

The CD4 transmembrane GGXXG and juxtamembrane (C/F)CV+C motifs mediate pMHCII-specific signaling independently of CD4-Lck interactions

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Abstract

CD4⁺ T cell activation is driven by 5-module receptor complexes. The T cell receptor (TCR) is the receptor module that binds composite surfaces of peptide antigens embedded within MHCII molecules (pMHCII). It associates with three signaling modules (CD3 $\gamma\epsilon$, CD3 $\delta\epsilon$, and CD3 ζ) to form TCR-CD3 complexes. CD4 is the coreceptor module. It reciprocally associates with TCR-CD3-pMHCII assemblies on the outside of a CD4⁺ T cells and with the Src kinase, Lck, on the inside. Previously, we reported that the CD4 transmembrane GGXXG motif and cytoplasmic juxtamembrane (C/F)CV+C motif found in eutherian CD4 (placental mammals) have constituent residues that evolved under purifying selection. Mutating these motifs together increased CD4-Lck association but reduced CD3 ζ , Zap70, and Plcy1 phosphorylation levels, as well as IL-2 production, in response to agonist pMHCII. Because these mutants preferentially localized CD4-Lck pairs to non-raft membrane fractions, one explanation for our results was that they impaired proximal signaling by sequestering Lck away from TCR-CD3. An alternative hypothesis is that the mutations directly impacted signaling because the motifs normally play a Lck-independent role in signaling. The goal of this study was to discriminate between these possibilities. Our results indicate that: intracellular CD4-Lck interactions are not necessary for pMHCII-specific signal initiation; the GGXXG and (C/F)CV+C motifs are key determinants of CD4-mediated pMHCII-specific signal amplification; the GGXXG and (C/F)CV+C motifs exert their functions independently of direct CD4-Lck association. These data provide a mechanistic explanation for why residues within these motifs are under purifying selection, and thus functionally important for CD4⁺ T cells in vivo. The results are also important to consider for biomimetic engineering of synthetic receptors.

eLife assessment

This study provides a **useful** description of two evolutionary conserved motifs in CD4 that contribute to the CD4-mediated enhancement of T cell receptor (TCR) signaling, which are notably acting independently of the CD4-LCK interaction. The supporting evidence is **solid** and supported using a T cell hybridoma in vitro system, but remains **incomplete** without experiments with primary T cells or the use of in vivo models to assess the importance of the CD4 motifs in T-cell biology.

Introduction

Delineating the mechanistic principles by which multi-module receptors drive complex biological processes is important both for our broad understanding of receptor biology, and for guiding biomimetic engineering of synthetic receptors for therapeutic purposes. For example, comparing the conventional chimeric antigen receptor (CAR) framework utilized in CAR-T cell therapy, which was informed by an early 1990's understanding of receptor biology, with the multi-module receptors that naturally drive T cell responses suggests that conventional CARs integrate with the T cell intracellular signaling machinery differently than their natural counterparts (Harris and Kranz, 2016 [↗](#); Wu et al., 2020 [↗](#)). These differences are likely to underlie differences in signaling output, leading us to posit that fully coopting T cell functions with synthetic receptors requires that they be designed to integrate with the T cell's intracellular signaling machinery in a way that mimics the native receptors (Kobayashi et al., 2020 [↗](#)). Achieving this goal requires a more complete understanding of the multi-module pMHC-specific receptors that mediate antigen-specific T cell activation. Understanding how CD4⁺ T cells respond is particularly important for informing biomimetic engineering of CARs given recent data showing that redirecting CD4⁺ T cells with CARs is important for long-lived therapeutic efficacy (Melenhorst et al., 2022 [↗](#)).

