

Single residue in CD28-costimulated CAR T cells limits long-term persistence and antitumor durability

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Chimeric antigen receptor (CAR) T cell therapies can eliminate relapsed and refractory tumors, but the durability of anti-tumor activity requires in vivo persistence. Differential signaling through the CAR costimulatory domain can alter the T cell metabolism, memory differentiation, as well as influence long-term persistence. CAR-T cells costimulated with 4-1BB or ICOS persist in xenograft models but those constructed with CD28 exhibit rapid clearance. Here, we show that a single amino acid residue in CD28 drove T cell exhaustion and hindered the persistence of CD28-based CAR-T cells and substituting this asparagine to phenylalanine (CD28-YMFM) promoted durable anti-tumor control. In addition, CD28-YMFM CAR-T cells exhibited reduced T cell differentiation and exhaustion as well as increased skewing towards Th17 cells. Reciprocal modification of ICOS-containing CAR-T cells abolished in vivo persistence and anti-tumor activity. This finding suggests modifications to the co-stimulatory domains of CAR-T cells can enable longer persistence and thereby improve anti-tumor response.

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Title: Single residue in CD28-costimulated CAR-T cells limits long-term persistence and anti-tumor durability

One sentence summary: CD28-costimulated CAR-T cells demonstrate increased persistence when mutated at one residue to resemble the ICOS YMFM motif.

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Conflict of Interest: The University of Pennsylvania has filed a patent application based on this work (application no. WO2018140725A1), and A.D.P. and S.G. are inventors.

ABSTRACT

Chimeric antigen receptor (CAR) T cell therapies can eliminate relapsed and refractory tumors, but the durability of anti-tumor activity requires *in vivo* persistence. Differential signaling through the CAR costimulatory domain can alter the T cell metabolism, memory differentiation, as well as influence long-term persistence. CAR-T cells costimulated with 4-1BB or ICOS persist in xenograft models but those constructed with CD28 exhibit rapid clearance. Here, we show that a single amino acid residue in CD28 drove T cell exhaustion and hindered the persistence of CD28-based CAR-T cells and substituting this asparagine to phenylalanine (CD28-YMFM) promoted durable anti-tumor control. In addition, CD28-YMFM CAR-T cells exhibited reduced T cell differentiation and exhaustion as well as increased skewing towards Th17 cells. Reciprocal modification of ICOS-containing CAR-T cells abolished *in vivo* persistence and anti-tumor activity. This finding suggests modifications to the co-stimulatory domains of CAR-T cells can enable longer persistence and thereby improve anti-tumor response.

Keywords: CAR-T Cells, Cancer Immunotherapy, Calcium Signaling, T Cell Exhaustion

INTRODUCTION

Chimeric antigen receptor (CAR)-T cell therapies mediate astonishing remissions of hematopoietic malignancies (1-3) and, in a few cases, solid tumors (4, 5). However, the anti-tumor response can be lost due to fleeting persistence of the CAR-T cells (6, 7), development of T cell dysfunction (8, 9), or tumor loss of the targeted antigen (10-12). For differences in CAR-T persistence, the prevailing hypothesis has been quantitative signal strength (13, 14), as strong T cell activation potentiates exhaustion (15-17). However, persistence differences can also result from qualitative signaling differences driven by the co-stimulatory domains of the CAR-T (CD28, 4-1BB, etc.) (18-22).

Second-generation CD28-based CAR-T cells lack durable persistence in responding patients compared to the persistence observed with 4-1BB-containing CARs. For instance, in a study (NCT02435849) that evaluated 4-1BB-based anti-CD19 CAR-T cells in pediatric acute lymphoblastic leukemia (ALL), the median duration of CAR transgene positive cells in the peripheral blood of the 60 patients with a favorable response was 168 days (1). Similarly, in a study (NCT02445248) that investigated 4-1BB-based anti-CD19 CAR-T cells in adults with diffuse large B-cell lymphoma, the median duration of CAR transgene positive cells was 289 days in patients that obtained CR/PR and 92 days in all patients (3). However, in a phase I trial of CD28-based anti-CD19 CAR-T cells in adult patients with ALL (NCT01044069), the median duration of CAR-T cell detection was only 14 days (23). While various other construct differences have been postulated to play a role in *in vivo* performance and persistence, including the CAR scFv and availability of target epitope, the CAR costimulatory domain has emerged as the primary predictive factor. The differential persistence of 4-1BB and CD28-costimulated CARs was first apparent in NSG xenograft models evaluating anti-CD19 and anti-mesothelin CAR-Ts (22, 24). Similarly, the persistence of CD28-based CARs is also dwarfed by the persistence of both 4-1BB- and ICOS-based anti-mesothelin CARs in xenograft models (19, 25). ICOS, a T cell costimulatory receptor of the B7-CD28 superfamily, which also includes inhibitory receptors CTLA-4 and PD-1, shares the signaling motif YMXM with CD28 in its intracellular domain. CARs containing the ICOS costimulatory domain

have yet to be evaluated clinically; however, mesothelin-specific ICOS-based CAR-T cells demonstrate robust persistence and anti-tumor efficacy in xenograft models (19, 25).

The shared YMXM motif of CD28 and ICOS, which is well characterized for binding of the SH2 domain of phosphoinositide 3-kinase, differs only in that X is asparagine (N) in CD28 and phenylalanine (F) in ICOS. The asparagine within the YMN_M motif of CD28 interacts with Grb2 and is responsible for NFAT activation and IL-2 promoter activity (26). Substitution of phenylalanine to asparagine in the YMF_M motif of ICOS to YMN_M induces Grb2 binding, IL-2 production, and NFAT activation upon stimulation of the ICOS receptor. Similarly, mutation of the YMN_M motif in CD28 to YMAM abolishes Vav1 activation, and reduces PLC γ 1 activation and calcium influx (27). Robust calcium signaling in T cells is associated with overactivation of NFAT to produce “partnerless NFAT”, which has been shown to drive T cells into states of exhaustion and dysfunction, specifically through the upregulation of NR4A transcription factors and TOX (28, 29). Therefore, we hypothesized that this single amino acid difference in the shared YMXM motif of ICOS and CD28 may explain the difference in persistence of T cells expressing CARs engineered with the intracellular domains from these two receptors and substitution of YMN_M to YMF_M may promote reduced exhaustion of CD28-costimulated CAR-T cells.

RESULTS

The persistence of CD28-based CARs can be enhanced through mutation of the Grb-2 interacting residue.

We utilized site-directed mutagenesis to generate mesothelin-specific CARs with single amino acid alterations in the YMXM motif – CD28-YMFM and ICOS-YMNM, to complement the existing CD28 (naturally YMNM) and ICOS (naturally YMFM) CARs (**Figure 1A**). These CARs target mesothelin through the SS1 scFv (30), contain the extracellular spacer region of CD8 α (which allows CAR dimerization), transmembrane domains of either CD28 or ICOS, respectively, and the CD3 ζ intracellular domain containing three functional immunoreceptor tyrosine-based activation motifs (ITAMs). A first generation CAR containing only the CD3z intracellular domain (z) or a truncated form of the CD3z intracellular domain (deltaz) were used as controls as indicated. Expression of the CAR receptors on normal donor T cells is similar amongst the variant designs (**Figure 1B**). In vitro characteristics and activities of the variant CD28-CAR-T cell products, including tonic signaling, inhibitory marker expression and T cell differentiation, as well as cytotoxicity and cytokine production, were indistinguishable (**Figure 1B-D**).

