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Design of TCR Structural Variants That Retain or Invert the Normal Activation Signal

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ABSTRACT

We designed variant human TCRs composed of the full-length TCR α/β or extracellular and transmembrane domains of the associated CD3 subunits fused to polypeptides derived from proteins thought to either enhance or inhibit normal T cell function. First, we showed that the C termini of both the TCR α- and β-chains can accommodate specific additional sequences, without abrogating complex formation or acute sensitivity of the receptor. Replacement of ITAMs with ITIM-containing intracellular domains inverted the TCR signal (i.e., created a ligand-dependent inhibitory receptor). The normal signaling function of the CD3 complex was transferable to the TCR by eliminating all CD3 ITAMs and grafting three to six ITAMs onto the C termini of the α/β-chains, with no effect on acute sensitivity. The observation that TCR variants of such diverse C-terminal composition can fold and function as signaling receptors demonstrates substantial structural and functional malleability of TCRs. These results add to knowledge about TCR structure–function with regard to acute signaling and may provide a route to use TCRs in different ways for T cell therapy. ImmunoHorizons, 2021, 5: 349–359.

INTRODUCTION

The TCR is an extraordinary signaling device with the capacity to trigger T cell response to cellular or artificial surfaces that display as few as one to two Ag molecules (1, 2). The mechanism by which the TCR achieves its high sensitivity is still controversial. Several models of receptor function have been proposed, none of which account for all aspects of TCR signaling (3). The high conservation of the primary and quaternary structures suggests a precisely tuned machine, in which exact geometries may be important to achieve sensitivity (4). At a minimum, it is thought that all TCR/CD3 subunits (α, β, γ, δ, ε, ζ) are required for function in human T cells, and this multisubunit structure is conserved over perhaps 300 million years of evolution, back to the divergence of mammals and avians (5).

Although lacking important structure-function details, TCR signaling is understood in outline. Ligand binding activates a cascade of phosphorylation events that initially center around the CD3 ITAMs, intracellular sequence motifs that are phosphorylated during signal transduction. Activated TCRs ultimately recruit a variety of kinases and adapter proteins within a microcluster of ligand-bound TCRs (6). The role of ITAMs in development of the TCR repertoire in vivo has been explored by removing individual or groups of ITAMs (7–12). Meanwhile a largely independent branch of research has demonstrated the modularity of T cell signaling components, notably through design of CARs, monomeric signaling molecules that use CD3ζ ITAMs, and other intracellular domains (ICDs) derived from costimulatory receptors [e.g., CD28, 4-1BB (13, 14)]. Elegant work in vitro has confirmed a quantitative relationship between the number of ITAMs and signal transduction using CAR
constructs (15). TCRs and CARs are also subject to negative modulation of their signal, notably by ITIM-containing inhibitory receptors such as PD-1. ITIMs are a family of phosphorylation-attracting domains that oppose ITAM signaling (see for review Refs. 16, 17). These motifs have been co-opted in the design of artificial monomeric inhibitory receptors (iCAR and Tmod) that produce ligand-dependent inhibition of CAR and TCR signaling (18, 19).

In this study, we set out to test the limits of TCR structure–function with respect to acute signaling. We concentrated on the ITAM and ITIM domains that are known to initiate and modulate acute response of T cells. Through a series of domain grafting and swapping experiments, we show that despite the TCR’s multisubunit, evolutionarily conserved structure: 1) the grafting and swapping experiments, we show that despite the TCR’s multisubunit, evolutionarily conserved structure: 1) the ITAM and ITIM domains that are known to initiate and modulate acute signaling of TCR and TCR/CD3 ICDs can be substantially altered without affecting complex formation or ligand binding; 2) these variants confer normal or divergent signaling outputs, depending on the ICDs; and 3) replacement of the 10 CD3 ITAMs with three to six ITAMs fused to the α/β TCR subunits forms a receptor with indistinguishable acute sensitivity compared with its wild-type TCR counterpart. These results add to knowledge about TCR structure–function vis-à-vis signaling and may provide a route to use TCRs in different ways for T cell therapy, for example, as peptide MHC (pMHC)-regulated inhibitory receptors.

**MATERIALS AND METHODS**

**Plasmid construction**

All variants of A*02:01-NY-ESO-1 directed TCR were derived from IG4 α95:LY/wtβ (20). TCRα/β fusions containing the NY-ESO-1–responsive activating and inhibitory constructs were created as previously described (19). Briefly, ICDs from CD28, CD4, CD8α, 4-1BB, various truncations of Lck, Fyn, ZAP70, and LAT were fused to TCRα/β with either S, GGS, (G4S)2, (G4S)3, or (G4S)4 as linkers. ITIM-fused TCRα/β constructs were created by fusing the ICDs of LILRB1 (LIR-1; R483-H649), programmed cell death protein 1 (PD-1; S192-L287), or KIR3DL2 (KIR; N365-F454) with a (G4S)4 linker for TCRα and (G4S)1 linker for TCRβ. For CD3 subunits directly fused to inhibitory domains, the extracellular domain and transmembrane domain of the CD3 subunits (CD3) were directly fused to LIR-1 (R483-H649). For CD3 constructs whose ITAMs were replaced by ITIMs, the conserved ITAM motif (YxxL/I) of each CD3 subunit was replaced with the region spanning LIR-1 ITIM3 and 4 (RESI 611-647). The rest of the CD3 backbone was left intact. All constructs used in this study are described in detail in Supplemental Table I. All constructs were assembled using Golden Gate Assembly.

