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Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function

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Abstract

Chimeric antigen receptors (CARs) link an antigen-recognition domain to CD28/CD3 ζ or 4-1BB/CD3 ζ intracellular signaling domains to redirect T cell specificity and function. T cells expressing CARs with CD28/CD3 ζ or 4-1BB/CD3 ζ signaling domains are effective at treating refractory B cell malignancies but exhibit differences in effector function, clinical efficacy, and toxicity that are assumed to result from the activation of divergent signaling cascades. We analyzed stimulation-induced phosphorylation events in primary human CD8⁺ CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cells by mass spectrometry and found that both CAR constructs activated similar signaling

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Author Contributions:

A.I.S., R.G.I., D.S., A.G.P., and S.R.R. designed the experiments. A.I.S., R.G.I., J.J.K., A.R., E.J.A., and U.J.V. conducted the experiments. V.V., C.L., J.R.W., J.J.K., and R.G. processed the data and wrote accompanying software. L.L. developed STII CAR technology and provided reagents. A.I.S. and R.G.I. performed formal data analysis. A.I.S. and S.R.R. wrote the manuscript. A.I.S., R.G.I., V.V., R.G., A.G.P. and S.R.R. edited the manuscript. A.G.P. and S.R.R. supervised all work.

Competing Interests:

S.R.R. is a founder, shareholder, and scientific advisor of Juno Therapeutics. R.G. is a consultant for Juno Therapeutics. STII CAR technology used in this study is licensed to Juno Therapeutics. A.I.S. and S.R.R. have filed a provisional patent application number 62/635,450 covering applications of mutant CD28 CARs for cellular therapy. The other authors declare that they have no competing interests.

Data and Materials Availability:

LC-MS/MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD007921. RNA-seq data have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number 109161. All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

intermediates. Stimulation of CD28/CD3 ζ CARs activated faster and larger-magnitude changes in protein phosphorylation, which correlated with an effector T cell–like phenotype and function. In contrast, 4–1BB/CD3 ζ CAR T cells preferentially expressed T cell memory–associated genes and exhibited sustained anti-tumor activity against established tumors in vivo. Mutagenesis of the CAR CD28 signaling domain demonstrated that the increased CD28/CD3 ζ CAR signal intensity was partly related to constitutive association of Lck with this domain in CAR complexes. Our data show that CAR signaling pathways cannot be predicted solely by the domains used to construct the receptor, and that signal strength is a key determinant of T cell fate. Tailoring CAR design based on signal strength may lead to improved clinical efficacy and reduced toxicity.

Introduction

Synthetic receptors that mimic natural T cell signaling cascades are being developed as immunotherapeutic reagents for cancer, autoimmunity, and infections. Chimeric antigen receptors (CARs), chimeric costimulatory receptors, and engineered T cell receptors (TCRs) can be introduced into T cells by gene transfer to redirect specificity and promote signaling pathways that initiate effector T cell functions (1). The most successful of these novel therapeutics to date are CARs, which are comprised of an extracellular antigen-specific single-chain variable (scFv) immunoglobulin fragment fused to intracellular signaling domains that activate T cells upon ligand binding (2). Although treatment with CAR T cells can result in the complete remission of relapsed or refractory B cell malignancies, it can also cause life-threatening toxicities including cytokine release syndrome (CRS) and neurotoxicity (3–12). Both efficacy and toxicity result from activation of intracellular signaling pathways mediated by CAR engagement.

T cell activation occurs following TCR engagement with antigen-specific peptide bound within the major histocompatibility complex (MHC). TCR binding stimulates intracellular phosphorylation of immunoreceptor tyrosine-based activating motifs (ITAMs) on the CD3 δ , ϵ , γ and ζ chains of the TCR (13). Combined with protein phosphorylation signals delivered in *trans* from costimulatory molecules and cytokines, these events alter T cell transcriptional programs, induce proliferation, promote cytotoxic functions, and stimulate cytokine release. Most CARs utilize a simplified format to recapitulate the signals necessary for T cell effector function and proliferation. CAR constructs combine the CD3 ζ endodomain in a single chain with a costimulatory domain from CD28 or 4–1BB. Both CD28/CD3 ζ and 4–1BB/CD3 ζ CAR T cells are effective for treating patients with B cell malignancies but may behave differently in vivo. CD28/CD3 ζ CAR T cells generally undergo intense proliferation within 7 days of transfer and seldom persist more than 60 days after infusion (7, 14). In contrast, 4–1BB/CD3 ζ CAR T cells reach peak number by 7–14 days after adoptive transfer and can persist for several months (8, 15). However, variability in patient cohorts, CAR structures, and clinical trial designs impede robust comparisons across trials. Studies comparing CD28/CD3 ζ and 4–1BB/CD3 ζ CAR T cells in vitro and in animal models partially explain these clinical differences by demonstrating that 4–1BB/CD3 ζ CAR T cells possess greater mitochondrial mass, a more memory T cell-like surface phenotype, and better retain effector functions in settings of chronic antigen stimulation (16, 17).

It is widely assumed that the differences in phenotype and function of CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cells are conferred by activation of divergent signaling pathways through the distinct CAR costimulatory molecule domains. Analyses of signaling pathways stimulated by 4-1BB/CD3 ζ or third-generation CD28/4-1BB/CD3 ζ CARs performed using phospho-flow cytometry, Western blot, or blot array do not identify differences between CD28/CD3 ζ and 4-1BB/CD3 ζ CAR signaling modules and only profile a small number of known signaling events to which there are experimentally-validated antibodies (18, 19). A more comprehensive, unbiased and quantitative approach to examine CAR signaling would be to use liquid chromatography-tandem mass spectrometry (LC-MS/MS). When applied to study TCR signaling, LC-MS/MS uncovered hundreds of novel phosphoprotein signaling events that were missed by antibody-based techniques (20–24).

In this study, we used LC-MS/MS to analyze CAR stimulation-induced signaling events in primary human CD8⁺ T cells that express clinically relevant CD28/CD3 ζ or 4-1BB/CD3 ζ CARs specific for CD19 or ROR1. We found that stimulation through CD28/CD3 ζ and 4-1BB/CD3 ζ CARs produced nearly identical protein phosphorylation events. Instead, stimulation of CD28/CD3 ζ CAR T cells prompted more rapid and intense phosphorylation of signaling intermediates and a more effector cell-like phenotype than stimulation of 4-1BB/CD3 ζ CAR T cells. CD28/CD3 ζ CAR T cells were less potent at eradicating disseminated lymphoma in a xenograft mouse model than 4-1BB/CD3 ζ CAR T cells. Increased basal phosphorylation of the CAR CD3 ζ chain and CAR-associated Lck contributed to the rapid kinetics and stronger signal strength of CD28/CD3 ζ CARs. Thus, the major distinction between CD28/CD3 ζ and 4-1BB/CD3 ζ CARs related not to divergent phosphoprotein signaling pathways, but rather signaling strength and kinetics, which in turn, affect T cell function and fate. These results may inform the design of new therapeutic receptors.

Results

CAR designs containing a Strep-tag II sequence enable selective activation of CAR signaling in primary T cells

TCR signaling has been studied using LC-MS/MS analysis of transformed Jurkat T cells stimulated with anti-CD3 monoclonal antibodies (mAb) (20, 21, 24). Jurkat cells were selected for signaling studies due to the ease at which they can be grown and manipulated with common molecular biology techniques. Primary T cells from human subjects are more appropriate for studying CAR signaling and we previously developed a method to activate CAR signaling in T cells without the need for ligand expressing stimulator cells (25). We modified two lentiviral vectors encoding CD19-specific and ROR1-specific 4-1BB/CD3 ζ CARs that are currently being tested in clinical trials by adding a nine-amino acid Strep-tag II (STII) sequence to the extracellular CAR hinge. For comparison, we cloned structurally identical CD19-specific and ROR1-specific CD28/CD3 ζ CARs containing the STII tag (Figure 1A). All constructs contained a truncated cell surface EGFR (EGFRt) marker downstream of a T2A ribosomal skip element for purification of CAR-expressing T cells (26). Inclusion of the STII sequence does not interfere with CAR T cell recognition or function, and STII CD28/CD3 ζ or 4-1BB/CD3 ζ CAR T cells are efficiently activated and

expanded in vitro by simulation with STII microbeads (Figure 1B) (25). Primary CD8⁺ T cells were transduced with each lentiviral vector, sorted for EGFRt expression, and expanded to $>1.6 \times 10^8$ cells with a single cycle of stimulation before subsequent analysis (Figure 1C). The abundance of CD28/CD3 ζ and 4-1BB/CD3 ζ CARs of each scFv specificity was similar on the cell surface as measured by staining with STII mAb (Figure 1D). CAR T cells also expressed similar amounts of CD45RO, CD62L, CD27, and CD28 (Figure 1E), which are markers associated with T cell memory. We only detected small frequencies of PD-1- or Tim-3-positive CAR T cells, suggesting the cells were not activated or exhausted after cell culture (Figure 1E). Accordingly, $>85\%$ of CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cells were in the G₀/G₁ cell cycle phase, indicating the cells were resting (Figures 1F).