Despite similar in vitro activity, noteworthy differences in CAR-T cell persistence and anti-tumor activity were detected after treatment of two xenograft models bearing well-established subcutaneous or intraperitoneal human pancreatic tumors. Treatment of subcutaneous Capan-2 tumors with either CD28- or CD28-YMFM CAR-T cells exerts a reduction in tumor size for 23 days, at which point tumors treated with CD28-based CAR-T cells resume growth while tumors treated with CD28-YMFM based CAR-T cells maintain durable tumor control (**Figure 2A-B**). Tumors treated with ICOS-based CAR-T cells maintain stable disease for 35 days and then initiate a steady reduction in size. Significant differences in CD4⁺ and CD8⁺ T cell persistence are observed in the peripheral blood 30 days after CAR-T cell treatment, where CD28-YMFM and ICOS-based CAR-T cells demonstrate robust persistence and counts of CD28-based CAR-T cells are similar to those of non-specific, untransduced T cells (**Figure 2C**). In a model of disseminated pancreatic tumor, ASPC-1 tumors were established through intraperitoneal injection and

treated with control T cells or CD28-, or CD28-YMFM-based CAR-T cells. Similar to the subcutaneous model, CD28- and CD28-YMFM CAR-T cells led to tumor reduction for 19 days and disease progression resumed for mice treated with CD28-based CAR-T cells (**Figure 2D**). Anti-tumor efficacy continued for CD28-YMFM-based CAR-T cells; eventually, tumor bioluminescence reached background levels of total flux ($\sim 10^6$ p/s).

Signaling through a CD28-based CAR containing the ICOS YMFM motif shows enhanced AKT phosphorylation with reduced pPLC γ , pVav and calcium flux.

To understand the mechanism behind the enhanced persistence of CD28, we studied early proximal and distal signaling events after antigen stimulation of mesothelin-specific CAR-T cells, which revealed increases in AKT activation in CD28-YMFM CAR-T cells relative to CD28 CAR-T cells and decreases in Vav1, PLC γ 1, and ERK activation (**Figure 3A-E**). AKT activation was significantly increased in CD28-YMFM CAR-T cells generated from four different healthy donors compared to CD28 CAR-T cells produced from the same donors (**Figure 3D-E**). Stimulation of endogenous ICOS has been shown to provide a stronger AKT activation than CD28 signaling through the recruitment of p50 α PI3K regulatory subunit, rather than p85 α , to the plasma membrane (31). A decrease in Vav1 phosphorylation was expected as activation of Vav1 by CD28 signaling requires Grb2 binding (32). Once activated, Vav1 signaling leads to NFAT activation and IL-2 production, as well as calcium release through PLC γ 1 and ERK activation (33). These signaling differences are consistent with the activity expected from altering the Grb2-binding domain of CD28. Additionally, we have previously demonstrated the detection of calcium release from CD28-based CAR-T cells when stimulated with cognate antigen (34) and now show that this calcium response is abrogated when CD28-YMFM CAR-T cells are stimulated by mesothelin; yet, all T cells demonstrate calcium release in response to TCR stimulation with OKT3 and the calcium ionophore ionomycin (**Figure 3F**). Taken together, the observed increase in AKT activation, and the loss of Vav1

phosphorylation and its downstream cascade of signaling events after stimulation of CD28-YMFM CAR-T cells suggest an alteration of the T cell signaling that differs from simply an attenuation of signal strength.

***In vivo* long-term signaling through 28z-YMFM CAR is associated with a transcriptional profile that resembles ICOS signaling.**

Initial signaling events demonstrated qualitative differences in signaling as expected from the literature. However, transcriptome analysis by RNA-sequencing of CAR-T cells six days after *in vitro* antigen recognition revealed that CD28-YMFM CAR-T cells had only 13 differentially expressed genes (<2- or >2-fold change) compared to CD28 CAR-T cells (**Figure 1E**). By contrast, there were 2173 differentially expressed genes when comparing ICOS and CD28 CAR-T cells. Thus, CD28 and CD28-YMFM CAR-T cell activities were unremarkable and significant phenotypic differences in persistence and anti-tumor activity only existed *in vivo*. Therefore, we established an *in vivo* model of chronic antigenic stimulation with subcutaneous Capan-2 tumors and harvested T cells from tumors one week and two weeks post treatment to assess gene expression. Analysis of T cell infiltration at early time points after *in vivo* administration (day 7 after treatment) demonstrated that all animals had a similar percentage of T cells infiltrating the tumor (**Figure S1**). Tumor measurements at two weeks demonstrated a reduction of tumor growth in all CAR-T cell treated groups (**Figure 4A**), but Ki67 expression on CD45⁺ cells showed that only CD28-YMFM and ICOS CAR-T cells were actively proliferating two weeks after treatment (**Figure 4B**), suggesting that CD28 CAR-T cells had become dysfunctional or inactive. Gene expression analysis of CAR-T cells two weeks after treatment showed clustering of CD28-YMFM with ICOS CAR-T cells, whereas the CD28 CAR-T cells were less related (**Figure 4C**). These differences were only pronounced after fourteen days of stimulation (**Figure 4D**), with 424 differentially expressed genes (DEGs) between CD28 and CD28-YMFM CAR-T cells, 604 DEGs between CD28 and ICOS CAR-T cells, and only 64 DEGs between CD28-YMFM and ICOS CAR-T cells, but few DEGs after seven days (**Supplemental Table 1-2**). We next compared the differentially expressed genes to those previously associated with T cell differentiation and exhaustion. Genes associated with central memory and Tscm differentiation, such as

Tcf7, *Fosb*, *Znf513*, were expressed higher in CD28-YMFM CAR-T cells than in CD28 CAR-T cells (**Figure 4E**), and the interferon-induced genes *Batf2*, *Cxorf2* and *Trim14* were expressed higher in both CD28-YMFM and ICOS CAR-T cells than in CD28 CAR-T cells. Similarly, *Pik3cd* or *Dusp6*, genes associated with reduced exhaustion or downregulated in effector cells, were expressed higher in CD28-YMFM CAR-T cells compared to CD28 or ICOS CAR-T cells. *Lxn*, which is upregulated in effector cells, had higher expression in CD28 CAR-T cells than CD28-YMFM or ICOS CAR-T cells. Consistently, gene set enrichment analysis (GSEA) revealed upregulation of exhaustion and differentiation pathways in CD28 CAR-T cells, while CD28-YMFM and ICOS CAR-T cells maintained a phenotype of less differentiated T cells (**Figure 4F**, and **Figure S2**). Interestingly, signaling through CD28-YMFM and ICOS polarized CAR-T cells towards a Th17 phenotype, supporting previous results that ICOS signaling has a key role in the maintenance of Th17 phenotype and function (**Figure 4G** and **Figure S2**).

Mutation of the YMFM motif in the ICOS signaling domain to YMNM (CD28 motif) impairs antitumor effect and T cell persistence.

The CD28 intracellular signaling domain contains other important signaling motifs besides the YMNM motif and it is important to study the contribution the Grb2-binding asparagine of the YMNM motif makes to the lack of CAR-T persistence in the absence of these other motifs. Thus, we utilized a reciprocal CAR design, substituting phenylalanine to asparagine in the YMFM motif of the ICOS-based CAR to generate the ICOS-YMNM CAR-T cells (**Figure 1A**). In vitro characterization of ICOS and ICOS-YMNM CAR-T cell cytotoxicity and cytokine secretion demonstrated no significant differences, as observed with in vitro characterization of the wildtype and variant CD28-based CAR-T cells (**Figure 5A-B**). However, treatment of a xenograft model with subcutaneous Capan-2 tumors revealed differences in anti-tumor activity and robust differences in CAR-T cell persistence (**Figure 5C-E**). In this model, ICOS-based CAR-T cells shrink the tumor size while ICOS-YMNM CAR-T cells only stabilize the disease. By 36 days after treatment, the persistence of ICOS CAR-T cells dramatically dwarfs that of ICOS-YMNM CAR-T cells, which resembles the persistence of first-generation CD3 ζ -only CAR-T cells (**Figure 5E**). These results

demonstrate reciprocal effects of the Grb2-binding residue within the YMXM motif and highlight the Grb2/Vav signaling pathways as targets to enhance CAR-T cell persistence in vivo.