CD4⁺ T cells are driven by 5-module receptors that recognize and drive responses to peptide antigens embedded within MHCII (pMHCII). Each naïve CD4⁺ T cell expresses a clonotypic receptor module, called the T cell receptor (TCR), that binds specifically to unique features of composite pMHCII surfaces (Kuhns and Badgandi, 2012 [↗](#); Kuhns and Davis, 2012 [↗](#)). The TCR lacks intracellular signaling domains and instead assembles with three signaling modules (CD3 γ , $\delta\epsilon$, and ζ) that have immunoreceptor tyrosine-based activation motifs (ITAMs), and other motifs, to connect TCR-pMHCII interactions to the intracellular signaling apparatus. CD4 is the coreceptor module. It binds pMHCII in a reciprocal fashion with TCR-CD3 on the outside of CD4⁺ T cells and interacts with the Src kinase, Lck, via an intracellular CQC zinc clasp motif and helix (Huse et al., 1998 [↗](#); Kim et al., 2003 [↗](#)). According to the widely accepted TCR signaling paradigm, co-engagement of pMHCII by TCR-CD3 and CD4 positions Lck and the CD3 ITAMs in the proper spatial proximity to enable Lck phosphorylation of the CD3 ITAMs to initiate signaling (Rudd, 2021 [↗](#)). However, when we tested this model directly, we found that CD4-Lck interactions are not key determinants of CD3 ζ ITAM phosphorylation (pCD3 ζ) (Lee et al., 2022 [↗](#)). Based on our work, as well as work from other labs, we consider the question of how intracellular pMHCII-specific CD4⁺ T cell signaling is initiated to be unresolved (Glassman et al., 2018 [↗](#); Horkova et al., 2023 [↗](#); Killeen and Littman, 1993 [↗](#); Xu and Littman, 1993 [↗](#)).

The answer to this question lies with how the 5 modules of pMHCII receptors evolved over 435 million years to refine pMHCII-specific signaling. In our recent publication, we computationally reconstructed CD4 evolution to identify residues and motifs in the extracellular, transmembrane, and intracellular domains that are of functional importance (Lee et al., 2022 [↗](#)). We then performed structure function analysis with mutants of key motifs to infer their function by

evaluating the phenotypes of the mutants. When we mutated the intracellular CQC clasp and IKRLH helix motifs that are known to mediate CD4-Lck interactions, we observed significant reductions in CD4-Lck association; yet we did not observe the expected impact on CD3 ζ ITAM phosphorylation (pCD3 ζ) predicted by the TCR signaling paradigm. In contrast, when we mutated the transmembrane GGXXG motif and cytoplasmic juxtamembrane (C/F)CV+C palmitoylation motif, we observed an increased frequency of CD4-Lck pairs that were preferentially localized to detergent soluble membrane domains. We also found evidence of reduced CD3 ζ , Zap70, and Plcy1 phosphorylation (pCD3 ζ , pZap70, and pPlcy1) compared with WT CD4. The reason that constituent residues of these motifs evolved under purifying selection appears to be due to reduced signaling in response to pMHCII when the motifs are altered. We took these data as evidence that the GGXXG and (C/F)CV+C motifs arose in eutherians (placental mammals) to tightly regulate pMHCII-specific signal initiation and postulated that the mutant CD4 molecules might sequester Lck away from TCR-CD3 to prevent CD3 ζ phosphorylation. However, an alternative hypothesis is that the GGXXG and (C/F)CV+C motifs impact pCD3 ζ levels independently of CD4-Lck interactions.

Here we took a reductionist approach to test these hypotheses by studying the impact of GGXXG and (C/F)CV+C motif mutants individually, or together, on proximal pMHCII-specific signaling in C-terminally truncated CD4 molecules (T1). Working with T1 eliminated motifs in the intracellular domain, including those that mediate CD4-Lck association. As expected from prior work, truncating CD4 relieved CD4-Lck interactions yet only slightly reduced IL-2 production (Lee et al., 2022 [↗](#)). Importantly, there was no impact on average pCD3 ζ levels or percent of cells with phosphorylated CD3 ζ , when compared with wild-type (WT) CD4, despite significantly reduced levels of CD4-Lck pairs. These data provide further evidence that the core tenet of the TCR signaling paradigm, wherein CD4 recruits Lck to CD3 ITAMs initiates signaling, needs revising. Importantly, T1 molecules bearing mutations in the GGXXG and (C/F)CV+C motifs individually or together reduced IL-2 production as well as pCD3 ζ levels and other TCR-proximal signaling events. The simplest interpretation of these data is that the GGXXG and (C/F)CV+C motifs are key determinants of pMHCII-specific signaling on their own, independent of CD4-Lck interactions.