We evaluated canonical T cell signaling events induced by STII ligation and found that cell-free STII microbead stimulation of CARs was similar to CAR antigen expressing tumor cells. ROR1-specific 4-1BB/CD3 ζ CAR T cells were incubated for 45 minutes with increasing amounts of STII microbeads or with ROR1-transduced K562 (K562/ROR1) tumor cells and CD3 ζ Tyr¹⁴² and SLP-76 Ser³⁷⁶ were measured by Western blot. At the highest bead to CAR T cell ratio, the phosphorylation of CD3 ζ and SLP-76 was grossly similar to that observed in lysates from CAR T cells stimulated with K562/ROR1 cells (Figures 1G and S1A). This bead to cell ratio was used for all subsequent experiments. Thus, STII microbead stimulation provided a precise method to selectively activate CAR signaling in primary T cells.

Mass spectrometry identifies common protein phosphorylation events after stimulation of CD28/CD3 ζ or 4-1BB/CD3 ζ CAR T cells

We performed tandem LC-MS/MS analysis to interrogate the signaling pathways activated in CD28/CD3 ζ or 4-1BB/CD3 ζ CARs in an unbiased manner. Human T cells expressing CD28/CD3 ζ or 4-1BB/CD3 ζ CARs were incubated with STII or uncoated (control) microbeads for 10 or 45 minutes (Figure 2A). CD19-specific CAR T cells generated from two different donors were used in two independent experiments, and a third independent experiment utilized ROR1-specific CAR T cells derived from one of the two donors (Figure 2B). Given that ROR1-specific and CD19-specific CARs displayed similar phenotypes across the two donors and were stimulated identically in a ligand-independent manner, the corresponding measurements were considered biological replicates. To provide relative quantitation of phosphopeptides within each experiment, we labeled each trypsin-digested lysate with a unique isobaric Tandem Mass Tag (TMT) and globally enriched for phosphopeptides by both pTyr immunoprecipitation and immobilized metal affinity chromatography (27) (Figure S1B). We identified a total of 26,804 phosphorylation sites across the three experiments corresponding to 4,849 proteins. Among phosphorylation sites, 571 (2.13%) were pTyr, 4,647 (17.33%) were pThr, and 21,586 (80.53%) were pSer (Figure 2C). Considering the stochastic limitations inherent in data-dependent acquisition shotgun proteomics (28), we found considerable overlap in the captured phosphoproteome between replicate experiments (Figure 2D). As previously described in murine T cells, 99% of phosphorylation sites detected in each experiment were present in both unstimulated and

stimulated T cell lysates, enabling quantitation of changes induced by CAR activation (22, 29).

We analyzed well described TCR phosphorylation events within the MS dataset to identify sites that were modified by CAR engagement (13). We calculated the $\log_2$ fold change ($\log_2FC$) value for all phosphorylation sites within each experiment by comparing stimulated samples to appropriate controls (i.e. CD28/CD3 ζ CAR 10 minute stimulation vs. CD28/CD3 ζ CAR 10 minute control). At 10 minutes, phosphorylation of CD3 ζ at Tyr⁸³, Tyr¹¹¹, and Tyr¹⁴² was increased in stimulated CAR T cells (Figure 2E). All CAR T cells displayed increased phosphorylation of CD28 at Tyr²⁰⁹ after 10 minutes, which demonstrated that 4-1BB/CD3 ζ CAR activation stimulated phosphorylation of endogenous CD28. After 45 minutes of stimulation, phosphoprotein signaling spread to downstream TCR nodes and we measured increased phosphorylation of PLC- γ 1 at Ser¹²⁴⁸ and BCL10 at Ser¹³⁸ (Figure 2E) (30, 31). We did not observe reproducible changes at either time point in the phosphorylation of CD3 δ , ϵ , or γ ITAMs. Because these sites are phosphorylated after TCR stimulation (13), our data indicated that CAR signaling only partially mimics endogenous TCR activation.

The MS data suggested that CD28/CD3 ζ CAR stimulation may initiate a greater magnitude $\log_2FC$ than 4-1BB/CD3 ζ CARs at both 10 and 45 minutes and we confirmed this finding by Western blot. Evaluation of cell lysates for CD3 ζ pTyr¹⁴², ZAP-70 pTyr³¹⁹ and PLC- γ 1 pTyr⁷⁸³ demonstrated that both CD28/CD3 ζ and 4-1BB/CD3 ζ CAR stimulation increased phosphorylation of these sites, but we observed more intense ZAP-70 and CAR CD3 ζ phosphorylation in stimulated CD28/CD3 ζ CAR T cells compared to 4-1BB/CD3 ζ CAR T cells (Figure 2F). We also detected a low level of basal CAR CD3 ζ phosphorylation in unstimulated CD28/CD3 ζ CD19 and ROR1 CAR T cells that was not present in 4-1BB/CD3 ζ CAR T cells. Western blot analysis of lysates from primary CD4⁺ T cells transduced with CD19 CARs and stimulated with STII microbeads also demonstrated similar patterns of basal CAR CD3 ζ phosphorylation as well as more rapid and robust phosphorylation of CD3 ζ and SLP-76 within CD28/CD3 ζ CAR T cells (Figure S2A). Constitutive phosphorylation of the CAR CD3 ζ domain or tonic signaling has been shown to occur with some CARs, including a CD19-specific CD28/CD3 ζ CAR that is being used in the clinic (32). Strong tonic signaling has been linked to sequences in the scFv that promote clustering of CAR molecules at the cell surface and results in the upregulation of T cell exhaustion markers (33, 34). Because we did not observe differences in PD-1 or Tim-3 expression, or evidence of CAR clustering in unstimulated CD28/CD3 ζ or 4-1BB/CD3 ζ CAR T cells (Figures 1E–F and Figure S2B), the low level of basal CAR CD3 ζ domain phosphorylation detected here may be distinct from more extreme tonic signaling observed in some CARs with different scFv specificities.

Phosphorylation events mediated by CD28/CD3 ζ or 4-1BB/CD3 ζ CARs differ in kinetics and magnitude

An advantage of shotgun MS is that it can quantitatively and temporally measure thousands of phosphorylation events outside of the canonical TCR signaling pathway to which there are no experimentally validated antibodies. We leveraged the *limma* statistical framework

and associated R package to identify phosphorylation sites that were increased or decreased in abundance after CD28/CD3 ζ and 4-1BB/CD3 ζ CAR ligation (35). We assigned a phosphorylation site to be CAR stimulation-responsive if it was detected in at least two of the three experiments, displayed an average $|\log_2\text{FC}| \geq 0.7$ between stimulated and unstimulated conditions at 10 or 45 minutes, and met a 5% FDR cutoff. A $\log_2\text{FC}$ cutoff of 0.7 was chosen because this represents approximately two standard deviations of the distribution of $\log_2\text{FC}$ values (Figure S3).

Using these cut-offs, 26 phosphorylation sites were identified as stimulation-responsive at 10 minutes after activation of CD28/CD3 ζ CAR T cells. These sites were enriched for proteins in the KEGG TCR signaling pathway including increased phosphorylation of p21 activated kinase 2 (PAK2) at Ser⁶⁴, CD8 alpha (CD8A) at Ser²³¹, protein kinase C θ (PKCT) at Ser³⁷⁰, and proto-oncogene vav (VAV1) at Ser⁷⁴⁸ and Thr⁷⁴⁹ (Figure 3A and Table S1). We also detected increased phosphorylation of neuroblast differentiation-associated protein (AHNAK) at Ser⁵⁸⁵⁷, which activates PLC- γ 1 and is required for T cell calcium (Ca²⁺) mobilization and effector functions (36, 37). In contrast, no sites met $\log_2\text{FC}$ and FDR criteria at 10 minutes after stimulation of 4-1BB/CD3 ζ CAR T cells (Figure 3A and Table S1). The lack of robust alterations in early protein phosphorylation in 4-1BB/CD3 ζ CAR T cells was consistent with Western blot data showing a very low level of phosphorylation of CAR CD3 ζ , ZAP-70, and PLC- γ 1 at 10 minutes after stimulation (Figure 2F).

After 45 minutes of stimulation, more intense protein phosphorylation had occurred, and we detected phosphorylation at 1,289 sites that met the $\log_2\text{FC}$ and FDR cutoffs in either CD28/CD3 ζ or 4-1BB/CD3 ζ CAR T cell samples. These included 1,279 phosphorylation sites from 743 gene products in CD28/CD3 ζ CAR T cells and 522 sites from 346 gene products in 4-1BB/CD3 ζ CAR T cells (Figures 3A and Tables S2–3). These data indicated that stimulation of CD28/CD3 ζ CAR T cells increased phosphorylation at a greater number of sites than did stimulation of 4-1BB/CD3 ζ CAR T cells. However, both CD28/CD3 ζ and 4-1BB/CD3 ζ CAR activation stimulated highly similar changes in the phosphorylation of activation-responsive sites (Figure 3B). Only 12 (0.93%) of the 1,289 phosphorylation sites that met the cut-offs in CD28/CD3 ζ CAR samples exhibited an opposite response after 4-1BB/CD3 ζ CAR stimulation, and only 43 (3.3%) sites exhibited a greater magnitude $\log_2\text{FC}$ after 4-1BB/CD3 ζ CAR stimulation. These observations were consistent with our earlier data suggesting signaling downstream of CD28/CD3 ζ CAR activation was more intense than downstream of 4-1BB/CD3 ζ CAR activation (Figure 3B and Table S2). Furthermore, these subsets of 12 and 43 phosphorylation sites did not map to currently defined 4-1BB signaling networks (38).