DISCUSSION

The persistence of CAR-T cells is tightly correlated to the durability of clinical remissions in patients with chronic lymphocytic leukemia (CLL) (7) and ALL (1). Persistence is perhaps even more critical for the clinical success of CAR-T cells in solid tumors, due to a multitude of immunosuppressive obstacles to overcome, the need to eliminate micrometastatic niches, as well as the kinetics required to eradicate significant masses of tumor. Substantial effort has been invested in developing novel approaches to enhance persistence and expansion of adoptively transferred CAR-T cells (35), including selection of specific T cell subsets (36-38), optimized T cell culture conditions (39, 40), combination therapies (41, 42), genetic modifications and alternative methods of integration to reduce tonic signaling (43, 44), introduction of ligands for costimulatory molecules (21), providing cytokine signaling (45-49) and the prevention of inhibitory signals (50, 51).

Here, we show that persistence and anti-tumor efficacy of CD28-based CARs targeting mesothelin can be improved by mutating a single amino acid. Because this point mutation does not increase the length of the genetic material to be included in viral vectors, this strategy can be easily combined with other genetic modifications to increase CAR-T cell therapeutic index. In the pancreatic cancer xenograft models presented here, loss of T cell persistence leads to tumor outgrowth. However, through the YMFM alteration within the CD28 signaling domain, CAR-T cells demonstrate robust persistence in vivo and durable tumor control. The asparagine in the endogenous CD28 signaling domain interacts with the SH2 domain of Grb2 (52) and this interaction is disrupted through the YMFM alteration (53). This decreased interaction increases AKT activation, reduces the activation of Vav1 and PLC γ 1, and reduces calcium influx in CAR-T cells stimulated with cognate antigen. These signaling alterations led to significant transcriptional changes that influence reduced T cell differentiation as well as protection from exhaustion. Importantly, the reciprocal alteration to ICOS with the YMNМ motif abolishes T cell persistence and anti-tumor activity, highlighting the influence this single amino acid has over control of CAR-T cell persistence and durable anti-tumor activity.

Alterations to the PYAPP motif within the CD28 costimulatory domain of CARs has been previously reported and was shown to decrease IL-2 secretion and Treg recruitment, reportedly through the elimination of CD28-Lck interactions (54). However, the CD28 PYAPP motif not only interacts with the SH3 domain of Lck (55), but also Filamin-A (56) as well as the SH3 domain of Grb2 (53, 57). In fact, the interaction with the Grb2 SH3 domain at the PYAPP motif is thought to reinforce the Grb2 SH2 domain interaction at the YMNM motif (53). T cell persistence was not evaluated in the prior report of CD28 alteration and it is unclear whether this alteration would enhance anti-tumor durability, especially since tumor growth was reduced but not eliminated in this model. However, it should be noted that ICOS-costimulated CAR-T cells were not as cytolytic *in vivo* as CD28-YMFM CAR-T cells and signaling interactions at motifs outside of the YMNM motif in CD28, such as the PRRP and PYAPP motifs, which are responsible for NF- κ B activation and Gads binding (58), may play an important role in this difference.

The decrease in Vav1 activation, which is mediated through C-terminal Grb2-SH3 and N-terminal Vav1-SH3 dimerization (59), likely influences the decrease in PLC γ 1 activation and calcium influx. Vav1 is recruited to a multiprotein complex that includes LAT, SLP76, and PLC γ 1 (60). PLC γ 1 hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and inositol 1,4,5-triphosphate (IP3), which mediates calcium release from the endoplasmic reticulum (61). Calcium signaling induces NFAT activation, which in turn can prompt the expression of a set of anergy and exhaustion-associated genes (28, 62, 63). In CD28-YMFM CAR-T cells in which calcium signaling upon CAR activation is reduced, a downregulation of exhaustion pathways in comparison to wildtype CD28 CAR-T cells is observed and may be due to the reduced activation of NFAT. Strategies to eliminate the exhaustion-related gene programs downstream of NFAT activation in CAR-T cells have demonstrated enhanced anti-tumor activity (64). The approach undertaken here suggests that alterations to the T cell signaling program upstream of NFAT activation and calcium release may also enhance anti-tumor activity and preserve T cell function. An alternative explanation for enhanced persistence of CD28-YMFM CAR-T cells is the increased Akt activation, which may lead to higher expression of anti-

apoptotic proteins. While this is an attractive hypothesis, no significant transcriptional changes of Bcl family members were observed and it remains unclear what role the enhanced Akt activation has on the persistence or anti-tumor activity of CD28-YMFM CAR-T cells.

The observation that CAR-T cells costimulated by ICOS were more enriched in Th17 cells than those costimulated by CD28 is well characterized as a function of ICOS signaling (65) and has been previously demonstrated in CAR-T cells through Th17-polarizing and non-polarizing culture conditions (19, 25). Interestingly, GSEA analysis also demonstrated that CD28-YMFM CAR-T cells exhibited a transcriptional profile more Th17-like than CD28 CAR-T cells. Th17 cells display a molecular signature consistent with stem-like properties, can give rise to Th1-like cells while also self-renewing to maintain a population of IL-17A-secreting cells, and demonstrate increased anti-tumor potency over Th1 cells when adoptively transferred in mouse models of melanoma (66, 67). In the absence of negative co-stimulation by CTLA4 or after anti-CTLA4 antibody blockade, CD4 T cells can differentiate into an ICOS⁺ Th1-skewed population with superphysiological cytokine output capabilities (68). On a molecular level, these results suggest that the ICOS YMFM motif is at least in part responsible for CD4 T cell differentiation towards a Th17 phenotype. It remains unclear whether the increased Akt activation, reduced activation of Vav1, or the combination of these factors in ICOS and CD28-YMFM CAR-T cells is responsible for driving Th17 differentiation. A TRAF-like motif has also been identified in ICOS and shown to be critical for the development of follicular helper T (T_{fh}) cells (69). This motif is not conserved in CD28-YMFM and, as a consequence, CD28-YMFM displayed less T_{fh} phenotype than CD28 CAR-T cells, suggesting that the YMFM motif does not play a role in T_{fh} development.

Achieving long-term durable persistence of CAR-T cells and associated anti-tumor activity will benefit patients with bulky disease and may be critical for CAR-T cell treatment success in solid tumors. This work reports a modification to a clinically relevant treatment model using a manufacturing process that is commercially validated and has the potential for rapid translation and enhancement of therapeutic efficacy. Utilization of the CD28-YMFM domain in other CAR constructs, including widely successful CD28-containing CD19 CARs, may also improve persistence and anti-tumor durability.

METHODS

Cell lines

All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). The Capan-2 (Pancreas adenocarcinoma) and ASPC-1 (Pancreas adenocarcinoma ascites metastasis) cell lines were cultured in DMEM/F12. All media was supplemented with 10% fetal calf serum (FCS). All tumor cell lines were authenticated in 2016 by the University of Arizona Genetics Core (Tucson, AZ) and regularly validated to be *Mycoplasma*-free.

Isolation, Transduction, and Expansion of Primary Human T Lymphocytes.