**Cell culture**

Jurkat cells encoding an NFAT luciferase reporter gene (BPS Bioscience) were maintained in RPMI media supplemented with 10% heat-inactivated (HIA) FBS (inactivated at 56°C for 1 h), 1% penicillin and streptomycin (pen/strep), and 0.4 mg/ml Geneticin. T2 cells were obtained from the American Type Culture Collection and maintained in IMDM supplemented with 20% HIA FBS and 1% pen/strep.

**Generation of CD3-knockout Jurkat NFAT luciferase reporter lines**

Guide RNAs (gRNA) for CRISPR knockout were obtained from Synthego. EnGen Spy Cas9 containing SV40 nuclear localization sequence at the N and C termini was purchased from New England Biolabs. Prior to transfection, single gRNA (sgRNA) was resuspended to 100 μM. The dissolved sgRNA was further diluted to 30 μM in 1× Tris-EDTA buffer purchased from Synthego. gRNA sequences targeting CD3δ are as follows: G*G*U*CCAGGAUGGUUUC CC, C*A*U*CACAGGGUACAGGAA, and C*C*U*CUAUG-GUAUCUGAG. gRNA sequences targeting CD3ε are as follows: U*U*U*CAGAUGCCAUCAUG, G*A*U*GAGGAUG AUAAAAACAU, and U*A*U*UAUGUCUGCUACCCAG. The asterisk (*) represents 2′-O-methyl analogs and 3′-phosphothioate internucleotide linkages reported to improve stability and knockout efficiency (21). First, each sgRNA targeting CD3δ or CD3ε was co-incubated with Cas9 protein at room temperature for 15 min. The final concentration of sgRNA was 3.6 μM and Cas9 2.6 μM in total 10 μl of volume of R buffer provided by the Neon Transfection Kit (Thermo Fisher Scientific). After the coincubation, the RNA protein complexes targeting CD3δ and CD3ε were combined. A total of 15 μl of the RNA protein complex was then combined with 15 μl of Jurkat NFAT luciferase reporter cells resuspended at 2e6/ml in the Neon R buffer (Thermo Fisher Scientific). Using the 10-μl format Neon Electroporation System, the Jurkat cells were transfected at 1400 V, 10 ms, three pulses. Transfected cells were immediately transferred to prewarmed RPMI 1640 supplemented with 20% HIA FBS and 0.1% pen/strep and incubated for 48 h at 37°C and 5% CO2 (19).

CD3δ/ε knockout cells were stained with PE-conjugated SK7 (BioLegend) to confirm lack of CD3 expression, expanded, and functionally validated (Fig. 3A). Briefly, CD3δ/ε knockout cells were transfected using the 100-μl format Neon Electroporation System as described above with TCRα and TCRβ subunits targeting A*02:01-NY-ESO-1 (clone IG4 α95:LY) (20) with either CD3δ alone, CD3ε alone, or with both CD3δ and CD3ε. Rescue of functional activity was measured by coculturing with peptide-loaded T2s as was done previously (19) and described below. The pool of CD3δ/ε knockout cells generated was then further engineered to remove CD3γ and CD3ζ as above and functionally validated (Fig. 3A).

CD3δ/ε knockout cells were stained with PE-conjugated SK7 (BioLegend) to confirm lack of CD3 expression, expanded, and functionally validated to be CD3δ/ε-null using genetic complementation as described (Fig. 3A). Briefly, CD3δ/ε knockout cells were transfected using the 100-μl format Neon Electroporation System as described above with TCRα and TCRβ subunits targeting A*02:01-NY-ESO-1 (clone IG4 α95:LY) (20) with either CD3δ alone, CD3ε alone, or with both CD3δ and CD3ε. Rescue of functional activity was measured by coculturing with peptide-loaded T2s as was done previously (19) and described below. The pool of CD3δ/ε knockout cells generated was then further engineered to remove CD3γ and CD3ζ as above and functionally validated (Fig. 3A). U*C*U*CACAGGAUGGAAA, C*A*G*AAGCCAAAAUAUCACA, and A*U*U*UUUU UUUAUCUUGUAGUAG are gRNA sequences used to target CD3 γ. G*A*A*ACUCUUUGUGUAGUGU, A*C*A*GUUGGCAU UACAGGUA, and A*A*A*GGAAAGGUGAAGGAG are gRNA sequences used to target CD3ζ.
**Jurkat cell assays**

Jurkat NFAT luciferase assays were carried out as previously described (19). Briefly, modified NY-ESO-1(v) peptide (SLLMWITQV) (Genscript) was serially diluted 3-fold starting at 100 μM and loaded onto 1e4 T2 cells resuspended in 15 μl of RPMI 1640, 1% BSA, and 0.1% pen/strep and incubated overnight. Jurkat NFAT luciferase cells were electroporated using the 10 μl format Neon Transfection Kit (1500 V, 10 ms, three pulses) with 1 μg of inhibitory TCRα/β variant DNAs and ITAM-null CD3 subunits with 0.5–1 μg of DNA of CD19 CAR activator per 1e6 cells. Cells were cultured in RPMI 1640 supplemented with 20% FBS and 0.1% pen/strep for ~18–24 h posttransfection. Jurkat cells were counted and resuspended in RPMI 1640 supplemented with 10% FBS and 0.1% pen/strep. A total of 1e4 resuspended Jurkat cells were cocultured with T2 cells loaded with varying amounts of NY-ESO-1(v) peptide for 4–6 h. Luciferase activity was measured using ONE-Step Luciferase Assay System (BPS Bioscience).