Finding relatively few differences in the phosphorylation events stimulated after activation of CD28/CD3 ζ and 4-1BB/CD3 ζ CARs was unexpected. When we questioned how proteins involved in the canonical T cell costimulatory signaling pathways were affected, we found that the phosphorylation of CD28 signaling intermediates VAV1, PIK3C2A, and PKCT was also increased after stimulation of both CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cells (Figure 3C) (39). This result appeared to agree with our earlier observations that endogenous CD28 was phosphorylated after activation of 4-1BB/CD3 ζ CAR T cells (Figure 2E). Within the 4-1BB signaling pathway, phosphorylation of lymphocyte-specific protein 1

(LSP1), a direct target of 4-1BB and TRAF2 signaling, was altered by stimulation of both CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cells (40). Thus, rather than activating divergent costimulatory pathways, as might be predicted from the distinct costimulatory domains encoded by the receptors, our data indicated that activation of CD28/CD3 ζ and 4-1BB/CD3 ζ CARs induced highly similar changes in intracellular protein phosphorylation that encompassed both canonical CD28 and 4-1BB signaling intermediates.

A map illustrating the major pathways and individual protein phosphorylation events affected by CD28/CD3 ζ and 4-1BB/CD3 ζ CAR stimulation includes proteins involved in canonical TCR signaling and MAPK signaling pathways (Figure 4, Table S3). In addition, stimulation of either CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cells affected phosphorylation of actin-cytoskeletal regulatory proteins, RNA processing proteins, nuclear pore proteins, and mitochondrial fission regulators such as DRP1 (41, 42). A list of CAR stimulation-responsive phosphorylation sites is provided in Tables S1–2. Many of these proteins have not been previously associated with TCR or CAR signaling.

Differences in the magnitude of CD28/CD3 ζ and 4-1BB/CD3 ζ CAR signaling persist across time

Phosphorylation events activated by CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cell stimulation were highly similar, but differed in intensity at the vast majority of sites. Signaling strength during T cell activation, measured as a composite of TCR affinity, co-stimulation and cytokine signals, also dictates clonal expansion and the effector capacities of a T cell response (43–45). To provide a holistic measure of CAR signal strength we sorted stimulation-responsive phosphorylation sites at 45 minutes from CD28/CD3 ζ and 4-1BB/CD3 ζ CAR samples by decreasing log₂FC. In line with results showing that stimulation of both CARs altered similar phosphorylation sites, 15 of the 20 most phosphorylated sites after stimulation were shared between CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cells (Table S4). However, phosphorylation of the top 20 sites increased from control samples by 11.15-fold on average in CD28/CD3 ζ CAR samples, but only 5.8-fold on average in 4-1BB/CD3 ζ CAR samples (Figure 3D). Stratifying CAR stimulation-responsive phosphorylation sites by signaling pathway further indicated that the average phosphorylation site within the KEGG TCR signaling pathway was modulated by 2.52-fold in CD28/CD3 ζ CAR samples, but only 1.69-fold in 4-1BB/CD3 ζ CAR samples (Figure 3E). To determine if 4-1BB/CD3 ζ CAR signals reached a similar intensity to that of CD28/CD3 ζ CARs at later times, we stimulated identically prepared CD8⁺ CAR T cells for 60, 120, or 180 minutes and measured phosphorylation of canonical and newly identified signaling intermediates (Table S4). Phosphorylation of PLC- γ 1 Tyr⁷⁸³ and DAPP1 Tyr¹³⁹ was apparent after stimulation of CD28/CD3 ζ but not 4-1BB/CD3 ζ CAR T cells (Figure 3F). These data suggest that 4-1BB/CD3 ζ CAR stimulation may not achieve the same signal intensity as CD28/CD3 ζ CAR T cells during this time frame.

Increased CAR signal intensity is associated with an effector cell-like phenotype and reduced in vivo anti-tumor activity

The strength of T cell activation and signal transduction influences transcriptional programs that regulate effector cell differentiation and memory formation (44). We used RNA-seq to

analyze changes in transcription within CD28/CD3 ζ or 4-1BB/CD3 ζ CD8⁺ CAR T cells at 6 hours after STII microbead stimulation. Consistent with the faster and more intense phosphoprotein signal, CD28/CD3 ζ CAR stimulation initiated more marked transcriptional changes. A comparison of stimulated to unstimulated CD28/CD3 ζ CAR T cells identified 4,894 differentially expressed genes at 6 hours, whereas 4-1BB/CD3 ζ CAR stimulation resulted in 197 differentially expressed genes. CD28/CD3 ζ or 4-1BB/CD3 ζ CAR stimulation increased expression of the canonical T cell activation marker CD69 to a similar degree (Figure 5A), but greater fold increases in expression of the effector molecules granzyme B (*GZMB*), interferon- γ (*IFNG*), interleukin-2 (*IL2*), tumor necrosis factor- α (*TNF*), macrophage inflammatory protein 1 α (*CCL3*), and macrophage inflammatory protein 1 β (*CCL4*) were observed in CD28/CD3 ζ CAR T cells (Figure 5B). Direct comparison of CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cells identified 1,673 differentially expressed genes after stimulation (Table S5). Of these, Krüppel-like factor 2 (*KLF2*), interleukin-7 receptor (*IL7R*), and Rho family-interacting cell polarization regulator 2 (*RIPOR2*, previously known as *FAM65B*) expression was decreased in CD28/CD3 ζ CAR T cells when compared to 4-1BB/CD3 ζ CAR T cells (Figure 5C). *KLF2* and *IL7R* are associated with memory T cell formation and are targets of the FOXO family of transcription factors (46–48). Consistent with this observation, *FOXO4* expression was reduced in stimulated CD28/CD3 ζ CAR T cells when compared to 4-1BB/CD3 ζ CAR T cells. qPCR confirmed that some of these T cell memory-associated genes were only differentially expressed in stimulated and not unstimulated CD28/CD3 ζ or 4-1BB/CD3 ζ CAR T cells (Figure 5D).

Because differences in TCR signal quantity affect T cell functions (44, 45), we measured CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cell effector functions in vitro. We activated CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cells with ROR1 and CD19 expressing K562 cells or STII microbeads, and measured cytokine production and proliferation. After 24 hours of co-culture, CD28/CD3 ζ CAR T cells secreted markedly more IFN- γ , IL-2, and TNF- α than 4-1BB/CD3 ζ CAR T cells (Figure 5E). By 72 hours, CD28/CD3 ζ CAR T cells had proliferated extensively (Figure 5F). These data suggested that stronger signaling by CD28/CD3 ζ CAR T cells correlated with increased short-term effector responses.

We also investigated the function of CD28/CD3 ζ and 4-1BB/CD3 ζ CAR T cells in vivo. For these studies, we used a defined 1:1 CD4:CD8 ratio CAR T cell product that displays superior control of xenograft Raji lymphoma in NOD/SCID/ $\gamma c^{-/-}$ (NSG) mice (49). Adoptive transfer of 3×10^6 CD28/CD3 ζ or 4-1BB/CD3 ζ CAR T cells into tumor bearing mice mediated complete tumor regression (Figure 5G). However, when fewer cells were transferred CD28/CD3 ζ CAR T cells were much less potent at eliminating Raji lymphoma cells than 4-1BB/CD3 ζ CAR T cells, and all CD28/CD3 ζ CAR T cell-treated mice died of tumor progression within 40 days (Figures 5G–H). Tumor progression occurred in mice treated at the lower CD28/CD3 ζ CAR T cell dose despite accumulation of CAR T cells to higher frequencies in tumor-involved bone marrow (Figure 5I). CD28/CD3 ζ CAR T cells in the bone marrow expressed higher levels of PD-1, Lag-3, and Tim-3 (Figure 5J), consistent with the acquisition of an exhausted phenotype. Taken together, the rapid and intense signaling mediated by CD28/CD3 ζ CAR activation correlated with an exhausted phenotype in CD8⁺ and CD4⁺ CAR T cells and reduced anti-tumor activity.