Blood samples were obtained from the Human Immunology Core of the University of Pennsylvania. Peripheral blood CD4⁺ and CD8⁺ T cells were negatively isolated using RosetteSep Kits (Stem Cell Technologies). Cells were cultured in RPMI 1640 media supplemented with 10% FCS, 100-U/ml penicillin, 100 µg/ml streptomycin sulfate, 10 mM HEPES in a 37°C and 5% CO₂ incubator. For stimulation, CD4⁺ and CD8⁺ T cells were cultured with activating beads coated with antibodies to CD3 and CD28 at a 1:3 cell to bead ratio. Approximately 24 h after activation, T cells were transduced with lentiviral vectors at an MOI of 5. For CD8⁺ T cells, human IL-2 (Chiron) was added every other day to a final concentration of 50 IU/ml. Cells were counted and fed every 2 days and once T cells appeared to rest down, as determined by both decreased growth kinetics and cell size, they were either used for functional assays or cryopreserved. All T cell functional assays were performed in media without cytokines.

Generation of CAR mutant constructs

CAR constructs targeting mesothelin with the SS1 scFv and costimulated by CD28, 4-1BB, and ICOS were previously described (24, 25). 28z-YMFM mutant was generated through site-directed mutagenesis of the SS128z construct using the oligonucleotides 28z-YMFM-F: CACAGTGACTACATGTTTCATGACTCCCCGCCG and 28z-YMFM-R: CGGCGGGGAGTCATGAACATGTAGTCACTGTG. ICOSz-YMNM mutant was generated through

site-directed mutagenesis of the SS1ICOSz construct using the oligonucleotides ICOSz-YMNM-F: CTAACGGTGAATACATGAACATGAGAGCAGTGAAC and ICOSz-YMNM-R: GTTCACTGCTCTCATGTTTCATGTATTACCGTTAG.

Western Blot

1 x 10⁶ T cells were stimulated with magnetic beads coated with a recombinant mesothelin as previously described (19). Cell pellets were collected and frozen at indicated time points after antigen recognition. Protein lysates from cells lines were prepared in a lysis buffer containing 2% SDS, 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, and 0.002% bromophenol blue. Equivalent amounts of protein were resolved by SDS-PAGE and processed by immunoblotting analysis. The following primary antibodies were used: anti-phospho-Erk1/2 (p44/42) (9101S/L), anti-Erk1/2 (9107S), anti-phospho-Akt (S473) (9271) and anti-Akt (2920) (Cell Signaling Technology, Beverly, MA), anti-Vav1 (sc-8039; Santa Cruz Biotechnology, Dallas, TX), anti-phospho-Vav1 (Y174) (ab76225), anti-phospho-PLC γ (Y783) (ab76031), and anti-PLC γ (ab41433) (Abcam, Cambridge, UK). Bands were visualized by enhanced chemiluminescence (ECL). Images were captured using an Odyssey Fc system (Li-Cor Biosciences; Lincoln, NE). Image processing and densitometry analysis were carried out using the Image Studio Lite software (Li-Cor Biosciences).

Calcium measurements

Calcium influx was measured by flow cytometry as previously described (34). Briefly, human CD4 T cells were electroporated with mRNA encoding CAR for SS1 Δ z, SS1z, SS1BBz, SS1ICOSz, SS128z, or SS128z-YMFM. Twenty-four hours after electroporation, T cells were loaded with 2 μ M Indo-1 AM for thirty minutes, washed, and resuspended in HBSS with 1mM Ca²⁺, 1mM Mg²⁺, and 1% FBS. Loaded T cells were acquired on a LSRIIFortessa equipped with a UV laser and filters at 395nm and 510nm for thirty seconds to collect baseline calcium measurements. In order to measure calcium responses to CAR stimuli,

cells were removed from the flow nozzle, stimulated through addition of recombinant mesothelin-Fc directly to the cell suspension, and returned to acquisition for six minutes. Subsequently, cells were stimulated with biotinylated OKT3 and avidin and returned to acquisition for 6 minutes to collect calcium response to TCR stimuli. Lastly, cells were treated with 1µg/mL of the calcium ionophore ionomycin to measure cellular calcium influx capability.

In vitro coculture experiments

1 x 10⁵ Tumor cells were seeded in 48-well plates and after 24 hours, 3x10⁵ T cells were added (E:T=3). For cytokine production assays, supernatants were collected 24 hours post-coculture and analyzed using the human cytokine 30-plex panel on the Luminex system (ThermoFisher, Waltham, MA) as previously described or assessed for human IL-2, TNF-α and IFN-γ using the DuoSet® ELISA Development Kit (R&D Systems, Minneapolis, MN).

Flow cytometry

For all experiments, T cell suspensions were stained with a fixable live/dead violet stain (L34955, Life Technologies) in PBS followed by surface antibody staining in FACS buffer. The Foxp3/Transcription Factor Staining Buffer set (Life Technologies) was used for Ki67 detection. The following antibodies were used: anti-CD45-PerCp-Cy5.5 (45-9459-42), anti-CD8-APC (17-0086-42), anti-CD4-PE (12-0048-42), TIM3-PerCPeF710 (46-3109-42), CD25-PECy7 (25-0257-42), Ki67-FITC (11-5699-42) (Life Technologies); anti-CD8-APC-H7 (560179), CCR7-FITC (150503) (BD biosciences, San Diego, CA); anti-CD4-BV510 (317444), PD-1-BV711 (329928) (Biolegend, San Diego, CA); CD27-PECy7 (A54823) and CD28-PE-CF594 (6607111) (Beckman Coulter). Expression of CAR proteins was evaluated using biotinylated goat anti-mouse IgG (115-065-072, Jackson ImmunoResearch, West Grove, PA) with streptavidin (APC or PE) (BD Biosciences). All experiments were conducted on a BD Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Eugene, OR).

Mice

NSG mice were purchased from The Jackson Laboratory and bred by the Stem Cell and Xenograft Core in the vivarium at the University of Pennsylvania. The mice were housed under specific pathogen-free conditions in microisolator cages and given ad libitum access to autoclaved food and acidified water.

In vivo assessment of anti-mesothelin CAR-T cells.

Xenograft tumors were established by subcutaneous injection of 5×10^6 Capan-2 cells in the presence of a 50% solution of Matrigel (BD Biosciences) in PBS. Capan-2 tumors were allowed to grow in NSG mice for 3 weeks. Mice were then treated with two intravenous injections of 10×10^6 CAR-T cells (50% chimeric receptor-positive, 1:1 CD4:CD8⁺ ratio) at day 0 and day 15. Tumor dimensions were measured with calipers, and tumor volumes calculated using the formula $V = 1/2 \times L \times W \times W$, where L is length (longest dimension) and W is width (shortest dimension). For the intraperitoneal xenograft model, 1×10^6 CBG+ ASPC-1 cells were injected intraperitoneally in NSG mice in 100 μ L PBS. Six days after tumor inoculation, tumor bioluminescence was measured through intraperitoneal injection of 3mg luciferin (Gold Biotechnology) and mice were randomized into treatment groups with average total flux ranging from $6.42 \times 10^7 - 1.33 \times 10^8$ p/s. Mice were treated the following day through an intravenous injection of 1×10^7 T cells, normalized to 30% CAR expression, in 100 μ L of PBS. Bioluminescent imaging was performed weekly in order to determine tumor burden. Peripheral blood was obtained from retro-orbital bleeding on indicated days after T cell injection and stained for the presence of human CD45, CD4, and CD8 T cells. After gating on the human CD45⁺ population, the CD4⁺ and CD8⁺ subsets were quantified using TruCount tubes (BD Biosciences). All experiments were performed in a blinded, randomized fashion.