Results

The goal of this study was to evaluate if the transmembrane GGXXG and juxtamembrane palmitoylation ((C/F)CV+C) motifs influence pMHCII-specific proximal signaling on their own rather than by sequestering CD4-Lck pairs away from membrane rafts (**Figure 1** [↗](#)). Accordingly, we used 58 α β ⁺ T cell hybridomas transduced to express the 5c.c7 TCR along with either the WT or mutant CD4 constructs described in **Table 1** [↗](#). The well-characterized 5c.c7 TCR recognizes the moth cytochrome c peptide 88-103 (MCC) in the context of mouse MHCII I-E^k. We used this experimental system because 58 α β ⁺ cells were used in seminal work that helped form the foundation of the TCR signaling paradigm (Glaichenhaus et al., 1991 [↗](#)). In that study, a loss of CD4-Lck interactions due to mutating the intracellular CQC clasp motif was linked with reduced signaling output as measured by IL-2 production. Furthermore, IL-2 production by these 58 α β ⁺ cells is CD4 dependent, indicating that relevant signaling pathways are intact.

WT and T1 equivalently enhance pMHCII-specific proximal signaling

We previously reported that the C-terminally truncated mouse CD4-T1 mutant (**T1: Table 1** [↗](#) and **Figure 1** [↗](#) arrow) had only a minor impact on IL-2 production relative to WT CD4 despite lacking most of the intracellular domain, including the CQC clasp and IKRLH motifs shown to mediate CD4-Lck association (Lee et al., 2022 [↗](#)). Because T1 maintains both the transmembrane GGXXG and juxtamembrane (C/F)CV+C motifs, we reasoned that it had utility for studying the contributions these motifs make to pMHCII-specific signaling in the absence of CD4-Lck interactions.