CD28/CD3 ζ and 4-1BB/CD3 ζ CARs differentially associate with endogenous CD28 and Lck

To interrogate possible causes of increased CD28/CD3 ζ CAR signaling kinetics and strength, we immunoprecipitated CAR complexes from unstimulated CD8⁺ T cells and probed for differences among associated T cell signaling proteins in the basal state. Western blot confirmed efficient CAR pull-down and showed association of endogenous CD28 and Lck with the CD28/CD3 ζ CAR, but only minimal CD28 and Lck association with the 4-1BB/CD3 ζ CAR (Figure 6A). Because basal CAR phosphorylation was conferred by the presence of the CD28 costimulatory domain and Tyr²⁰⁶, Tyr²⁰⁹, and Tyr²¹⁸ were intensely phosphorylated after CAR stimulation, we constructed CD28/CD3 ζ CARs with tyrosine to phenylalanine mutations at these residues (Figure 6B). CD19 and ROR1 CARs with mutations of Y218F (Y1) or all three tyrosines (Y3) were efficiently expressed in T cells and led to vigorous IFN- γ production and proliferation in response to co-culture with ROR1⁺ or CD19⁺ tumor cells (Figures 6C–D). We observed partial (Y1) or complete (Y3) abrogation of basal CAR CD3 ζ phosphorylation in CARs containing Y to F mutations as compared to CD28/CD3 ζ CARs, however Y1 and Y3 CARs still phosphorylated SLP-76 and PLC- γ 1 with similar kinetics and intensity as the wild-type CD28/CD3 ζ CAR after STII microbead stimulation (Figure 6E). Immunoprecipitation of each CAR indicated that Lck association was not abrogated by the Y1 and Y3 mutations, although endogenous CD28 did not associate with the Y3 CAR (Figure 6F). Taken together, these results suggested that neither basal CAR CD3 ζ phosphorylation nor endogenous CD28 association were responsible for the increased signal kinetics and strength of CD28/CD3 ζ CARs.

Constitutive Lck activation promotes T cell effector functions (50, 51). Therefore, we tested whether association of Lck with CD28/CD3 ζ CARs mediated rapid and robust phosphorylation of signaling intermediates after CAR activation. We generated proline to alanine mutations at the Lck binding site of CD28 (Figure 7A) (52, 53).

Immunoprecipitation of the wild-type and mutant CD28/CD3 ζ CARs showed that Lck association was absent in CARs with proline to alanine mutations (Figures 7B and S4). Signaling analyses of these constructs demonstrated mutation of proline residues alone in CD28P CARs partially abrogated basal CAR CD3 ζ phosphorylation but did not reduce signal intensity (Figure 7C). However, simultaneous mutation of proline and tyrosine residues in Y3P CARs fully abrogated basal CAR phosphorylation and reduced the magnitude of SLP-76 and PLC- γ 1 phosphorylation after stimulation (Figure 7C). Thus, differences in CAR signal intensity between CD28/CD3 ζ CARs and 4-1BB/CD3 ζ CARs were in part related to greater Lck association with CD28/CD3 ζ CARs. These data suggest that CAR signal intensity could be altered by mutating tyrosine and proline residues in the CD28 signaling domain.

Discussion

The adoptive transfer of CD28/CD3 ζ or 4-1BB/CD3 ζ CD19 CAR T cells is an effective therapy for patients with certain B cell malignancies, but can cause serious toxicities that originate from CAR signaling after tumor recognition (1). However, CD28/CD3 ζ or 4-1BB/CD3 ζ CAR signaling modules confer differences in effector functions and metabolic profiles, and both types of CAR T cells can cause serious toxicities that originate from CAR

signaling after T cell activation (16, 17). Crafting safer and more effective CAR T cells might be accomplished by modifying CAR design, but optimization is hindered by the rudimentary understanding of how signaling by synthetic CARs directs T cell functional outputs and cell fate decisions.

We used mass spectrometry to study changes in the phosphoproteome of T cells after CAR ligation and captured 26,804 unique phosphorylation sites that describe changes induced by CD28/CD3 ζ and 4-1BB/CD3 ζ CARs in detail. Our experiments utilized structurally-identical CARs specific for two target antigens being pursued in clinical trials. In contrast to the current perception of how costimulatory domains function in CARs, our data show that, in the space of this dataset, CD28/CD3 ζ and 4-1BB/CD3 ζ CAR activation initiated nearly identical directional changes in protein phosphorylation. Notably, both CARs altered phosphorylation of proteins involved in canonical TCR, CD28, and 4-1BB signaling pathways. Only 12 of 1,289 (0.93%) phosphorylation sites exhibited opposite responses to stimulation when comparing CAR constructs at 45 minutes after activation, and these few sites are not directly linked to either CD28 or 4-1BB signaling pathways. Therefore, our data refute the notion that CD28/CD3 ζ and 4-1BB/CD3 ζ CARs activate divergent signaling cascades and indicate that one cannot predict the signaling cascades initiated synthetic CARs by focusing solely on the domains encoded by the receptor polypeptide. Rather, synthetic CARs initiate a plethora of signals that both encompass and surpass those of the natural molecules on which the synthetic receptors are based.

Instead, our data demonstrate that a major difference between CD28/CD3 ζ and 4-1BB/CD3 ζ CARs is that CD28/CD3 ζ receptors signal with markedly increased kinetics and intensity. Within 45 minutes, CD28/CD3 ζ CAR stimulation altered the phosphorylation state of hundreds of proteins involved in TCR signaling, MAP kinase signaling, actin-cytoskeleton rearrangement, and RNA processing pathways. Alterations in protein phosphorylation after 4-1BB/CD3 ζ CAR stimulation affected identical proteins as CD28/CD3 ζ CAR stimulation but proceeded more slowly and with reduced intensity. Subsequent analysis of CAR binding partners demonstrated more Lck in immunoprecipitates from unstimulated CD28/CD3 ζ CAR T cells than 4-1BB/CD3 ζ CAR T cells. Mutational analysis of the CAR CD28 signaling domain identified residues responsible for basal CAR CD3 ζ phosphorylation as well as Lck association, and showed that abrogation of these features reduced CAR signal intensity. Thus, future analyses of novel CAR designs should consider how interactions with potential binding partners might affect signaling.

Our data also showed that the more rapid and intense phosphoprotein signaling in CD28/CD3 ζ CAR T cells induced an effector-cell like transcriptional profile and more robust T cell cytokine production early after CAR ligation, but less potent anti-tumor function in an in vivo tumor model of disseminated lymphoma. These data are consistent with prior findings that intense TCR signaling and IL-2 production promote differentiation of short-lived effector CD8⁺ T cells (54, 55), and also support a model by which TCR and costimulatory signals sum linearly to impact cell fate (45). Some amount of CD28 signaling is beneficial for T cell memory formation (56), and we found the 4-1BB/CD3 ζ CARs used in this study phosphorylated endogenous CD28 and activated the CD28 signaling pathway. However, encoding a fully-functional CD28 signaling domain on a CAR polypeptide chain

may yield excessive stimulation that increases the incidence of cytokine release syndrome, promotes T cell exhaustion and reduces persistence. Indeed, analyses of acute lymphoblastic leukemia patients who were in morphologic relapse at the time of treatment with CD28/CD3 ζ CAR T cells showed poor long-term CAR T cell persistence and low overall survival due to frequent relapse with CD19⁺ disease (57).

CARs are often currently selected to maximize measures in vitro T cell effector functions and proliferation, and our findings indicate that these selection criteria may not be optimal for predicting in vivo efficacy. Future optimizations to CAR design should consider both the signaling domains contained within the receptor, interactions with potential binding partners, and how modifications of costimulatory domains might tailor overall signal strength for downstream T cell functional outputs. Mutating the CAR CD28 signaling domain is a logical approach for reducing excessive signal strength and further studies of mutated CAR signaling domains are warranted. However, variability in CAR structural features outside of the costimulatory CD28 or 4-1BB and CD3 ζ signaling domains may limit the universality of this approach. Most CARs possess a unique combination of scFv, hinge, and transmembrane domain and this structural variation can affect CAR signaling and may alter association with endogenous T cell signaling molecules. For instance, the CAR CD28 transmembrane domain utilized in this study may have influenced association with endogenous CD28 and Lck. Further immunoprecipitation analysis of CARs with CD8 α transmembrane domains and other structural features could answer this question (3).

Our approach evaluating CD28/CD3 ζ and 4-1BB/CD3 ζ CAR signaling using a cell free stimulation method has the advantage of identifying phosphorylation events induced by the synthetic receptors in isolation. While this approach did not integrate the multitude of other events that may occur when CAR T cells encounter tumor cells in vivo (58), it could be extended to interrogate cross-talk between CAR signaling and costimulatory or inhibitory pathways by immobilizing various ligands onto the magnetic beads. Our approach was also not designed to compare TCR and CAR signaling. A direct comparison of TCR and CAR ligation-induced phosphoprotein signaling is likely to be valuable and is a focus of ongoing research. Thus, MS analysis can provide important insights into CAR signaling that will impact next-generation CAR designs.