**RESULTS**

**C termini of the TCRα and β subunits can be fused to activating and inhibitory domains without affecting acute sensitivity**

We first tested the feasibility of fusing exogenous sequences to the C termini of a benchmark NY-ESO-1 TCR (22); G5 linkers of varying lengths were also tested. A variety of constructs derived from signaling molecules thought to enhance or inhibit TCR function were tested in this context (Supplemental Table I). A subset of these ICDs fused to the C terminus of TCRα or TCRβ did not harm acute TCR-mediated NFAT luciferase response in Jurkat cells when cocultured with peptide-loaded T2 cells (Fig. 1); in particular, domains from CD28, CD4, 4-1BB, LCK, FYN, and LAT produced functional TCRs with minimal shift of EC₅₀ when fused to the C terminus of either or both TCR chains. These domains encompassed both globular and extended structures. Surprisingly, no definitive rules were observed regarding which domains were accommodated well by the TCR complex, but some trends were apparent; for example, longer (GGGGS)x3 or x4 linkers were preferred to shorter S or (GGS)x1 (Fig. 1B), and β-chain was slightly more preferred (Fig. 1C). Furthermore, the NY-ESO-1 TCRα and β fused via their C termini to KIR, PD-1, or LIR-1 ITIM domains did not harm the TCR EC₅₀ in this context (Fig. 2, Supplemental Fig. 1A). To summarize these results, 1) C termini of TCRs tolerated significant engineering; 2) β-chain fusions were potentially more stable and sensitive, as were longer G4S linkers; and 3) ITIM-containing domains did not produce detectable change in dose-response.

**Creation of CD3-deficient Jurkat cells as an assay system for TCR/CD3 variants**

The lack of EC₅₀ shift in TCRs fused to inhibitory domains in wild-type Jurkat cells suggested that the 10 endogenous CD3 ITAMs may overpower any inhibitory effect exerted by ITIMs. To explore other TCR designs, including inverter TCRs (invTCRs) that might invert the normal activation signal, it was necessary to generate a Jurkat cell line deficient in CD3 expression as the basis for an assay. Jurkat cells express CD3, which poses a problem for assessment of the functional effect of added subunits because of the possibility of structurally mixed TCR complexes. To provide a clean assay background devoid of wild-type CD3 subunit function, we used CRISPR/Cas9 with a pool of gRNAs to target the genomic CD3 loci in Jurkat cells (see Materials and Methods). After transfection of the gRNAs/Cas9 RNA protein complex, Jurkat cells were grown and cloned by single-cell dilution. The sequence relatedness and complexity of the CD3-encoding loci hindered assessment of genetic knockout by standard Sanger sequencing methods. We therefore did a complementation test to detect residual subunit function in two of the clones (clones 24 and 32). CD3 subunits without activating intracellular ITAM domains (∆ITAMCD3) were expressed in selected knockout clones. If the clone lacked functional CD3 subunits, then expression of ∆ITAMCD3 (null) subunits would lead to TCR/CD3 surface expression but no NFAT luciferase response when stimulated by A*02:01-NY-ESO-1. More specifically, we searched for functional complementation of individual CD3 subunits by transfecting all combinations of CD3 subunits, with one subunit missing. Clones 24 and 32 were confirmed as CD3-null on this basis and were selected for detailed study of the invTCRs (Fig. 3A, Supplemental Fig. 1B).

**Reconstitution of invTCR function in CD3-deficient Jurkat cells**

Having generated a ∆CD3 Jurkat cell line, we sought to confirm our hypothesis that endogenous CD3 ITAMs suppress the inhibitory effects of TCRα/β-ITIM fusions. We therefore coexpressed either wild-type NY-ESO-1 TCRα and β (wild-type TCRα/β) or NY-ESO-1 TCRα and β fused via their C termini to KIR, PD-1, or LIR-1 ITIM domains as above (Fig. 2, Supplemental Fig. 1A), along with ∆ITAMCD3 subunits, in CD3-null Jurkat cells (Fig. 3B). It was only possible to measure an inhibitory signal from ITIMs in our system indirectly by its effect on activation (19). To test for ligand-dependent inhibition of an activating signal, we coexpressed a CD19 CAR to serve as the activating receptor. We thus determined the IC₅₀ of the ITIM-fused TCRα/β in the presence of CD19⁺ T2 cells. The different TCRα/β subunits expressed if and only if coexpressed with ∆ITAMCD3 subunits (Fig. 3C, top panel). As expected, wild-type TCRα/β, when combined with ∆ITAMCD3, showed no activation/inhibition upon ligand binding (Fig. 3B, Supplemental Fig. 1C, black closed circle). TCRα/β-PD-1, TCRα/β-KIR, and TCRα/β-LIR-1, in contrast, showed reproducible, ligand-dependent inhibition of CD19 CAR activation (Fig. 3B, https://doi.org/10.4049/immunohorizons.2100033
Supplemental Fig. 1C, green, light blue, and purple). These results confirm that the CD3-null Jurkat cells are functionally compromised for CD3 function, ITAMs dominate ITIMs, and TCRα/β can signal as an inhibitory receptor via C-terminally fused ITIM domains if ITAMs are eliminated from the complex.

We next sought to improve the potency of the invTCR in CD3-deficient Jurkat cells. To do this, we focused on the ICD of LIR-1, possibly the most-potent member of the inhibitory class of T cell modulators (19, 23). LIR-1 ITIM-containing CD3 subunits were expressed in clone 32 cells, along with NY-ESO-1 TCRα and β, resulting in surface expression of TCR/CD3 (Fig. 4A). We also expressed TCR/CD3 chains with all four different CD3 subunits fused to ITIM-containing domains. We determined the IC50 values of the inverter TCR-CD3 complex against CD19 CAR, as above. Expressing LIR-1–fused CD3δ/ε or CD3γ/ζ showed similar inhibition to fully reconstituted invTCR-CD3 complex (Fig. 4A, Supplemental Fig. 1D). The reconstituted TCR IC50 was compared with previously characterized inhibitory constructs (“blockers”) that use either an Ftcr (TCR ligand-binding domain) or scFv against HLA-A*02:01-NY-ESO-1 pMHC (19). The blocking activity in the presence of CD19 CAR was similar among the scFv, Ftcr, and invTCR constructs (Fig. 4B, Supplemental Fig. 1E). Thus, the switch of ITAMs for ITIMs converted the NY-ESO-1 TCR into a ligand-dependent inhibitory receptor similar to, but no more potent than, other blocker constructs composed of CAR backbones tested previously.