Ex vivo analysis of tumor infiltrating T cells

Capan-2 tumors were harvested at days 7 and 14 after T-cell injection and processed using the gentleMACS Dissociator (Miltenyi). For flow cytometry analysis, cells were filtered to ensure a single cell suspension and stained for viability followed by staining for murine CD45 and human CD45, EpCAM and Ki67. To analyze the transcriptional profile of tumor infiltrating T cells, T cells were isolated by Ficoll–Paque density gradient centrifugation (GE Healthcare). Remaining Dead cells were removed using the Dead Cell Removal kit (Miltenyi) according to manufacturer’s instructions.

Differential expression analysis

Frozen CD8+ T cells in RLT buffer were sent to BGI Genomics for RNA extraction, library preparation and sequencing. Raw data files for in vitro stimulations found in Figure 1E were uploaded to GEO (accession number GSE145007). FPKM and counts of ex vivo analyses found in Figure 4 and Supplemental Figure 2 were attached as Supplemental Tables 1 and 2. We removed poor quality raw reads as follows: 1) Remove reads with adaptors; 2) Remove reads in which unknown bases are more than 10%; 3) Remove low quality reads (the percentage of low quality bases is over 50% in a read, where a low quality bases are ones with sequencing quality < 5). After filtering, we used Bowtie2 (70) to map reads to the human reference genome, GRCh38, with parameters: -q --phred64 --sensitive --dpad 0 --gbar 99999999 --mp 1,1 --np 1 --score-min L,0,-0.1 -p 16 -k 200. FPKM and counts were calculated using RESM (71).

RNA-seq transcript count data was normalized using DESeq2 (72) and utilized to calculate differential expression between 28z, 28z-YMFM, and ICOSz CARs at day 7 and day 14, also using DESeq2. Genes were identified as significantly differentially expressed if their adjusted P-value was < 0.05 and their absolute log₂ fold change was greater than 1 (i.e. non-log₂ transformed fold change greater than 2). Multidimensional scaling (MDS) analysis was performed on the significant differentially expressed genes among the three CARs using Euclidean distances and the R function cmdscale() with parameters set as k = 2 dimensions, and eigen values = TRUE. Gene set enrichment analysis (GSEA) of immunology related

gene lists from MSigDB C7 (73-75) was performed using three log₂ fold change ranked lists representing the expression patterns of three different CARs (28z-YMFM vs 28z, ICOSz vs 28z, ICOSz vs 28z-YMNM). Analysis was performed using the fgsea R Bioconductor package (76) and the msigdb R CRAN package.

Statistical Analysis

All statistical analyses were performed with Prism software version 6.0 (GraphPad). Statistically significant differences were tested by the specific tests indicated in the figure legends. The number of animals per group in each experiment was determined on the basis of previous statistical analyses by our group. Symbols indicate statistical significance as follows: *P < 0.05; **P < 0.01, and ***P < 0.001. A P value less than 0.05 was considered significant. Where used for statistical evaluations, figure legends clarify whether ANOVA was 1-way or 2-way.

Study Approval

The present studies in mice were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania, Philadelphia, PA, USA.

List of Supplementary Material

1. Supplementary Figure 1. **T cell infiltration in tumors from NSG animals treated with SS1-CART.**
2. Supplementary Figure 2. **Long-term signaling through 28z-YMFM CAR delays T cell differentiation and exhaustion and drives Th17 polarization.**

AUTHOR CONTRIBUTIONS

S.G. and A.D.P. designed the study, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. A.M. analyzed the RNA-seq data. V.C. M. performed the Western blots and analysis. C.S., A.W., F.L. manufactured cells and performed in vivo experiments.

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Figure 1

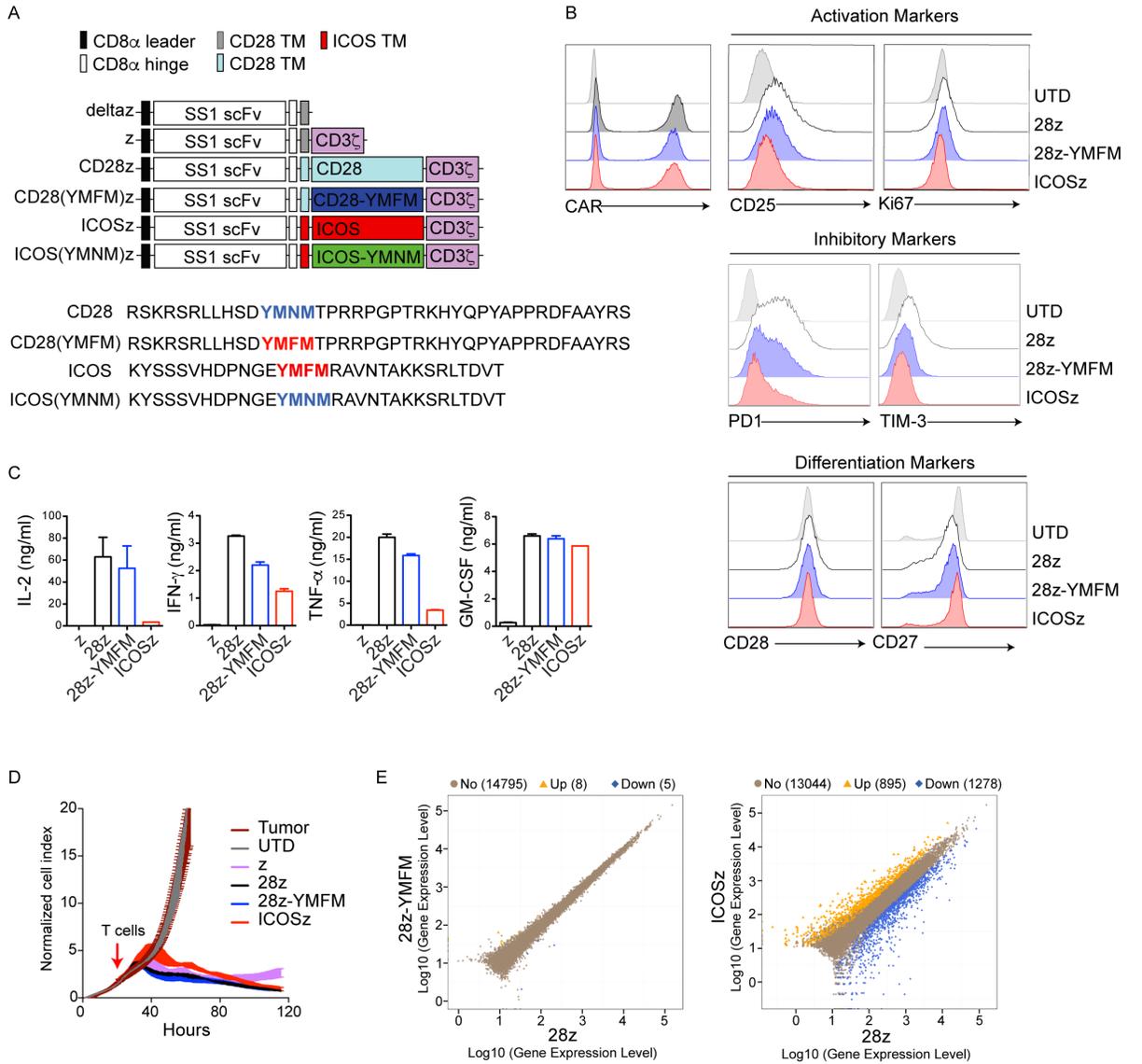


Figure 1. Short-term signaling through 28z-YMFM CAR closely resembles CD28 signaling. (A) *Upper panel*, Schematic representation of a panel of chimeric receptors that contain the SS1 scFv and differ in the intracellular domain. The CD28(YMFM)z mutant contains the CD28 transmembrane and intracellular domain with a point mutation in the YMN motif [the asparagine (N) is mutated to phenylalanine (F)]. The ICOS(YMNM)z mutant contains the ICOS transmembrane and intracellular domains with a point mutation in the YMFM motif [the phenylalanine (F) is mutated to an asparagine (N)]. *Lower panel*, the full amino acid sequences for CD28 and ICOS intracellular domains and its corresponding mutants are shown. (B) Characterization of tonic signaling in CAR-T cells during primary expansion. Representative histograms showing the expression of the CAR protein and markers of activation, inhibition and differentiation in CAR-T cells at 14 days after stimulation with anti-CD3/CD28 beads. (C) CAR-T cells were cocultured with mesothelin+ Capan-2 cells. Supernatants were obtained 24 hours later, and cytokine production was analyzed by Luminex. Representative of 2 donors. (D) A real-time cytotoxicity assay (xCELLigence) was used to evaluate the lysis of Capan-2 tumor cells when