Materials and Methods

Acquisition of peripheral blood T cells from healthy donors

Healthy adults (>18 years-old) were enrolled in an Institutional Review Board-approved study for peripheral blood collection. Informed consent was obtained from all enrollees. Researchers were blinded to all personally-identifiable information about study participants and were provided only donor age and a nondescript donor ID number. 400cc of peripheral blood was collected by venipuncture and mononuclear cells (PBMC) were isolated by density gradient using Lymphocyte Separation Media (Corning). CD4⁺ and CD8⁺ T cells were isolated using the EasySep Human CD4⁺ and CD8⁺ T Cell Isolation Kits (Stem Cell Technologies); For the three shotgun mass spectrometry experiments, CD8⁺CD62L⁺ T cells were further enriched by staining with CD62L-PE (DREG-56, ThermoFisher) followed by

the EasySep Human PE Selection Kit (Stem Cell Technologies). Isolations were performed in accordance with manufacturer's instructions.

Cell Culture

293T LentiX cells (Clontech) were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum, 1mM L-glutamine (Gibco), 25mM HEPES (Gibco), and 100U/mL penicillin/streptomycin (Gibco). K562 (CCL-243) and Raji (CCL-86) cells were obtained from ATCC and cultured in RPMI-1640 (Gibco) supplemented with 5% fetal bovine serum, 1mM L-glutamine, 25mM HEPES, and 100U/mL penicillin/streptomycin. Primary human T cells were cultured in CTL medium consisting of RPMI-1640 supplemented with 10% human serum, 2mM L-glutamine, 25mM HEPES, 100U/mL penicillin/streptomycin, 50 μ M β -Mercaptoethanol (Sigma), and 50 U/mL human IL-2 (Prometheus). All cells were cultured at 37°C and 5% CO₂, and tested bi-monthly for the absence of mycoplasma using MycoAlert Mycoplasma Detection Kit (Lonza).

Generation of chimeric antigen receptors (CARs) and recombinant lentiviral vectors

CD19-specific and ROR1-specific CAR constructs have been previously described (49, 59). For work in this manuscript, a single Strep-tag II sequence and two G₄S linkers were inserted between the FMC63 or R12 single chain variable fragment and IgG4 hinge (25). These were linked to the 27 amino acid transmembrane domain of human CD28 (UniProt: P10747) and to a signaling module comprising either (i) the 41 amino acid cytoplasmic domain of human CD28 with an LL→GG substitution located at positions 186–187 of the native CD28 protein (60), or (ii) the 42 amino acid cytoplasmic domain of human 4-1BB (UniProt: Q07011), each of which was linked to the 112 amino acid cytoplasmic domain of isoform 3 of human CD3 ζ (UniProt: P20963–3). Mutant CD28/CD3 ζ CARs with tyrosine to phenylalanine substitutions at CD28 UniProt positions 206, 209, and 218 and/or proline to alanine substitutions at CD28 UniProt positions 208 and 211 were generated by site-direct mutagenesis. All CAR constructs were linked by T2A sequence to a truncated epidermal growth factor receptor (EGFRt), codon-optimized, and cloned into a HIV7 lentiviral vector. For fluorescence microscopy, the CD3 ζ endodomain was directly fused to eGFP. To make CAR antigen-expressing K562 cells, amino acids 1–325 of human CD19 (UniProt: P15391) were cloned into a HIV7 lentiviral vector and amino acids 1–937 of human ROR1 (UniProt: Q01973) were cloned into a mp71 retroviral vector which was a gift of Dr. Wolfgang Uckert, Max Delbrück Center for Molecular Medicine. All cloning was performed by PCR, enzyme digest, and/or Gibson assembly. Plasmids were verified by capillary sequencing and restriction digest.

Lentivirus preparation and transduction

To prepare CAR T cells, LentiX cells were transiently transfected with the HIV7 CAR vector, as well as psPAX2 (Addgene Plasmid # 12260) and pMD2.G (Addgene Plasmid # 12259) packaging plasmids. One day later (day 1), primary T cells were activated using Dynabeads Human T-Activator CD3/CD28 (ThermoFisher) and cultured in CTL supplemented with 50 U/mL IL-2. The next day (day 2), lentiviral supernatant was harvested from LentiX cells, filtered using 0.45 μ m PES syringe filters (Millipore), and added to activated T cells. Polybrene (Millipore) was added to reach a final concentration of

4.4µg/mL and cells were spinoculated at 800×g and 32°C for 90 minutes. Viral supernatant was replaced 8 hours later with fresh CTL supplemented with 50 IU/mL IL-2. Half-media changes were then performed every 48 hours using CTL supplemented with 50 U/mL IL-2. Dynabeads were removed on day 6; CD8⁺EGFRt⁺ transduced T cells were FACS-sorted on a FACSARIA II (BD Biosciences) on day 9.

To prepare K562/CD19 cells, LentiX cells were transiently transfected with psPAX2, pMD2.G, and an HIV7 lentiviral vector encoding CD19. To prepare K562/ROR1 cells, LentiX cells were transiently transfected with MLV g/p, 10A1, and a mp71 retroviral vector encoding ROR1. To prepare Raji/ffluc cells, LentiX cells were transiently transfected with psPAX2, pMD2.G, and an HIV7 lentiviral vector encoding GFP and firefly luciferase. Two days later, viral supernatant was filtered using a 0.45µm PES syringe filter, and added to K562 or Raji cells. Five days later, transduced cells were stained monoclonal antibodies specific for CD19 (HIB19, Biolegend) or ROR1 (2A2, Miltenyi Biotec) and FACS-sorted on a FACSARIA II to greater than 97% purity.

T cell expansion for mass spectrometric and functional analyses

FACS-sorted CD8⁺EGFRt⁺ cells were expanded over a single stimulation cycle prior to MS and/or functional analyses. CD19-specific CAR T cells were expanded by co-culture with irradiated CD19⁺ lymphoblastoid cell lines (LCL) in a 1:7 (T cell:LCL) ratio and assayed 8 days after stimulation. ROR1-specific CAR T cells were expanded using a rapid expansion protocol containing purified OKT3, irradiated LCL, and irradiated PBMC, and assayed 11 days after stimulation. During expansion, cultures were fed with fresh CTL media containing 50 IU/mL IL-2 every 2–3 days.

Flow cytometry and cell phenotyping

T cells were stained with a 1:100 dilution of fluorophore-conjugated monoclonal antibodies specific for human CD4 (RPA-T4), CD8 (SK1), CD27 (M-T271), CD28 (CD28.2), CD45 (HI30), CD45RO (UCHL1), CD62L (DREG-56), CD223 (3DS223H), CD279 (eBioJ105), or CD366 (F38–2E2) purchased from BD Biosciences, ThermoFisher, or Biolegend. T cells were also stained with isotype control fluorophore-conjugated antibodies when appropriate. Cetuximab (anti-EGFR, Bristol Myers Squibb) and 3E8 (anti-STII, FHCRC) were biotinylated using the EZ-Link Sulfo-NHS-Biotin kit (ThermoFisher) followed by cleanup with a Zeba Spin Desalting Column (ThermoFisher) and used to stain T cells in conjunction with Streptavidin-APC (ThermoFisher). DNA content staining was performed by fixing T cells with 70% ice-cold ethanol, permeabilizing cells with 1% Triton-X (Sigma), degrading RNA with 100µg/mL RNase A (ThermoFisher), and staining DNA with 20µg/mL Propidium Iodide (ThermoFisher). All data was collected on a FACSCanto II or FACSARIA II (BD Biosciences).

STII and control microbead preparation

1mL Streptavidin Coated Magnetic Particles (Spherotech) was washed once in excess 1× PBS supplemented with 100 U/mL penicillin/streptomycin (PBS+P/S) using a benchtop magnet. STII microbeads were prepared by resuspending beads in 1mL PBS+P/S and then slowly adding 16.67µg STII biotin mAb (GenScript) while vortexing. Beads were incubated

overnight at 4°C on a 3D orbital shaker, washed three times with excess PBS+P/S using a benchtop magnet, and resuspended in 4mL PBS+P/S. To make control beads, 1mL Streptavidin Coated Magnetic Particles were washed once using a benchtop magnet and resuspended in 4mL PBS+P/S. Beads were stored at 4°C.

Cell stimulations, protein lysates, RNA isolation

CAR T cells were washed and resuspended in warm CTL medium at a concentration of 2×10^7 cells per mL. Cells were then incubated with STII or control microbeads at a ratio of 30 μ L beads per 1×10^6 cells in a 37°C water bath. After the allotted time, cells were quickly washed twice using ice-cold PBS and lysed in a 6M Urea, 25mM Tris (pH 8.0), 1mM EDTA, 1mM EGTA solution supplemented with protease (Sigma) and phosphatase inhibitors (Sigma) at a 1:100 dilution, hereon referred to as lysis buffer. Lysates were sonicated for 15 seconds prior to centrifuging at 10,000 $\times$ g and 4°C for 10 minutes. Beads were removed during lysate clearing. After 6 hours of stimulation, RNA isolations were performed using a NucleoSpin RNA kit (Macherey-Nagel) according to manufacturer's instructions. Beads were removed using a benchtop magnet prior to cell lysis and RNA extraction.