We next compared the LIR-1–based invTCR to a more native-like inhibitory domain (Fig. 4C). Inhibitory curves were again generated using the CD19 CAR to mediate activation in clone 32 cells. To generate a more native-like structure with regard to ICD sequence length, the CD3 ITAMs were replaced by 36 (LIR-1 611-646) residues spanning LIR-1’s ITIM 3 and 4 (orange). We included these ITIMs because of their importance in SHP-1 binding (24). CD3 ICDs range from 45 to 112 residues, whereas the full-length LIR-1 ICD is 168 residues. The ITAM3/4 domain constructs were in this range, 61–168 residues (see Materials and Methods). Both LIR-1 sequence-containing invTCRs blocked well (Fig. 4C, Supplemental Fig. 1F). This result suggests that LIR-1 611-646 is sufficient to recruit SHP-1 and this inhibitory function is transferable.

**FIGURE 1.** A*02:01 NY-ESO-1 directed TCR C-terminal modifications have negligible effect on acute TCR activity.

(A) Fluorescently labeled HLA-A*02:01-NY-ESO-1(v) tetramer complexes (probe) binding, normalized to the benchmark TCR, is plotted on the y-axis versus relative EC50 on the x-axis (see Materials and Methods). Dotted lines align to benchmark TCR values. (B) Replotted from data shown in (A) to compare different linker lengths. Singular short or long data points derived from either TCRα or β C-terminal fusions paired with corresponding wild-type subunit. (Short, short), (long, long), and (short, long) data points derived from both subunit replacement with C-terminal fusion constructs. (C) Replotted from data shown in (B) to compare TCRα versus TCRβ fusions. Fusions to TCR β are better tolerated, and longer linkers are preferred. Data are representative of one to three independent experiments. Outliers were repeated, whereas constructs similar to or worse than benchmark TCR were abandoned after the first trial.

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Charge-swapped and ITAM-shuffled TCR/CD3 complexes function in Jurkat cell assays

In the process of further exploration of invTCRs, we uncovered additional structure-function relationships within the TCR/CD3 complex. These findings followed from efforts to demonstrate activity of the invTCR against a TCR as opposed to a CAR. As mentioned above, one challenge is the intermingling of wild-type and ITIM-fused CD3 subunits, potentially creating a complex mixture of structures with differing ratios of ITIMs and ITAMs. Conclusions about structure-function relationships of the invTCR are clouded by these hybrid molecules. We thus attempted to create a structure that could assemble and function independently of a wild-type receptor. The CD3 complex and its assembly with the TCR depends on charge-pair interactions within the membrane-spanning domains of the subunits (25, 26). We used this structural information to design charge-swapped subunits intended to attract correct subunit partners but repel corresponding wild-type subunits (Fig. 5A). We focused particularly on the charge-pair interactions between TCRβK288 and CD3εD137/yE122 because they appeared to be the most amenable to engineering within the TCR and CD3 portions of the complex. The TCRα-R253–CD3εD15 (Supplemental Fig. 2A) and TCRα-K258–CD3εD111/yD137 interactions proved more difficult to disrupt and rescue. Furthermore, when these two mutations were combined, expression and activity were never fully recovered (Supplemental Fig. 2C). In contrast, the positively charged K288 residue of TCRβ, which interacts with corresponding negatively charged residues of CD3ε (D137) and CD3γ (E122), was more tractable. These charge pairs were interchanged between subunits to generate distinct TCRβ–CD3εγ complexes.

We tested the design premise of a charge-swapped twin TCR by expressing the twin subunits, otherwise wild type in sequence, in the presence of NY-ESO-1 TCRα/β. EC_{50} values were measured for these constructs in Jurkat cells (Fig. 5A). In wild-type Jurkat cells, either TCRα/β or TCRα and K288D TCRβ were coexpressed. As expected, TCRα/β showed robust function upon ligand binding (Fig. 5B, black closed circle). However, replacing the β subunit with the K288D mutant dramatically reduced the functional activity of the TCR, with ~700-fold right shift compared with wild type (Fig. 5B, black closed circle versus open circle). This functional activity could be rescued by coexpression of the matching CD3γ mutant E122R or E122K (Fig. 5B, gray and blue compared with open circle). Surprisingly, expression of CD3γ E122K without its charge-swapped partner CD3ε D137K/R completely rescued activity of the K288D TCRβ in the presence of the wild-type CD3ε, ε, and γ subunits, suggesting some flexibility in the interactions within the membrane-spanning domains of the subunits.
FIGURE 3. Generation of ΔITAMCD3 Jurkat NFAT luciferase reporter line for inverter TCR evaluation.