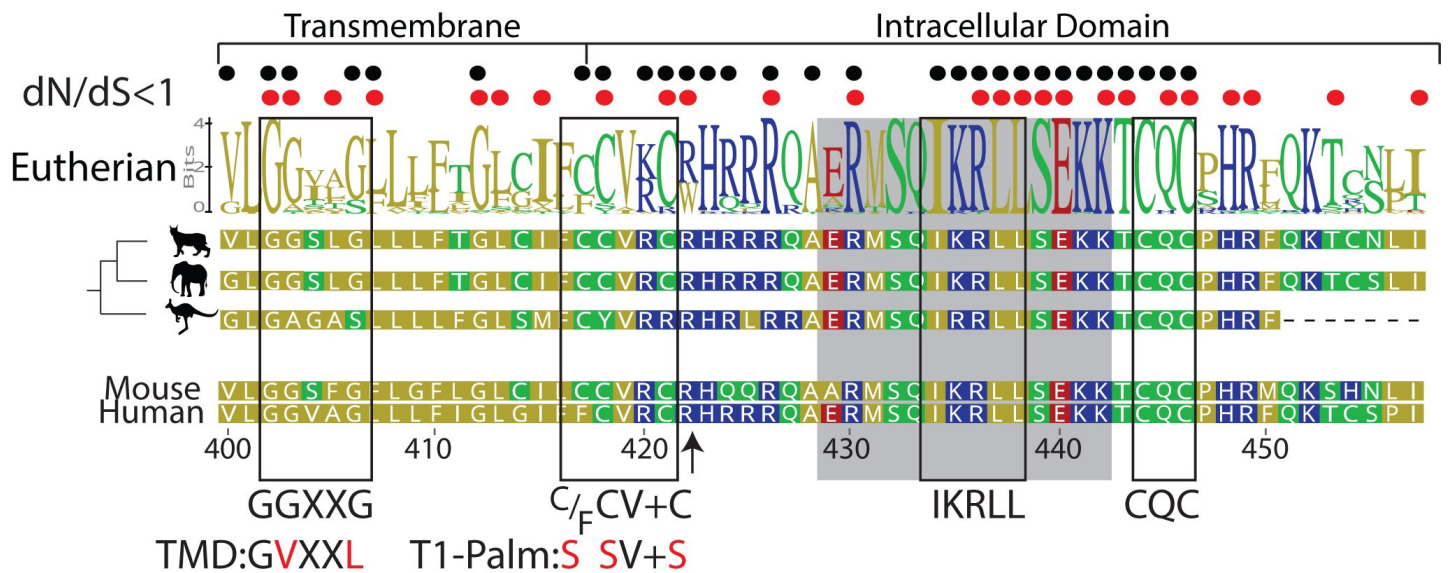


Figure 1.

Computational reconstruction of CD4 evolution

The maximum likelihood phylogenetic tree clusters mammalian CD4 sequences. The tree highlights ancestral reconstructions of CD4 sequences from marsupials (kangaroo silhouette), Atlantogenata (elephant), and Boreoeutheria (wildcat). The logoplot of extant eutherian (Atlantogenata and Boreoeutheria) CD4 sequences show sequence conservation over evolutionary time. In these plots, the height of symbols indicates the relative frequency of each amino acid at that specific position. The mouse CD4 numbering (uniprot) is used as a reference, and residues are color-coded based on sidechain polarity. Evolutionary insertion or deletion events are indicated by dashes (-). Most recent common ancestor (MRCA) sequences are shown at each node in the tree (Node 1-4). As in our previous study (Lee et al., 2022), the ratio of synonymous (dS) and nonsynonymous (dN) substitution rates was calculated. Black dots indicate dN/dS ratios that are significantly below 1 across the entire dataset. Red dots indicate residues under purifying selection in the mammalian only dataset. Previously identified motifs are indicated by boxes, while the intracellular domain helix is shaded gray. The arrow at position 422 indicates where CD4 was truncated (CD4-T1), while TMD and T1-Palm show the mutations studied in this study.

Motif location/known function	Mutant Names	Mutated Motif	Residue Mutations
C-terminally truncated CD4	T1	Ends at R422	R422 is the last residue
Extracellular D1 C"-strand (binds pMHCII)	T1 ^{Δbind}	GKGVLR	K68D, V70D, L71S, I72D, R73S
TMD/Protein or cholesterol interactions	T1-TMD	GGxxG	G403V, G406L
Juxtamembrane/Palmitoylation	T1-Palm (2C)	CV+C	C418S, C421S
Juxtamembrane/Palmitoylation	T1-Palm (3C)	CCV+C	C471S, C418S, C421S
TMD+Palm/raft localization	T1-TP (2C)	GGxxG, CV+C	G403V, G406L, C418S, C421S
TMD+Palm/raft localization	T1-TP (3C)	GGxxG, CCV+C	G403V, G406L, C417S, C418S, C421S

Table 1.