Protein digestion, TMT labeling, and phosphotyrosine (pTyr) peptide immunoprecipitation

Protein was quantified in lysates by Micro BCA Assay (ThermoFisher), and lysates were diluted to 2mg/mL using lysis buffer. Lysates were reduced in 24mM TCEP (ThermoFisher) for 30 minutes at 37°C with shaking, followed by alkylation with 48mM iodoacetamide (Sigma) in the dark at room temperature for 30 minutes. Lysates were then diluted with 200mM Tris (pH 8.0), to a urea concentration of 2M. Lys-C (Wako) was dissolved in 25mM Tris (pH 8.0) at 200ug/mL and added to lysates at 1:100 (enzyme:protein) ratio by mass and incubated for 2 hours at 37°C with shaking. Samples were further diluted with 200mM Tris (pH 8.0) to a urea concentration of 1M before adding trypsin at a 1:50 trypsin:protein ratio. After 2 hours, a second trypsin aliquot was added at a 1:100 trypsin:protein ratio. Digestion was carried out overnight at 37°C with shaking. After 16 hours, the reaction was quenched with formic acid to a final concentration 1% by volume. Samples were desalted using Oasis HLB 96-well plates (Waters) and a positive pressure manifold (Waters). The plate wells were washed with $3 \times 400\mu$ L of 50% MeCN/0.1% FA, and then equilibrated with $4 \times 400\mu$ L of 0.1% FA. The digests were applied to the wells, then washed with $4 \times 400\mu$ L 0.1% FA before being eluted drop by drop with $3 \times 400\mu$ L of 50% MeCN/0.1% FA. The eluates were lyophilized, followed by storage at -80°C until use. For TMT labeling (ThermoFisher), desalted peptides were resuspended in 50 mM HEPES at 1mg/mL based on starting protein mass. TMT reagents were resuspended in 257 μ L MeCN and transferred to the peptide sample. Samples were incubated at room temperature for 1 hour with mixing. Labeling reactions were quenched by the addition of 50 μ L of 5% hydroxyl Amine (Sigma) and incubated for 15 minutes at room temperature with mixing. The independent labeling reactions were then pooled together and lyophilized. The labeled peptides were desalted as above and then lyophilized and stored at -80°C. Immunoprecipitation of pTyr peptides was performed using PTMScan P-Tyr-1000 kit (Cell Signaling). The enriched pTyr peptide fraction was purified using a C18 Spin Tip (ThermoFisher), lyophilized, and stored at -80°C until analysis. The flow-through fraction was desalted, lyophilized, and stored at -80°C.

Basic (high pH) reverse phase liquid chromatography

The desalted and pTyr peptide-depleted flow-through was fractionated by high-pH reverse phase (RP) liquid chromatography. 4mg of the protein digest was loaded onto a LC system consisting of an Agilent 1200 HPLC with mobile phases of 5mM NH_4HCO_3 (pH 10) (A) and 5mM NH_4HCO_3 in 90% MeCN (pH 10) (B). The peptides were separated by a 4.6mm $\times$ 250mm Zorbax Extend-C18, 3.5 μm , column (Agilent) over 96 minutes at a flow rate of 1.0mL/min by the following timetable: hold 0% B for 9 minutes, gradient from 0 to 10% B for 4 minutes, 10 to 28.5% B for 50 minutes, 28.5 to 34% B for 5.5 minutes, 34 to 60% B for 13 minutes, hold at 60% B for 8.5 minutes, 60 to 0% B for 1 minute, re-equilibrate at 0% B for 5 minutes. Fractions were collected at 1 minute intervals from 0–96 minutes by the shortest path by row in a 1 mL deep well plate (ThermoFisher). The high pH RP fractions were concatenated into 24 samples by every other plate column starting at minute 15 (e.g.: sample 1 contained fractions from wells B10, D10, F10, etc.). The remaining fractions were combined such that fractions from 12 to 14 minutes were added to sample 1, all fractions after 86 minutes were added to sample 24, and all fractions from 0 to 11 minutes were combined into sample 'A'. 95% of every 12th fraction of the 24 samples was combined (1,13; 2,14; ...) to generate 12 more samples, which were dried down and stored at -80°C prior to phosphopeptide enrichment by immobilized metal affinity chromatography.

Immobilized metal affinity chromatography (IMAC)

IMAC enrichment was performed using Ni-NTA-agarose beads (Qiagen) stripped with EDTA and incubated in a 10mM FeCl_3 solution to prepare Fe^{3+} -NTA-agarose beads. Fractionated lysate was reconstituted in 200 μL of 0.1% TFA in 80% MeCN and incubated for 30 minutes with 100 μL of the 5% bead suspension while mixing at room temperature. After incubation, beads were washed 3 times with 300 μL of 0.1% TFA in 80% MeCN. Phosphorylated peptides were eluted from the beads using 200 μL of 70% ACN, 1% Ammonium Hydroxide for 1 minute with agitation at room temperature. Samples were transferred into a fresh tube containing 60 μL of 10% FA, dried down and re-suspended in 0.1% FA, 3% MeCN. Samples were frozen at -80°C until analysis.

Nano-liquid chromatography-tandem mass spectrometry

Phosphopeptide-enriched samples were analyzed by LC-MS/MS on an Easy-nLC 1000 (ThermoFisher) coupled to an LTQ-Orbitrap Fusion mass spectrometer (ThermoFisher) operated in positive ion mode. The LC system, configured in a vented format consisted of a fused-silica nanospray needle (PicoTip emitter, 50 μm ID $\times$ 20cm, New Objective) packed in-house with ReproSil-Pur C18-AQ, 3 μm and a trap (IntegraFrit Capillary, 100 μm ID $\times$ 2 cm, New Objective) containing the same resin as in the analytical column with mobile phases of 0.1% FA in water (A) and 0.1% FA in MeCN (B). The peptide sample was diluted in 20 μL of 0.1% FA, 3% MeCN, and 8.5 μL was loaded onto the column and separated over 210 minutes at a flow rate of 300 nL/min with a gradient from 5 to 7% B for 2 minutes, 7 to 35% B for 150 minutes, 35 to 50% B for 1 minute, hold 50% B for 9 minutes, 50 to 95% B for 2 minutes, hold 95% B for 7 minutes, 95 to 5% B for 1 minute, re-equilibrate at 5% B for 38 minutes. A spray voltage of 2000 V was applied to the nanospray tip. MS/MS analysis occurred over a 3 second cycle time consisting of 1 full scan MS from 350–1500 m/z at

resolution 120,000 followed by data dependent MS/MS scans using HCD activation with 27% normalized collision energy of the most abundant ions. Selected ions were dynamically excluded for 45 seconds after a repeat count of 1.

Immunoprecipitation

Protein G Dynabeads (ThermoFisher) were incubated with anti-STII antibody (GenScript) for 60 minutes, cross-linked for 30 minutes using 20mM dimethyl pimelimidate (ThermoFisher) diluted in 200 mM triethanolamine (Fisher Scientific), quenched with 150mM monoethanolamine (Fisher Scientific), and washed three times with 1× PBS. T cells were lysed in NP40 Cell Lysis Buffer (ThermoFisher) supplemented with protease and phosphatase inhibitors. Lysates were incubated on ice for 15 minutes and then centrifuged at 10,000×g and 4°C for 10 minutes. Immunoprecipitations were performed according to manufacturer's instructions where Dynabeads were incubated with equal masses of cleared lysates for 90 minutes at room temperature.

Western blotting

Equal masses of protein lysate or equal volumes of immunoprecipitation eluents were loaded into NuPAGE Gels (ThermoFisher). After protein transfer onto nitrocellulose membranes (ThermoFisher), membranes were blocked with Western Blocking Reagent (Sigma). Membranes were stained with primary and secondary antibodies diluted in SuperBlock (ThermoFisher) supplemented with 0.1% Tween. The following antibodies were used: anti-human CD247 (8D3, BD Biosciences), anti-human CD247 pTyr142 (K25–407.69, BD Biosciences), anti-ZAP-70 pTyr319 (65E4, Cell Signaling), anti-SLP-76 (polyclonal, Cell Signaling), anti-SLP-76 pSer376 (D9D6E, Cell Signaling), anti-PLC-g1 (D9H10, Cell Signaling), anti-PLC-g1 pTyr783 (D6M9S, Cell Signaling), anti-DAPP1 (D9K4O, Cell Signaling), and anti-DAPP1 pTyr139 (D7G4G, Cell Signaling), anti-Lck (D88, Cell Signaling), anti-CD28 (D2Z4E, Cell Signaling), anti-mouse HRP (polyclonal, Cell Signaling), and anti-rabbit HRP (polyclonal, Cell Signaling). Typical antibody dilutions ranged from 1/10,000 to 1/2,500. Band intensities were quantified using ImageJ (NIH), normalized to total protein, loading control or immunoprecipitation input, and then re-normalized to a control sample.