treated with CAR-T cells (E:T=3:1) over a 120-hour period. (E) CAR-T cells from 2 different healthy donors were stimulated with immobilized recombinant mesothelin. Transcriptome analysis by RNA-sequencing was performed 6 days following antigen recognition. Scatter plot show the gene expression levels in 28z-YMFM or ICOSz CAR-T cells versus 28z CAR-T cells. The number of up- (orange) and down- (blue) regulated genes is indicated.

Figure 2

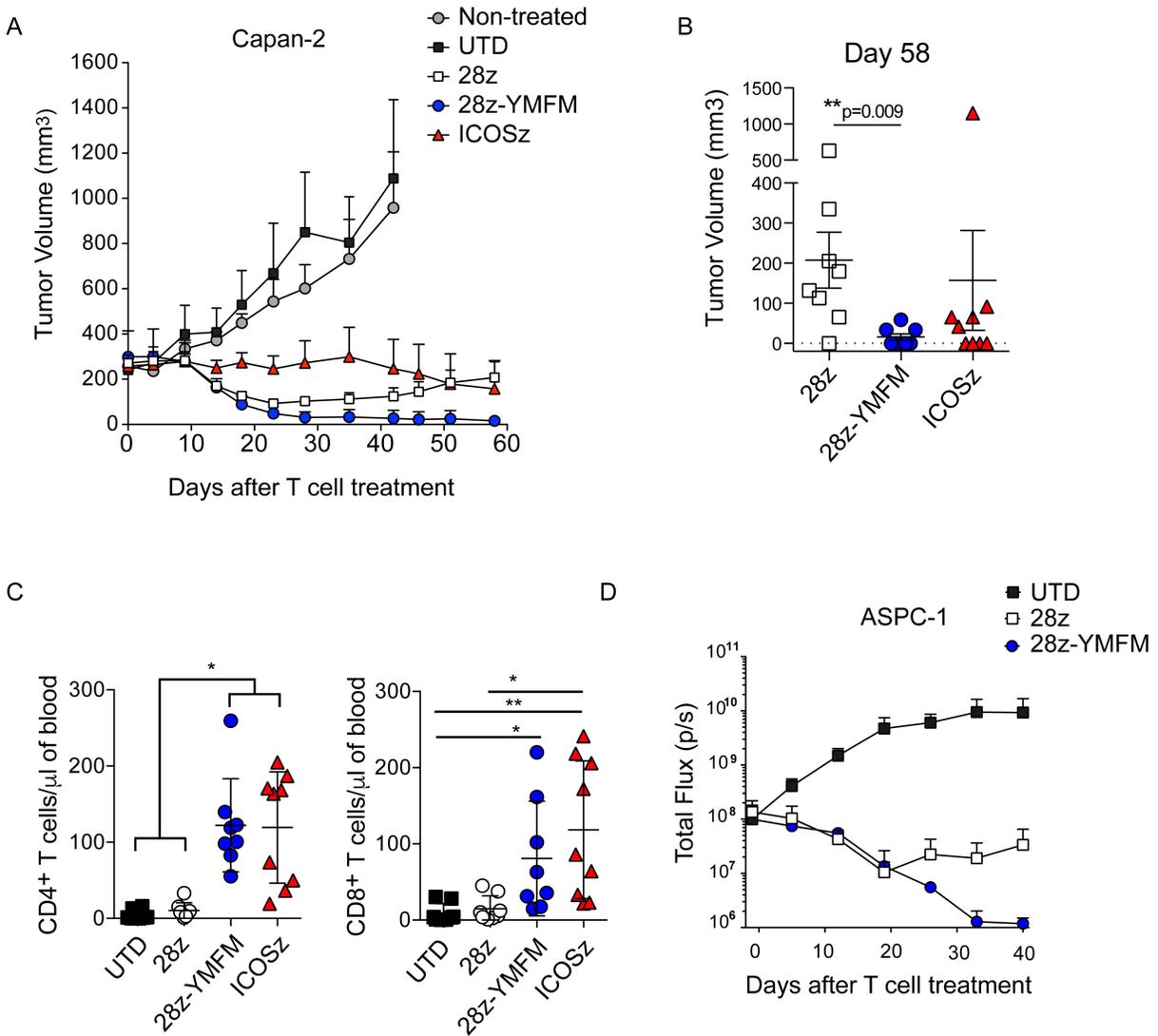


Figure 2. The persistence of CD28-based CARs can be enhanced through mutation of the Grb-2 interacting residue. (A-B) NSG mice bearing s.c. Capan-2 tumors were treated 20 days after tumor implantation with two doses of control T cells (untransduced, UTD) or CAR-T cells (n=8-9) on day 0 and day 15. (A) Tumor volume was analyzed at indicated time points. (B) Tumor volume on day 58 for individual animals is plotted. Error bars represent \pm SEM (n = 8 tumors). P value was calculated by Kruskal-Wallis multiple comparisons test. (C) The concentration of CD4⁺ and CD8⁺ T cells was determined in the blood of treated animals 30 days after T cell injection. Error bars represent \pm SEM (n = 8-9). **P < 0.01, and ***P < 0.001. P values were calculated by Kruskal-Wallis multiple comparisons test. (D) NSG mice bearing intraperitoneal CBG⁺ ASPC-1 tumors were treated 7 days after tumor implantation with 10×10^6 CAR-T cells. Bioluminescence was analyzed at the indicated time points (n=4).