(A) CD3 subunits that lack activating intracellular ITAM domains (ΔITAM CD3) were expressed in complete functional knockout clone CD3 KO Jurkat. Electroporate TCRαβ + wtCD3 subunits

Electroporate TCRαβ + various combinations of ΔITAM CD3 subunits

(B) TCRαβ + ΔITAMCD3δ/ε/γ/ζ

TCRαβ-PD-1 + ΔITAMCD3δ/ε/γ/ζ

TCRαβ-KIR + ΔITAMCD3δ/ε/γ/ζ

TCRαβ-LIR-1 + ΔITAMCD3δ/ε/γ/ζ

CD19 CAR only

CD19 CAR + wtTCRαβ

CD19 CAR + TCRαβ-PD-1

CD19 CAR + TCRαβ-KIR

CD19 CAR + TCRαβ-LIR-1

(C) CD19 CAR

CD19 CAR + wtTCRαβ

CD19 CAR + TCRαβ-PD-1

CD19 CAR + TCRαβ-KIR

CD19 CAR + TCRαβ-LIR-1

organization of subunits in the TCR to balance the charges in the transmembrane segments (Fig. 5B, black open circle versus magenta).

We next constructed an invTCR equivalent to the charge-swapped twin TCR (Fig. 5C). We first tested whether the mutant invTCR containing LIR-1 ITIM3/4 in a CD3γ/ζ backbone was functional against a CD19 CAR. As expected, the transmembrane charge-swapped mutant invTCR complex blocked CD19 CAR-mediated activation in the presence of CD19+ target cells (Fig. 5D and Supplemental Fig. 2E). We then sought to test this mutant invTCR against a wild-type TCR in wild-type Jurkat cells. If successful, this construct would allow us to measure the blocking potency of an invTCR on a second TCR. Unfortunately, the level of subunit mixing of a charge-swapped TCRβ with wild-type CD3γ/ζ subunits was too high to separate activation from blockade when dosed with the invTCR target ligand (Supplemental Fig. 2D). We speculate that introduction of additional variants to enhance the specificity of subunit interactions would ultimately resolve this difficulty.

In light of these results, we took an alternative approach, which ultimately succeeded in testing the hypothesis that invTCRs may inhibit TCR/CD3 activating receptors. This approach also yielded another remarkable result: namely, that acute signaling can be transferred from the CD3 ITAMs to the TCR α/β-chains. To do this, we attempted to create a modified, active TCR and its reciprocal invTCR using a pool of CD3-null subunits shared between them. The concept was to generate an active TCR variant in which the ITAMs were supplied not by CD3 but by fusion to the TCRα- and β-chain C termini (Fig. 6A). Surprisingly, this design not only functioned but also produced TCR variants with identical EC50 compared with wild type coexpressed with wild-type CD3 subunits (Fig. 6B, Supplemental Fig. 3A, 3B). Thus, three to six ITAMs shuffled from CD3 to TCR were sufficient to produce a receptor with indistinguishable acute-signaling behavior in Jurkat cells.

The ITAM-shuffled TCR/CD3 enabled us to test if the invTCR could inhibit activity of a TCR variant, as we had shown for a CAR. To achieve this, we used α/β-chains fused to ITIM, not ITAM, domains (Fig. 6A). These constructs were cotransfected into Jurkat cells with ΔITAMCD3 subunits to form invTCR complexes. In addition, the TCRα/β ITAM fusions were cotransfected to generate activating receptors in which the CD3-null subunits were shared with both the ITAM-shuffled activator and the invTCR. Surface expression of both the ITAM-shuffled TCR and invTCR was comparable to previous experiments (Fig. 6C). The IC50 of the NY-ESO-1 invTCR

32. Each ΔITAMCD3 subunit was omitted to see if the remaining endogenous subunits would rescue functional activity. The complete set of wild-type CD3 subunits were coexpressed as a positive control (black). (B) Inhibitory effects of constructs with PD-1 (green), KIR (light blue), or LIR-1 (CD purple) fused to the TCRαβ in clone 32. Note the maximal inhibition is never complete and somewhat variable. This is at least partly caused by a fraction of activator-only Jurkat cells. (C) Surface expression of CD19 CAR and NY-ESO-1 invTCR.
Since the invention of CARs, it has been known that ICDs from CD3 subunits and other activating immune receptors can be stitched together to serve in T cells as signaling components of monomeric membrane proteins with Ab-based ligand-binding domains (13, 14). These first-, second-, and third-generation CARs convert Ag binding into signals that stimulate T cell proliferation and cytotoxicity much like native TCRs (see Ref. 27 for review). Second- and third-generation CARs have been used in the clinic as components of cell therapeutics and achieved noteworthy success (28–31). Most engineering for therapeutic purposes has been restricted to CARs, as opposed to TCRs, given the CAR’s tolerance for alterations (32). TCRs have been typically used in their native form or with minor sequence changes that involve introduction of stabilizing disulfide bonds or swapping of mouse constant domains to facilitate desirable H-L-chain pairing in human cells (33–35). Notable exceptions are: 1) chimeric TCRs with an scFv fused to the N terminus of the TCR/CD3 subunits, creating a TCR/CD3 hybrid receptor that transduces non-pMHC ligand binding into TCR signaling (36) and 2) single-variable-domain TCRs that function in the context of a TCR-like CD3 complex to signal (37). These studies demonstrate the resilience of the N terminus of the TCR with regard to structural perturbations. However, they also reveal that the extraordinary sensitivity of the TCR cannot be simply co-opted by grafting a different ligand-binding domain onto the structure. The ligand-binding domain itself, rather than the receptor signaling sequences and structures, appears to play a dominant role in dictating receptor sensitivity. The observation that TCRs can accommodate charge-swapped subunits that preserve net charge neutrality, and even a situation in which one transmembrane residue is switched from negative to positive (CD3ζ E122K), further suggests more plastic structure-function behavior than might be expected from the high conservation of TCR/CD3 primary sequences in the transmembrane segments and ICDs.