RNA sequencing (RNA-seq)

RNA was extracted from 24 samples from three donors. Total RNA integrity was checked using an Agilent 4200 TapeStation (Agilent Technologies) and quantified using a Trinean DropSense96 spectrophotometer (Caliper Life Sciences). RNA-seq libraries were prepared from total RNA using the TruSeq RNA Sample Prep Kit v2 (Illumina) and a Sciclone NGSx Workstation (PerkinElmer). Library size distributions were validated using an Agilent 4200 TapeStation. Additional library quality control, blending of pooled indexed libraries, and cluster optimization were performed using a Qubit 2.0 Fluorometer (ThermoFisher). RNA-seq libraries were pooled (6–8-plex) and clustered onto a flow cell lane. Sequencing was performed using an Illumina HiSeq 2500 in rapid mode employing a paired-end, 50 base read length (PE50) sequencing strategy.

Quantitative PCR

RNA was extracted and total RNA integrity was verified using an Agilent 4200 TapeStation (Agilent Technologies). 500ng RNA was used to prepare cDNA with SuperScript III (ThermoFisher). 15ng cDNA was added to a reaction with TaqMan Universal Master Mix II with UNG (ThermoFisher) and one of the following ThermoFisher TaqMan assay probes: Hs00172973_m1 (*FOXO4*), Hs00902234_m1 (*IL7R*), Hs00360439_g1 (*KLF2*), or Hs99999907_m1 (*B2M*). Reactions were run in duplicate or triplicate on one 384-well plate.

Ct values were calculated by dividing mean Ct of technical replicates from *FOXO4*, *IL7R*, and *KLF2* probes by the mean Ct of the housekeeping gene $\beta 2$ microglobulin (*B2M*). CD28/CD3 ζ versus 4–1BB/CD3 ζ ratios for each donor were calculated and subjected to a log₂ transformation.

In vitro functional assays

CAR T cells were co-cultured with K562, K562/CD19, or K562/ROR1 cells at a T cell to tumor cell ratio of 2:1. In some experiments, CAR T cells were also incubated with control or STII microbeads at a ratio of 30 μ L beads per million cells. Cytokine concentrations in cellular supernatant were quantified by ELISA (ThermoFisher) 24 hours after stimulation. T cell proliferation was quantified by staining CAR T cells with a 0.2 μ M solution of carboxyfluorescein succinimidyl ester (CFSE) dye (ThermoFisher) and incubating CAR T cells with K562/CD19, K562/ROR1 cells, control beads, or STII microbeads for 72 hours.

Transfer of T cells in NOD/SCID/ γ c^{-/-} (NSG) mice

Six- to eight-week-old male or female NSG mice were obtained from the Jackson Laboratory or bred in-house. Mice were engrafted via tail vein with 5×10^5 CD19⁺ Raji/ffluc cells, and 7 days later, injected intravenously with PBS or a defined product of purified CD8⁺ and CD4⁺ CD19-specific CAR T cells mixed together in a 1:1 ratio. Bioluminescence imaging was performed as described (49). Mice were either followed for survival or sacrificed on day 20 for analysis of T cell frequencies and phenotypes by flow cytometry. Peripheral blood was extracted, red blood cells were lysed using ACK Lysing Buffer (ThermoFisher), and remaining cells were stained with fluorochrome-labeled mAbs. Bone marrow was isolated from hind-limbs by mechanical disruption followed by red blood cell lysis, and staining with fluorochrome-labeled mAbs. Mice handlers were blinded to group allocation. The Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee approved all experimental procedures.

Fluorescence microscopy

CD8⁺ T cells from healthy donors were transduced as previously described. Instead of FACS-purification on day 9, cells were imaged on a Deltavision Elite microscope (GE Healthcare). At least 8 cells were visualized per condition. Raw images were subjected to a linear adjustment of brightness and contrast using ImageJ (NIH).

Shotgun mass spectrometry data analysis

Raw MS/MS spectra from each replicate experiment were searched together against the reviewed Human Universal Protein Resource (UniProt) sequence database (release 2016_01)

with common laboratory contaminants using the MaxQuant/Andromeda search engine version 1.6.0.1 (61). The search was performed with a tryptic enzyme constraint for up to two missed cleavages. Variable modifications were oxidized methionine, phosphorylated serine, phosphorylated threonine, and phosphorylated tyrosine. Carbamidomethylated cysteine was set as a static modification. Peptide MH⁺ mass tolerances were set at 20 ppm. The overall FDR was set at 1% using a reverse database target decoy approach.

For the three TMT experiments, phosphopeptide site localization was determined by MaxQuant and converted to phosphorylation sites using Perseus version 1.6.0.7 (62). At this step, reverse hits and potential contaminants were excluded from further analysis. Data normalization was performed by scaling each TMT channel to the channel median, followed by a log₂ transformation. Stimulation vs. control ratios were calculated by subtracting the appropriate control channels from stimulated channels. Due to incomplete MS sampling, some phosphorylation sites (features) were only found in one or two replicate experiments, and a much smaller minority (< 1%) of sites were not found in every TMT channel.

Differential expression analyses over phosphorylation sites were performed using the limma statistical framework and associated R package (35, 63). For these analyses, we chose to keep only features that had values in at least two experiments and all TMT channels, leaving us with 14,490 quantified phosphorylation sites. A linear model was fitted to each phosphorylation site, and empirical Bayes moderated t-statistics were used to assess differences in expression/abundance. Contrasts comparing stimulation vs control treatments were tested. Intraclass correlations were estimated using the duplicate correlation function of the limma package to account for measures originating from the same patients and the same antigens (64). An absolute log₂fold change cutoff (stimulation versus control) of 0.7 and a false discovery rate (FDR) cutoff of 5% were used to determine differentially expressed phosphorylation sites. Analyses of signaling networks and KEGG Pathways were performed using StringDB.

RNA sequencing data analysis

Image analysis and base calling were performed using Illumina's Real Time Analysis v1.18 software, followed by 'demultiplexing' of indexed reads and generation of FASTQ files, using Illumina's bcl2fastq Conversion Software v1.8.4. The RNA-seq data were aligned to the human genome (UCSC Human Genome Assembly GRCh38 reference) using STAR, and gene quantification was performed using RSEM (65, 66). Genes with less than 10 non-zero read counts (taking into account technical replicates) were discarded, leaving 18,498 expressed genes. All libraries passed the quality control criteria (libraries with more than 200,000 reads, 12,000 detected genes and an exon range > 60%). Raw count data were imported into R. edgeR was used to calculate the normalization factors to scale the raw library sizes, followed by a voom transformation from the limma Bioconductor package (67, 68). It transforms count data to log₂counts per million and estimates the mean-variance relationship to compute appropriate observation-level weights. Linear models with subject random effects were again used for differential gene expression analysis as described above in *Shotgun mass spectrometry data analysis*. Contrasts comparing treatments (control versus stimulation) or CARs (CD28/CD3 ζ versus 4-1BB/CD3 ζ) were tested. An absolute log₂fold

change cutoff of 1 and a FDR cutoff of 1% were used to determine differentially expressed genes.

Analysis of T cell phenotype, function, and in vivo experiments

FlowJo version 9 (Treestar) was used to analyze flow cytometry files and calculate proliferation indices. Prism version 7 (GraphPad Software) was used to plot data and calculate statistics. P values meeting an $\alpha = 0.05$ level of significance are indicated in the figures. The precise statistical tests used are indicated in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Both CD28/CD3ζ and 4-1BB/CD3ζ CAR T cells can be activated through an engineered STII hinge.

(A) Schematic of CARs incorporating a STII sequence in the extracellular hinge. CARs contained either the CD19-specific FMC63 scFv or ROR1-specific R12 scFv. (B) Schematic of CAR T cell activation through the STII hinge using magnetic beads coated with antibody against STII. (C) Flow cytometry analysis of CD8 and EGFRt staining on singlet CD19 CAR T cells after expansion. Dot plots are representative of 3 independent experiments. The frequency of positive cells are means from all experiments. (D and E) Flow cytometry analysis of STII staining of cell surface CAR (D) or CD45RO, CD62L, CD27, CD28, PD-1, and Tim-3 phenotypic marker staining (E) on sort-purified CD19-specific or ROR1-specific singlet CD8⁺ CAR T cells after expansion. Histogram plots of CD28/CD3ζ CAR T cells (red), 4-1BB/CD3ζ CAR T cells (blue), or isotype control staining (grey) are representative of 4 independent experiments. The frequency of positive cells are means from all experiments. (F) Flow cytometry analysis of the DNA content of CAR T cells after

expansion. Histograms are representative of 4 independent experiments. The frequency of cells in G₀/G₁ gate are means from all experiments. (G) Western blot analysis for CD3 ζ , CD3 ζ pTyr¹⁴², SLP-76, and SLP-76 pSer³⁷⁶ in lysates of ROR1 4-1BB/CD3 ζ CAR T cells after 45 minutes of co-culture with varying quantities of STII microbeads, K562 cells, or K562/ROR1 cells. Blots and Fold change (log₂FC) of normalized band intensity values are representative of 2 independent experiments. The indicated P values were calculated by paired two-tailed t test (E).