Figure 3

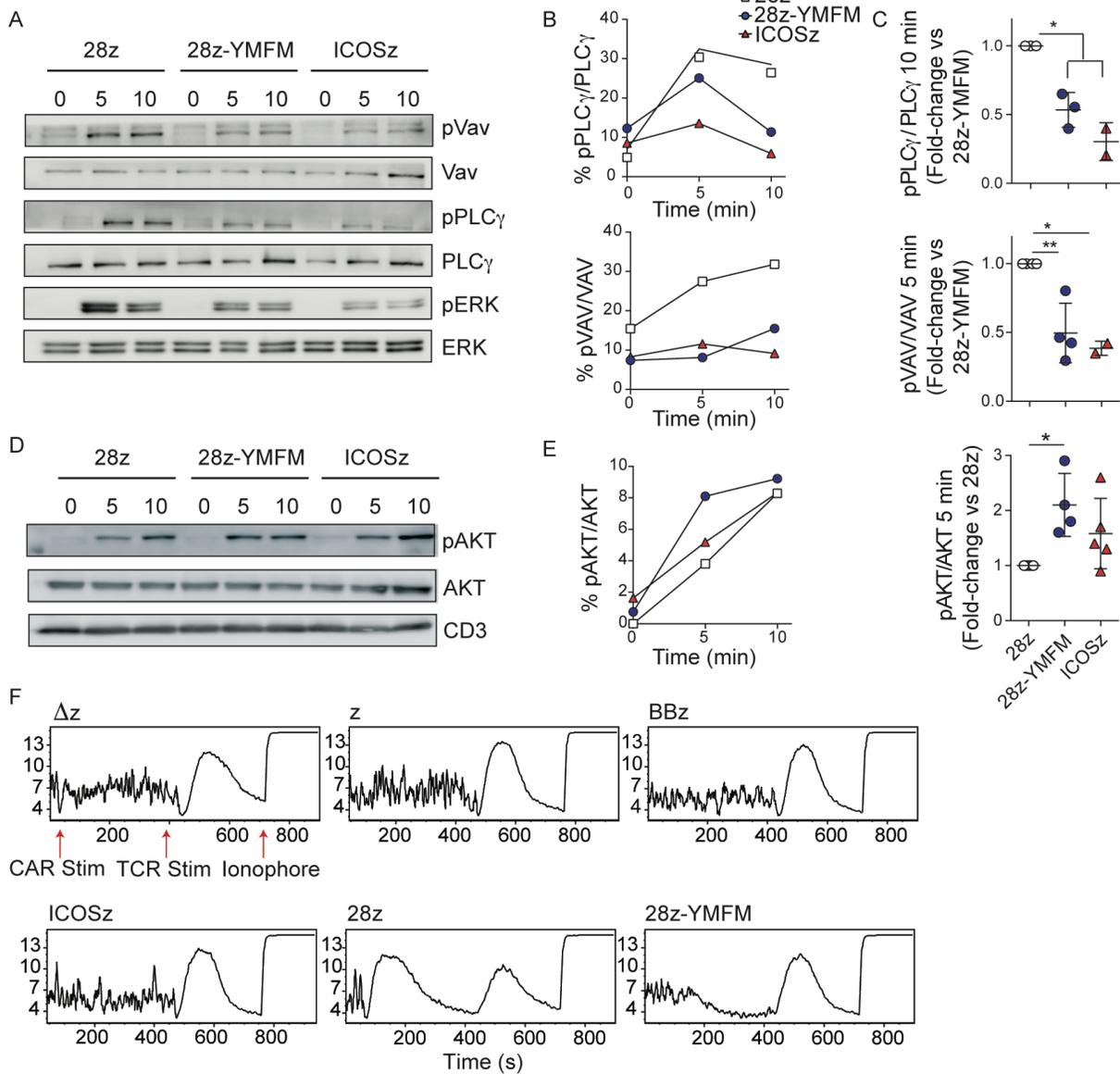


Figure 3. Signaling through a CD28-based CAR containing the ICOS YMFM motif shows enhanced AKT phosphorylation with reduced pPLC γ , pVav and calcium flux. (A-E) CAR-T cells were stimulated with magnetic beads coated with recombinant mesothelin. Cell lysates were obtained at 5- and 10-minutes following antigen encounter and phosphorylation levels for Vav, PLC γ , ERK and AKT were analyzed by Western Blot (A, D) and densitometry (B, E). Basal phosphorylation was evaluated without stimulation (minute 0). **(C)** T cells from 2-5 different healthy donors were stimulated as in (A-E), and AKT, VAV and PLC γ phosphorylation were analyzed by densitometry. The mean \pm SD from four independent experiments is shown. P values were calculated by 1-way ANOVA with Tukey post hoc test. *P < 0.05, **P < 0.01. **(D)** Calcium influx was measured in CAR-T cells at baseline for 30 seconds, then after stimulation with mesothelin-coated magnetic beads for 6 minutes, followed by stimulation with biotinylated OKT3 and avidin for 6 minutes, and treated with ionomycin. The mean Indo-1 ratio of violet/blue fluorescence emission is displayed on the y-axis and the time of sample collection in seconds is displayed on the x-axis. Representative of 3 different experiments using 3 different normal donors.

Figure 4

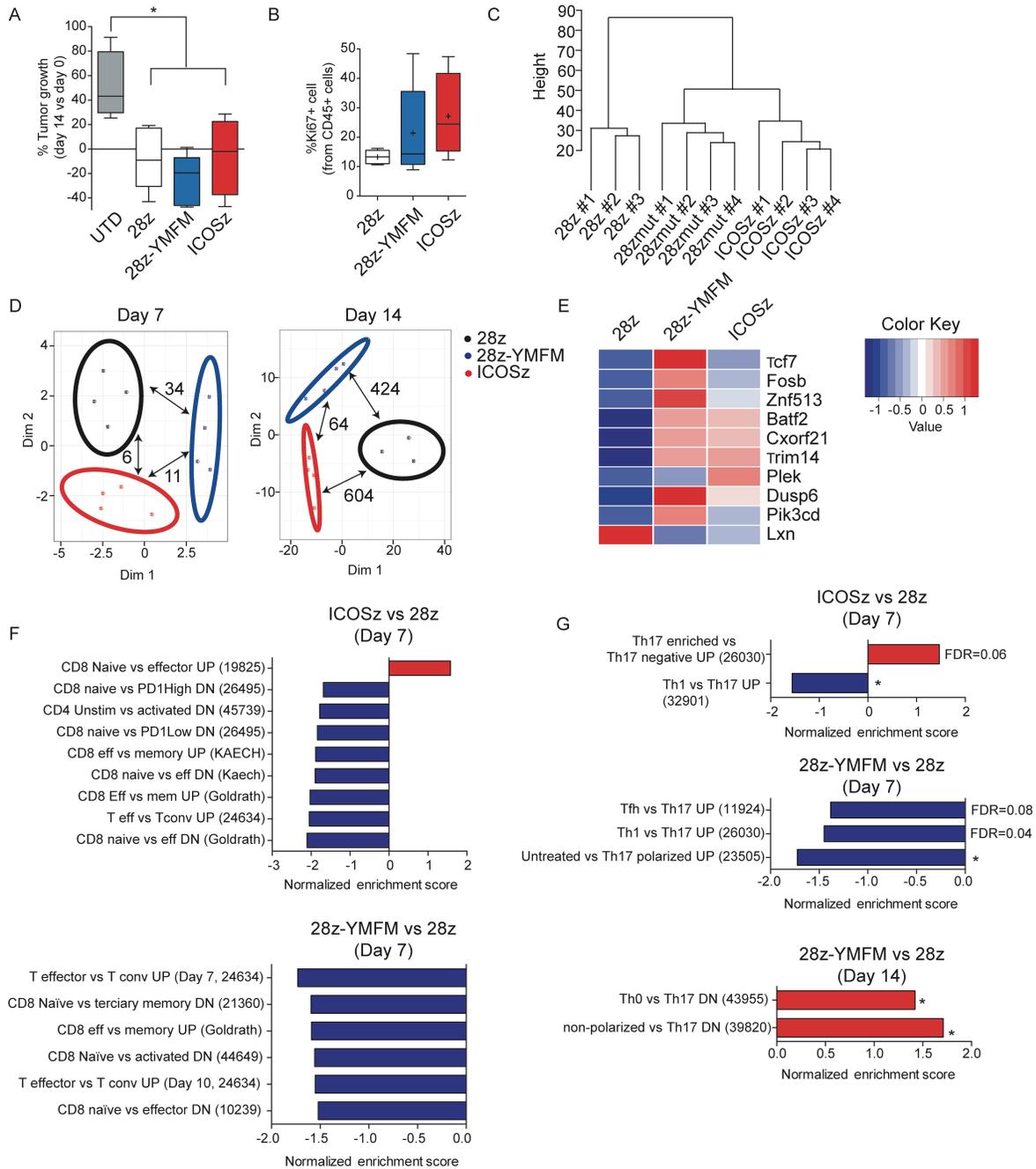


Figure 4. *In vivo* long-term signaling through 28z-YMFM CAR is associated with a transcriptional profile that resembles ICOS signaling. NSG mice bearing s.c. Capan-2 tumors were treated with CAR-T cells and tumor growth was monitored. T cells were isolated from tumors at day 7 and 14 after treatment for analysis. **(A)** Box plots of the change in tumor volume on day 14 versus baseline (n=4-5). *P < 0.05 by 1-way ANOVA with Tukey post hoc test. **(B)** The expression of CD45 and Ki67 on T cells isolated on day 14 after treatment was analyzed by flow cytometry. For A and B, box plots show median (line), mean (plus)