We sought to build TCRs with distinct signaling properties: for example, which invert ligand-binding signals that normally activate T cell response. We were surprised by how tolerant clone 32 along with TCRαβ against A*02:01-NY-ESO-1. To determine the IC50 of the inverter TCR, they were coexpressed along with activating CD19 CAR, which reacts with CD19 expressed on the surface of T2s. (B) The reconstituted inverter TCR IC50 was compared with previously characterized inhibitory Fcr- and scFv-LIR-1 fusions against A*02:01-NY-ESO-1 pMHC (19). Inhibitory activity against CD19 CAR was very similar between scFv-LIR-1, Fcr-LIR-1, and invTCR. (C) Two types of ICDs were appended to inverter CD3 domains, and their IC50s were compared: LIR-1 ICD (purple) or the region that contains ITIM 3 and 4 in the native CD3 backbone (orange).

**FIGURE 4.** Reconstitution of inverter TCR in ΔITAMCD3 Jurkat NFAT luciferase cell line. (A) Inverter CD3 subunits were generated by replacing the CD3 ICD with the ICD of LIR-1. These inverter CD3 subunits were expressed in (73 nM) in the context of the ITAM-shuffled TCR (Fig. 6C, Supplemental Fig. 3C, square) was similar to its IC50 measured with the CD19 CAR activator (168 nM) (Fig. 6C, triangle), demonstrating that invTCRs inhibit TCRs as they do CARs.

**DISCUSSION**
the TCR structure is with regard to C-terminal modification, accommodating a variety of C-terminal fusions, including globular domains and extended sequences. Potential activating domains were selected from the set of proteins known to participate directly in T cell activation via the TCR, spanning the main arms of downstream signaling (38). Interestingly, none of the TCR fusions improved acute sensitivity. This suggests that recruitment of the proteins tested in our TCR-fusion experiments, including CD28, is not rate-limiting for TCR activation in vitro. In contrast, it is known that CARs with ITAM-containing ICDs derived from CD3ζ or FcRIγ are not sufficient to fully activate proliferation in acute primary human and mouse T cell assays (39–41). The addition of a costimulatory domain in tandem provides either a quantitatively or qualitatively different signal that boosts acute response in both primary T and Jurkat cells (41). Notably, our assays were performed in Jurkat cells, which express many, but not all, of the proteins used to create the fusions. Although Jurkat cells do not contain all the components identified through study of normal T cell biology [e.g., PTEN, SYK, SHP-1, CTLA-4 (42)], we have shown that acute receptor sensitivity measured in Jurkat cells correlates with primary T cells (32). Thus, the lack of acute effect in Jurkat cells may be explained by the parent molecules’ availability for recruitment into the signaling complex. Alternatively, the fusion proteins may not be positioned in an optimal way to function in acute signaling. Indeed, it is possible that the fusions compromise the function of TCRs in some fashion, which only a subset of fusions are able to partially offset.

The result that the acute signaling function of CD3 can be totally replaced by ITAMs on the TCR α/β-chains is remarkable but consistent with data from careful comparisons of CARs and TCRs that reveal very little difference in response properties when corrected for functional sensitivity to Ag. We should point out that this statement may be controversial, but we believe that many studies focus on binding affinity, rather than functional sensitivity. We and others have shown that these parameters are only weakly correlated in CARs, as in TCRs ([12, 32]; see Ref. 43 for review). Our results are consistent with quantitative experiments of James (15) who investigated sensitivity in Jurkat cells using a CAR-like scaffold. He showed that a single ITAM could mediate some activation, with a big inflection in the response curve at three ITAMs/T CR. His results are extended in this study to include TCRs with different ITAM numbers with respect to sensitivity.

FIGURE 5. Construction of charge-swapped twin TCRs. (A) Cartoon representation of engineered TCR transmembrane interactions. In the native TCR–CD3 complex, the positively charged K288 of TCRβ interacts with corresponding negatively charged residues of CD3ζ (D137) and CD3γ (E122). These charge-pairs were mutated to K288D and D137/K/R and E122/K/R to generate distinct TCR complexes. (B) Jurkat NFAT luciferase activity of charge-swapped TCRβ–CD3ζ/γ complex. In wild-type Jurkat cells, either wild-type TCRα/β or wild-type TCRα and K288D TCRβ were coexpressed with or without corresponding CD3ζ/γ mutants. Rescue of NFAT response was measured. (C) Cartoon representation of ITIM3/4-substituted transmembrane domain mutant TCR–CD3ζ/γ complex. TCRα–CD3ζ transmembrane mutation (cyan star) and TCRβ–CD3γ transmembrane mutation (black star) are described in dotted boxes. (D) Jurkat NFAT luciferase activity of charge-swapped invTCRα/β–CD3ζ/γ complex. In clone 32 Jurkat cells, CD19 CAR was expressed with or without charge-swapped TCRβ with ITIM-fused charge-swapped CD3ζ/γ. Inhibitory activity was measured by coculturing with NY-ESO-1(v) peptide-loaded T2 cells.
To induce inhibitory rather than activating signals, we substituted ITIM sequences for the CD3 ITAM domains. We showed that we could create an invTCR with sensitivity toward a CD19 CAR within the range of other potent blocker modules seen before but not as sensitive as the best activating TCRs (19). Allen and colleagues (44, 45) first described a phenomenon they called altered-peptide ligands (APLs), whereby pMHC agonists could be converted into nonagonists—even antagonists—by single amino acid substitutions in the peptide. They subsequently explained this behavior by differences in off-rates of the TCR/pMHC complexes; faster off-rate variants produced lower activation (46). However, the details of the APL mechanism with respect to signaling are still imperfectly understood (47). Some have postulated that APLs trigger a negative signal from the engaged TCR, but there is evidence against this view (48). We believe that the invTCRs described in this study are examples of TCRs that directly transform ligand binding into a negative signal, mediated via the ITIM mechanism of T cell checkpoint control.