Figure 2. CAR T cells signal through endogenous T cell signaling proteins.

(A and B) Human CAR T cell treatment conditions and experimental groups. (C to E) Tandem MS/MS analysis of phosphorylated peptides from lysates of CAR T cells stimulated as in (A). The total number of pSer, pThr and pTyr peptides identified (C) and the Venn diagram of the overlap among phosphorylation sites (D) are pooled from 3 independent experiments. The fold change ($\log_2$ FC) in phosphorylation at the indicated times at sites involved in canonical TCR signaling (E) are means $\pm$ range from 2 or 3 independent experiments. (F) Western blot analysis for CD3 ζ , CD3 ζ pTyr¹⁴², ZAP-70 pTyr³¹⁹, and PLC-

γ 1 pTyr⁷⁸³ in lysates from CD19-specific CD28/CD3 ζ or 4-1BB/CD3 ζ CAR T cells at the indicated times after stimulation. Blots are representative of the 3 independent experiments. Fold change ($\log_2$ FC) of normalized band intensity are means $\pm$ SD from all experiments. The indicated P values were calculated by repeated-measures one-way ANOVA with Tukey's multiple comparisons test comparing CD28/CD3 ζ and 4-1BB/CD3 ζ CAR samples (F).

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Figure 3. The kinetics and strength of signaling vary after stimulation of CD28/CD3 ζ or 4-1BB/CD3 ζ CAR T cells.

(A) Volcano plots of fold change ($\log_2FC$) and false discovery rate (FDR) for phosphorylation sites identified by tandem MS/MS in Fig 2. Green dots indicate sites with increased phosphorylation and red dots indicate sites with decreased phosphorylation after CAR stimulation in at least 2 experiments. (B) Comparison of stimulation-responsive phosphorylation sites identified by tandem MS/MS in Fig 2 at 45 minutes after activation in either CD28/CD3 ζ and 4-1BB/CD3 ζ CAR samples. Green dots specify sites that exhibited opposite responses after CD28/CD3 ζ or 4-1BB/CD3 ζ CAR activation, whereas red dots indicate sites phosphorylated to a greater extent after stimulation of 4-1BB/CD3 ζ CAR T cells in at least 2 tandem MS/MS experiments in Fig 2. (C) The fold change in phosphorylation sites on known CD28 and 4-1BB signaling pathway members at 45 minutes after CAR T cell stimulation. Data are means $\pm$ range from 2 or 3 tandem MS/MS experiments in Fig 2. (D) The fold change in the 20 most phosphorylated sites identified by

tandem MS/MS in Fig 2. Data are means from all experiments. (E) The absolute fold change of phosphorylation sites on known KEGG TCR signaling pathway proteins identified by tandem MS/MS in Fig 2. Data are means from all experiments. (F) Western blot analysis for CD3 ζ , DAPPI, DAPPI pTyr¹³⁹, SLP-76, SLP-76 pSer³⁷⁶, PLC- γ 1, and PLC- γ 1 pTyr⁷⁸³ in lysates from ROR1 CAR T cells stimulated with STII microbeads for the indicated times. Blots are representative of 3 independent experiments. Fold change of normalized band intensity are means $\pm$ SD from all experiments. The indicated P values were calculated by unpaired two-tailed t test (D and E).

Figure 4. Stimulation of CD28/CD3 ζ or 4-1BB/CD3 ζ CAR T cells alters protein phosphorylation across similar signaling pathways and cellular compartments.

Map of select proteins differentially phosphorylated after 45 minutes of CAR T cell stimulation from analysis of all tandem MS/MS experiments in Fig 2.

Figure 5. Increased CD28/CD3ζ CAR signal intensity is associated with an effector cell-like phenotype and reduced in vivo anti-tumor activity.

(A to C) RNA-Seq analysis of total RNA expression in CD28/CD3ζ or 4-1BB/CD3ζ CAR T cells with and without stimulation. The fold change values of the indicated transcripts (A and B) are means ± SD from 3 independent experiments. Transcripts in (B) met an FDR of 1% for differential expression between CD28/CD3ζ and 4-1BB/CD3ζ CAR T cells.

Volcano plot analysis (C) indicates genes with increased expression in CD28/CD3ζ CAR T cells (green) or increased expression in 4-1BB/CD3ζ CAR T cells (red). (D) qRT-PCR analysis of IL7R, KLF2, and FOXO4 expression in CD28/CD3ζ and 4-1BB/CD3ζ CAR T cells. Fold change data are means ± SD from 3 biological replicates. (E) ELISA analysis of cytokine production 24 hours after co-culture of ROR1-specific CAR T cells with K562/ROR1 cells. Data are means ± SD of 3–4 independent experiments. (F) Flow cytometry analysis of T cell proliferation as measured by CFSE dye dilution at 72 hours after STII microbead stimulation. Histogram plot of unstimulated CD28/CD3ζ CAR T cells (grey), unstimulated 4-1BB/CD3ζ CAR T cells (black), stimulated CD28/CD3ζ CAR T cells (red),

and stimulated 4-1BB/CD3 ζ CAR T cells (blue). The proliferation index of cells are means from 5 independent experiments ($p = 0.0747$). **(G to J)** At 7 days after Raji/ffluc engraftment, NSG mice were treated with a single infusion of the indicated dose of CAR T cells. Survival analyses (G) of 6, 9, or 15 mice per group are pooled from 2–3 independent experiments. Bioluminescence images of Raji/ffluc tumor burden in mice at the indicated time points (H) are representative of all experiments. (I and J) Flow cytometry analysis of CAR T cell frequency in bone marrow or peripheral blood (I), or abundance of PD-1, Lag-3, or Tim-3 on CAR T cells in the bone marrow (J) on day 20. Frequency and MFI data are means $\pm$ SD of 10 mice per group from 2 independent experiments. The indicated P values were calculated by one sample t test with null hypothesis $H_0 = 0$ (D), paired two-tailed t test (E and F), log-rank (Mantel-Cox) test (G), or unpaired two-tailed t test (I and J).

Figure 6. CD28/CD3 ζ and 4-1BB/CD3 ζ CARs differentially associate with endogenous Lck and CD28.

(A) Western blot analysis for Lck, CD28, and CD3 ζ in whole cell lysates (L) and STII immunoprecipitated fractions (IP) from unstimulated ROR1-specific CAR T cells. Blots are representative of 3–4 independent experiments. (B) Schematic of mutations made to the CAR CD28 signaling domain. (C) Flow cytometry analysis of ROR1-specific CAR T cell proliferation as measured by CFSE dye dilution at 72 hours after co-culture with K562/ROR1 cells. Histograms of untransduced T cells (grey), or 4-1BB/CD3 ζ (blue), CD28/CD3 ζ (red), Y1 (green), and Y3 CAR T cells (purple) after stimulation are representative of 3 independent experiments. (D) ELISA analysis of IFN- γ production by ROR1-specific 4-1BB/CD3 ζ (blue), CD28/CD3 ζ (red), Y1 (green), or Y3 CAR T cells (purple) after co-

culture with K562/ROR1 cells for 24 hours. Fold change data are means $\pm$ SD of 3 independent experiments. (E) Western blot analysis for CD3 ζ , CD3 ζ pTyr¹⁴², SLP-76, SLP-76 pSer³⁷⁶, PLC- γ 1, and PLC- γ 1 pTyr⁷⁸³ in lysates from ROR1-specific CAR T cells stimulated for the indicated times with STII microbeads. Blots are representative of 3 independent experiments. Fold change of normalized band intensity are means $\pm$ SD from all experiments. (F) Western blot analysis for Lck, CD28, and CD3 ζ in whole cell lysates (L) and STII immunoprecipitated fractions (IP) from resting CAR T cells. Blots are representative of at least 3 independent experiments. Fold change of normalized band intensity are means $\pm$ SD from 3 (CD28) or 4 (Lck) independent experiments. The indicated P values were calculated by repeated-measures one-way ANOVA with Tukey's multiple comparisons test comparing samples at equivalent time points (E and F).

Figure 7. Mutations that reduce Lck binding diminish CD28/CD3 ζ CAR signal intensity. (A) Schematic of mutations made to the CAR CD28 signaling domain. (B) Western blot analysis for Lck and CD3 ζ within whole cell lysates (L) and STII immunoprecipitated fractions (IP) from resting CAR T cells. Blots and fold change in the normalized band intensity values are representative of 2 independent experiments. (C) Western blot analysis for CD3 ζ , CD3 ζ pTyr¹⁴², SLP-76, SLP-76 pSer³⁷⁶, PLC- γ 1, and PLC- γ 1 pTyr⁷⁸³ within lysates from CAR T cells stimulated for the indicated times with STII microbeads. Blots are representative of 3 independent experiments. Fold change in the normalized band intensity values are means $\pm$ SD from all experiments. The indicated P values were calculated by repeated-measures one-way ANOVA with Tukey's multiple comparisons test comparing samples at equivalent time points (C).