and 25th to 75th percentile (box). The end of the whiskers represents the minimum and the maximum of all of the data. Error bars represent \pm SEM ($n = 4-5$). $p > 0.05$. **(C)** Dendrogram showing the relatedness of gene expression patterns for each individual animal. **(D)** MDS analysis of differentially expressed genes between the 3 sets of CAR-T cells isolated from Capan-2 xenograft tumors ($n=3-4$) on day 7 and day 14. The number of differentially expressed genes ($FDR < 0.05$, $FC < 2$ or $FC > 2$) between a pair of CARs is indicated with pair-connecting arrows. **(E)** Heatmap showing differential expression of genes associated with T-cell differentiation and exhaustion. Genes reported to be preferentially expressed in early stages of T cell differentiation (Tcf7, Fosb, Znf513, Batf2, cxorf2, trim14), genes upregulated in effector cells (Lxn) or downregulated in effector or exhausted T cells (Dusp6 and Pik3cd) are plotted. **(F, G)** Normalized enrichment score of selected up- or down-regulated gene sets associated with T cell differentiation **(F)** or Th17 polarization **(G)** as determined by GSEA using MSigDB C7 gene ontology sets. For all pathways, the false discovery rate (FDR) $q \leq 0.02$ (unless otherwise indicated). Reference numbers for immunology related gene lists from MSigDB C7 are indicated in parantheses.

Figure 5

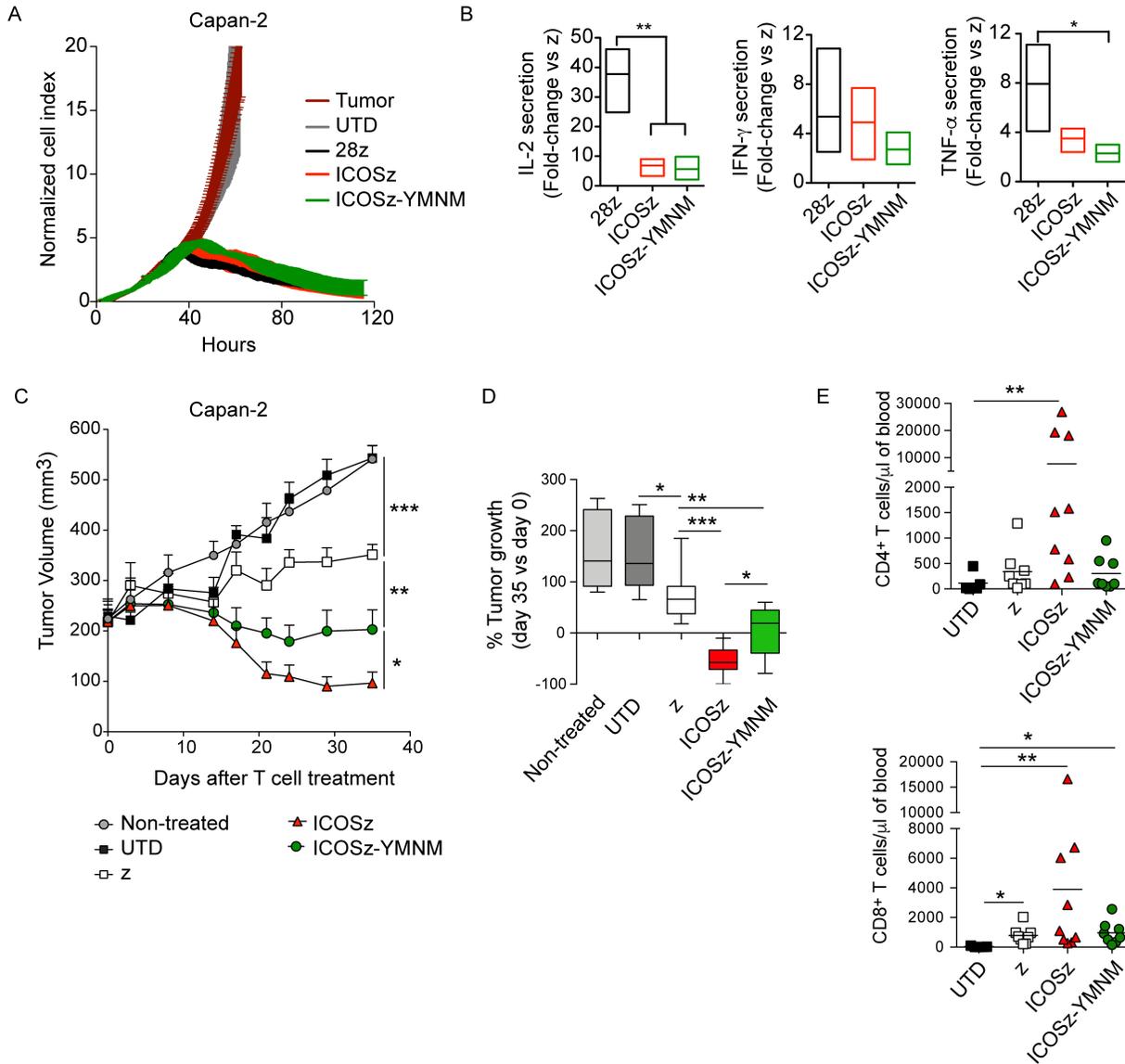


Figure 5. Mutation of the YMFM motif in the ICOS signaling domain to YMNM (CD28 motif) impairs antitumor effect and T cell persistence. (A) A real-time cytotoxicity assay (xCELLigence) was used to evaluate the lysis of Capan-2 tumor cells when treated with CAR-T cells (E:T=3:1) over a 120-hour period. (B) CAR-T cells were cocultured with mesothelin⁺ Capan-2 cells. Supernatants were obtained 24 hours later, and cytokine production was analyzed by ELISA. Fold-change cytokine production in second-generation versus first-generation (z) CAR-T cells was analyzed in 3-4 healthy donors. P values were calculated by 1-way ANOVA with Tukey post hoc test. (C-E) NSG mice bearing s.c. Capan-2 tumors were treated 30 days after tumor implantation with two doses of CAR-T cells (n=6-9). (C) Tumor volume was analyzed at indicated time points. Statistical analysis was performed by 2-way ANOVA followed by Dunnett's multiple comparisons. (D) Box plots of the change in tumor volume on day 35 versus baseline (n=6-9). Box plots show median (line) and 25th to 75th percentile (box). The end of the whiskers represents the minimum and the maximum of all of the data. P values were calculated with two-tailed Mann-Whitney tests. (E) The concentration of CD4⁺ and CD8⁺ T cells was determined in the blood of treated animals 36

days after T cell injection. P values were calculated by Kruskal-Wallis multiple comparisons test. *P < 0.05; **P < 0.01, and ***P < 0.001.