Apart from the focus on in vitro sensitivity and not T cell development, our study has limitations. We measured acute sensitivity of variant TCRs in Jurkat cells, and it is possible that some of the constructs tested in this study (e.g., the fusions to CD28) may provide benefit in long-term contexts for primary T cells, including cell therapy where exhaustion is thought to limit efficacy in vivo (49). The studies focused on a single TCR, the ultrasensitive optimized clinical NY-ESO-1 TCR. It is possible that some of the results may not generalize to other TCRs or to more native situations in which expression levels are regulated endogenously. Finally, we studied the response of Jurkat cells using the NFAT promoter fusion and did not assess other signaling pathways involved in TCR function. In addition, we were not able to achieve the sensitivity level on the inhibitory side with invTCRs, which wild-type TCRs display routinely for activation.

Ease of engineering has prompted use of CARs in dual-signal integrators for synthetic cellular logic gates (50). The potential of creating two TCRs whose subunits do not comingle using charge-swapped transmembrane domains may provide an avenue for TCRs to be used in these logic systems. TCRs have the virtue that they arise in the body with exquisite sensitivity and selectivity against pMHC Ags. The capacity of TCRs to accommodate C-terminal fusions offers opportunities to modify TCR behavior in cis, for example, through inverting specific pMHC signals. Such behavior, if optimized, may prove useful for AND NOT signal integration in which the inhibitory signal is derived from pMHCs, such as minor histocompatibility Ags (6, 19). If the results generalize to other TCRs (e.g., to class-II–restricted TCRs), modified TCRs might provide an

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**FIGURE 6. Characterization of C-terminally ITAM- or ITIM-fused TCRα/β.**

(A) Cartoon representation of ITAM-shuffled KRAS TCRα/β (KRAS TCRα/β-ITAM) expressed with or without ITIM-shuffled TCRα/β (NY-ESO-1 TCRα/β-ITIM) in cells of the presence of ΔITAMCD3 subunits. (B) NFAT luciferase response of wild-type TCRα/β coexpressed with either ΔITAMCD3 (open circle) or wild-type CD3 (closed circle) and ITAM-shuffled TCRα/β coexpressed with ΔITAMCD3 subunits (triangle) in Jurkat cells. (C) IC₅₀ curves of ITIM-shuffled NY-ESO-1 TCRα/β against either CD19 CAR or ITAM-shuffled KRAS TCRα/β. To test ITIM-shuffled NY-ESO-1 TCRα/β activity against ITAM-shuffled KRAS

TCRα/β transfected Jurkat cells were cocultured with T2s expressing both A*02:01 and A*11:01 loaded with 1 μM KRAS G12D peptide and titrating amounts of NY-ESO-1 peptide.
alternative means to modulate response to specific Ags. For example, invTCRs might be used to inhibit reactivity toward specific self-antigens in a subset of a patient's T cells in an Ag-dependent manner without eliminating portions of the TCR repertoire. From a practical perspective, the invTCR will likely need further development for use in primary T cells because the presence of some ITAMs in the complex (i.e., from wild-type CD3ζ and CD3γ) prevents signal inversion (i.e., creates an activator; Supplemental Fig. 2D). With CAR activators, this problem can be eliminated by removal of wild-type CD3 expression in the host cell. If invTCRs are paired with TCRs as opposed to CARs, proper subunit segregation will need to be enforced, for example, by improved specificity of the charge-pair interactions described in this article to explore invTCR function.

In conclusion, by exploring C-terminal and transmembrane variants of the TCR, we have uncovered a surprising degree of structure-function flexibility. Specifically, we have shown that 1) the C termini of the TCR can be fused to additional sequences, preserving acute function; 2) the ITAM domains can be eliminated from all CD3 subunits and fused to the TCRs/β subunits to create a receptor that is very similar to the wild-type TCR in acute assays; 3) ITAMs dominate ITIMs if they are present in the same TCR/CD3 complex, but if the ITAMs are replaced by ITIMs, signaling is inverted and the TCR becomes a ligand-gated inhibitory receptor; and 4) conserved, charged residues in the transmembrane domains of the TCR/CD3 complex can be swapped to create functional TCRs, a further indication of the robust mechanism that operates in acute TCR signaling. These results suggest opportunities for design of TCR-based receptors that extend well beyond those that have been used in T cell therapeutics to date.

DISCLOSURES

The authors are members of a company (A2 Biotherapeutics) focused on the cell therapy modality for cancer.

