific tumor cell lysis by CD27 costimulated FR-specific CAR T cells.** (A, B) CAR-transduced T cell lytic function was tested in bioluminescent killing assay using 96 well Microplate. FR CAR T cells killed FR+ SKOV3 and A1847 cells but showed no killing of the FR− C30 and AE17 cells at the indicated E:T ratio for ~20 h, where untransduced T cells (UNT) and CD19 CAR...
T cells served as negative controls. Results are expressed as a mean and SD of triplicate wells from 1 of at least 3 separate experiments. (C) T cells were co-cultured with CFSE-labeled tumor cells at a 2:1 ratio in the absence of exogenous IL-2. After 5 days of co-culture, cells were collected, stained with CD3, and analyzed for CFSE dilution by FACS analysis. Both FR-2 and 27z CAR-transduced T cells kill SKOV3 cells, as evidenced by the lack of CD3neg CFSEpos cells, but not C30 cells. Non-transduced T cells and CD19-27 CAR-transduced T cells did not kill ovarian cells. (D) In all extended coculture experiments, both FR-2 and 27z CAR-transduced T cells eliminated the CD3neg CFSEpos FRpos tumor cells (SKOV3, A1847, OVCAR4, and AE17.FR) but not FRneg tumor cells (C30 and AE17).

**Figure 4. CD27 costimulation enhances CAR T-cell survival and expansion in vitro.** (A) CFSE-labeled FR-2 and 27z CAR-transduced T cells undergo cellular division when cocultured for 5 days with FRpos SKOV3 cells but not the FRneg C30 cells. Untransduced T cells (UNT) and CD19-27z CAR T cells did not dilute CFSE when cocultured with either FRpos SKOV3 cells or FRneg C30 cells. (B) FR CAR T cell expansion was measured in response to stimulation with FRpos target cells at a 1:2 ratio in the absence of exogenous IL-2. Viable (trypan blue exclusion) cell counting results showed that FR-27z CAR T cells underwent increased numerical expansion after 10 days, compared to FR-2 CAR T cells. Counts reflect mean ± SD for triplicate cultures of two independent assays. (C) Bcl-XL expression by FR- or CD19-specific CAR CD8 T was examined after 3 days of coculture with SKOV3 or C30 cells. Bcl-XL expression was preferentially increased in FR-27z CAR T cells populations, compared with FR-2 CAR+ T cells after stimulation with FRpos tumor cells. One representative FACS analysis is shown (n=3). (D) CD27 costimulation protects against antigen-induced cell death. Apoptotic T cells were detected by simultaneous staining with Annexin V-FITC(AV) and 7-AAD-PerCP-cy5.5 at 72h after indicated tumor cell stimulation. Apoptosis was quantified in percentages of living
(AV+/7AAD−), early apoptotic (AV+/7AAD−), late apoptotic (AV+/7AAD+), and necrotic (AV−/7AAD+) cells. One representative experiment of three is shown.

**Figure 5. Comparison of FR-specific CARs with CD27, CD28 and 4-1BB costimulatory endodomain.** (A) FR-specific CAR expression on primary human T cells. Shown, CD8+ T cells can efficiently express FR-specific CARs with or without CD27, CD28 and 4-1BB costimulatory endodomains as measured by flow cytometry. (B) CAR-transduced T cell show lytic function in a bioluminescent killing assay. FR CAR-T cells killed FR+ SKOV3 but did not kill FR-negative C30 cells at the indicated E:T ratio over ~20 h. Untransduced T cells (UNT) served as negative controls. Mean and SD of triplicate wells from 1 of at least 3 independent experiments is shown. (C) Bioluminescence images show fLuc+ SKOV3 tumors in NSG mice immediately prior to and three weeks after first intravenous injection of 10 x 10⁶ CAR-T cells on days 40 and 45 after tumor inoculation. (D) Tumors in mice treated with FR-27z, -28z, -BBz CAR-T cells regressed; tumors treated with untransduced T cells (UNT) did not regress 3 weeks post-first T cell dose (p<0.001). Arrows indicate timing of T cell infusion. FR-z CAR-transduced T cells only slowed tumor growth (p=0.015). Prior to infusion, CAR+ T cell frequency (~50%) and surface scFv expression level (range 315-398 MFI) was equalized. (E) Circulating human CD4+ and CD8+ T cell counts. In contrast to CD28, CD27 and 4-1BB signaling enhances the survival of circulating human CD4+ and CD8+ T cells in vivo 3 weeks following T cell infusion (27z vs z, p=0.029; BBz vs z, p=0.024; 28z vs z, p=0.20). Mean cell concentration (cells/μl) ± SD for all evaluable mice in each treatment group is shown. (F) Surface CAR expression on persisting FR-specific human CD3+ T cells from blood of treated mice measured by flow cytometry. Mean CAR+ expression frequency per CD3+ T cell and SD per group is shown.
Figure 1

A  CAR construct:

MOv19 scFv

FR-z

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FR-27z

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Intracellular domains

Anti-CD19 scFv

CD19-27z

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:CD8a Leader
:CD8a hinge
:CD8a TM

B

CD3+ T cells

CD8a Leader

CD4+ T cells

CD8+ T cells

CD3-APC-Cy7

CD8a hinge

CD8-FITC

CD8a TM
Figure 2

A

Ovarian Cancer lines

Breast Cancer lines

Control cell lines

Folate Receptor alpha-FITC

B

UNT

IFN-γ (pg/mL)

FR expression level

High

Low

Negative

Positive

CD19-27z CAR

FR-z CAR

FR-27z CAR

Cell lines
Figure 3
Figure 4

A

SKOV3(FR+)

C30(FR-)

UNT

FR-z CAR

FR-27z CAR

CD19-27z CAR

CFSE

B

FR+

FR-

Fold Expansion

Day post stimulation

C

SKOV3(FR+)

C30 (FR-)

FR-z CAR

FR-27z CAR

CD19-27z CAR

Bcl-XL-PE

D

SKOV3(FR+)

C30(FR-)

7-AAD-PerCP-Cy5.5

Annexin-V-FITC
Figure 5

A

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B

E:T Ratio

Specific lysis (%)

Day of infusion

Day 21 p.i.

C

D

Tumor Volume (mm³)

Days post first T cell infusion

E

Peripheral blood T cell counts (cells/µl)

F

CAR expression (%)

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