ACKNOWLEDGMENTS

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REFERENCES

15. James, R. J. 2018. Tuning ITAM multiplicity on T cell receptors can control potency and selectivity to ligand density. Sci. Signal. 11: eaan1088
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<th>Construct</th>
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<tr>
<td>NY-ESO1 1G4 TCRbeta S57C (G4S) CD4 [394-433] GGGGS NM_000616 <strong>Fuse full length NY-ESO1 1G4 TCRbeta S57C with aa394-433 of CD4 ICD separated by a GGS linker</strong></td>
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<td>NY-ESO1 1G4 TCRbeta S57C (G4S) CD8 [183-214] GGGGS NM_001768 <strong>Fuse full length NY-ESO1 1G4 TCRbeta S57C with aa183-214 of CD8 ICD separated by a GGS linker</strong></td>
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<tr>
<td>NY-ESO1 1G4 TCRbeta S57C (GGS) CD4 [394-433] GGGGS NM_000616 <strong>Fuse full length NY-ESO1 1G4 TCRbeta S57C with aa394-433 of CD4 ICD separated by a GGS linker</strong></td>
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<td>NY-ESO1 1G4 TCRbeta S57C (GGS) CD8 [183-214] GGGGS NM_001768 <strong>Fuse full length NY-ESO1 1G4 TCRbeta S57C with aa183-214 of CD8 ICD separated by a GGS linker</strong></td>
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<td>NY-ESO1 1G4 TCRbeta S57C (G4S) Lck [2-509, Y505A] GGGGSGGGGSGGGGSGGGGS NM_005356 <strong>Fuse full length NY-ESO1 1G4 TCRbeta S57C with aa2-509 of LCK (Y505A) separated by a (G4S)3 linker</strong></td>
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<td>NY-ESO1 1G4 TCRbeta S57C (G4S) LAT [35-233] GGGGS NM_001014987 <strong>Fuse full length NY-ESO1 1G4 TCRbeta S57C with aa35-233 of LAT ICD separated by a (G4S)3 linker</strong></td>
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<td>NY-ESO1 1G4 TCRbeta S57C (G4S) ZAP70 [318-600] S/GGGGS NM_001014987 Fuse full length NY-ESO1 1G4 TCRbeta S57C with aa318-600 ZAP70 Interdomain B protein kinase domain separated by a GGS linker</td>
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Supplemental Table 1. Comprehensive list and description of all constructs used in this study. Constructs starting with S indicates activating receptors. Constructs starting with I describe inhibitory receptors, or constructs related to inhibitory receptor characterization. Relevant accession numbers and linker information are included as described.
Supplemental Figure 1. Characterization of inverter TCR constructs and ΔITAM CD3 Jurkats

(A) Jurkat NFAT luciferase cells were transfected with either TCRα or TCRβ was fused to ICD of PD1 (green), KIR (light blue), or LIR-1 (purple). Single subunit fusions were paired with corresponding wild-type subunit. (B) CD3 subunits that lack activating intracellular ITAM domain (ΔITAM CD3) were expressed in complete functional knockout Clone 32. The complete set of wild-type CD3 subunits were co-expressed as a positive control (black). (C) Inhibitory effects of constructs with PD-1 (green), KIR (light blue), or LIR-1 ICD (purple) fused to the TCRα/β in Clone 32 against CD19 CAR. (D) Inverter CD3 subunits were generated by replacing the CD3 ICD with the ICD of LIR-1. These inverter CD3 subunits were expressed in Clone 32 along with TCRα/β against A*02:01-NY-ESO-1. To determine the IC50 of the inverter TCR, they were co-expressed along with activating CD19 CAR, which reacts with CD19 expressed on the surface of T2s. (E) The reconstituted inverter TCR IC50 was compared to previously characterized inhibitory Ftc-r- and scFv-LIR-1 fusions against A*02:01-NY-ESO-1 pMHC. (F) Two types of ICDs were appended to inverter CD3 domains and their IC50s were compared: LIR-1 ICD (purple) or the region that contains ITIM 3 and 4 in the native CD3 backbone (orange).
Supplemental Figure 2. NFAT response of charge-swapped TCR constructs. Mutant (m) and wild-type (wt).

(A) Cartoon representation of charge-swapped residues on the TM of TCRα and CD3ζ. TCRαR253D or R253E were paired with corresponding charge-swapped CD3ζ subunits and NFAT luciferase response was measured in Jurkat cells. In the case of CD3ζ, additional disulfide mutations were made (-CC) to discourage pairing with endogenous subunits. (B) The TCRβK288E mutant was paired with the corresponding CD3ε and CD3γ charge-swapped subunits and ligand-dependent NFAT luciferase response was measured in Jurkat cells. (C) Both charged residues in TCRα that interact with CD3ζ(R253) and CD3ε(K258) were charge-swapped and co-expressed with corresponding CD3ζ and CDε mutants. Ligand-dependent NFAT luciferase response was measured in wild-type Jurkat cells. (D) NY-ESO-1 TCRα(R253D)/TCRβ(K288D) were co-expressed with either wild-type CD3γζ or CD3ε(E122K)/CD3ζ(D15K) in the presence of ΔITAMCD3δ/ε. The degree of cross-activation between mutant TCRαβ and wild-type CD3 subunits was measured by ligand-dependent NFAT luciferase response in Clone 32 Jurkat cells. (E) mutant inverter TCR comprising NY-ESO-1 TCRα(R253D)/TCRβ(K288D) were co-expressed with CD3ε(E122K)/CD3ζ(D15K)-LIR-1 in the presence of ΔITAMCD3δ/ε to measure blocking activity against CD19 CAR.
Supplemental Figure 3. NFAT response of ITAM-shuffled TCRα and/or TCRβ in Clone 32 cells expressing Δ ITAMCD3 subunits.

(A) CD3ζ ICD was fused to TCRα and/or TCRβ of A*02:01 NY-ESO-1(v) targeting TCR. NFAT luciferase responses of Jurkat cells against NY-ESO-1(v) peptide-loaded T2 cells were evaluated in the presence of ΔITAMCD3 subunits. (B) CD3ζ ICD was fused to TCRα/β of HLA-A*11:01 KRAS G12D targeting TCR. NFAT luciferase response of transfected wild-type Jurkat cells against KRAS G12D peptide-loaded T2s expressing A*11:01 was measured. (C) Inhibition of ITAM-shuffled KRAS TCR by ITIM-shuffled NY-ESO TCR. T2s expressing both A*11:01 and A*02:01 were loaded with constant amount of KRAS G12D peptide (1 uM) with titrating amount of NY-ESO-1 peptide.