Motifs and mutants analyzed in this study

Accordingly, we generated 5c.c7⁺ 58αβ⁺ cells expressing WT CD4, T1, or T1 combined with our previously reported Δbind mutant, which disrupts binding to pMHCII in the CD4 D1 domain as well as signaling output (**Table 1** [↗](#) and **Figure 2** [↗](#) – **figure supplement 1** [↗](#)) (Glassman et al., 2016 [↗](#); Glassman et al., 2018 [↗](#); Parrish et al., 2015 [↗](#)). The goals were to determine: 1) if T1 significantly impairs pMHCII-specific proximal signaling events, relative to WT, as expected based on the TCR signaling paradigm because it lacks the CQC and IKRL motifs that mediate CD4-Lck interactions; 2) use T1^{Δbind} to determine the overall contribution of pMHCII-dependent assembly of CD4 with TCR-CD3 to proximal signaling. For IL-2 production, we observed a slightly reduced magnitude of the response that was consistent across a range of MCC peptide concentrations for T1 compared to WT (**Figure 2A** [↗](#)). We also observed reduced pMHCII-specific endocytosis for T1 compared to WT (**Figure 2** [↗](#) – **figure supplement 2** [↗](#)). Importantly, the T1^{Δbind} mutant did not produce IL-2 or undergo pMHCII-specific endocytosis. These data indicate that, in our system, extracellular CD4-pMHCII engagement is critical for IL-2 production while the CD4 intracellular domain C-terminal of R422 can be deleted.

Next, we evaluated the impact of these mutations on proximal signaling events measured by flow cytometry in response to antigen presenting cells (APCs) expressing agonist MCC:I-E^k after background subtraction of signal levels in response to APCs expressing null Hb:I-E^k (**Figure 2** [↗](#) – **figure supplement 3** [↗](#)). Our analysis of five independently generated pairs of WT and T1 cell lines showed variable difference in pCD3ζ levels. For three pairs of lines, the T1 response was higher than the WT and for two it was lower (**Figure 2B** [↗](#)). The averages of the T1 response normalized to the paired WT controls showed no difference between the population. There were also no differences for the average percent of T1 cells with response levels to MCC that were above the null Hb peptide when normalized to their paired WT controls. Similar results were observed for pZap70 and pPlcy1 levels (**Figure 2** [↗](#) – **figure supplement 4** [↗](#)). These data, summarized in **Table 2** [↗](#), provide additional evidence that CD4-Lck interactions via the CQC clasp and IKRL motifs are not key determinants of pMHCII-specific early signal events or IL-2 production in this experimental system.

In contrast, the T1^{Δbind} mutant significantly reduced pCD3ζ, pZap70, or pPlcy1 levels compared to the T1 control but did not obviously impact the percent of cells responding to agonist MCC:I-E^k (**Figure 2C** [↗](#) and **Figure 2** [↗](#) – **figure supplement 5** [↗](#)). Because the antibodies used to detect pCD3ζ, pZap70, and pPlcy1 recognize single phosphorylated tyrosines, these data indicate that CD4 enhances the number of TCR-CD3 complexes per cell with phosphorylated CD3ζ molecules at Y142 (same in human and mouse), as well as the number of phosphorylated Zap70 and Plcy1 molecules per cell, in a manner that is dependent on TCR-CD3 and CD4 co-engagement of pMHCII but independent of the intracellular domain C-terminal of R422. A summary of these results can be found in **Table 3** [↗](#).

The GGXXG and (C/F)CV+C motifs influence IL-2 production independently of Lck

Having established that T1 contributes to early pMHCII-specific signaling (e.g. pCD3ζ) as well as signaling output (i.e. IL-2), we generated 5c.c7⁺ 58αβ⁺ cells expressing WT, T1, or the truncated T1 wherein the GGXXG and/or (C/F)CV+C motifs are also mutated (**Table 1** [↗](#) and **Figure 3** [↗](#) – **figure supplement 1** [↗](#)). To study the function of the GGXXG motif alone, we used our previously described TMD mutant (GGXXG to GVXXL) wherein bulky side chains replace the glycines that compose a flat surface on the transmembrane helix that could mediate protein:protein or protein:cholesterol interactions (Fessler, 2016 [↗](#); Parrish et al., 2015 [↗](#); Song et al., 2014 [↗](#); Teese and Langosch, 2015 [↗](#); Wacker et al., 2013 [↗](#)). We called these mutants T1-TMD. To test the function of the palmitoylation motif, which contains the core CVRC (418-421) sequence in mouse and humans, we made note of a broader (C/F)CVRC motif in eutherians wherein the majority of ortholog sequences had CCVRC, as found in mouse, and a minority had FCVRC as found in humans.

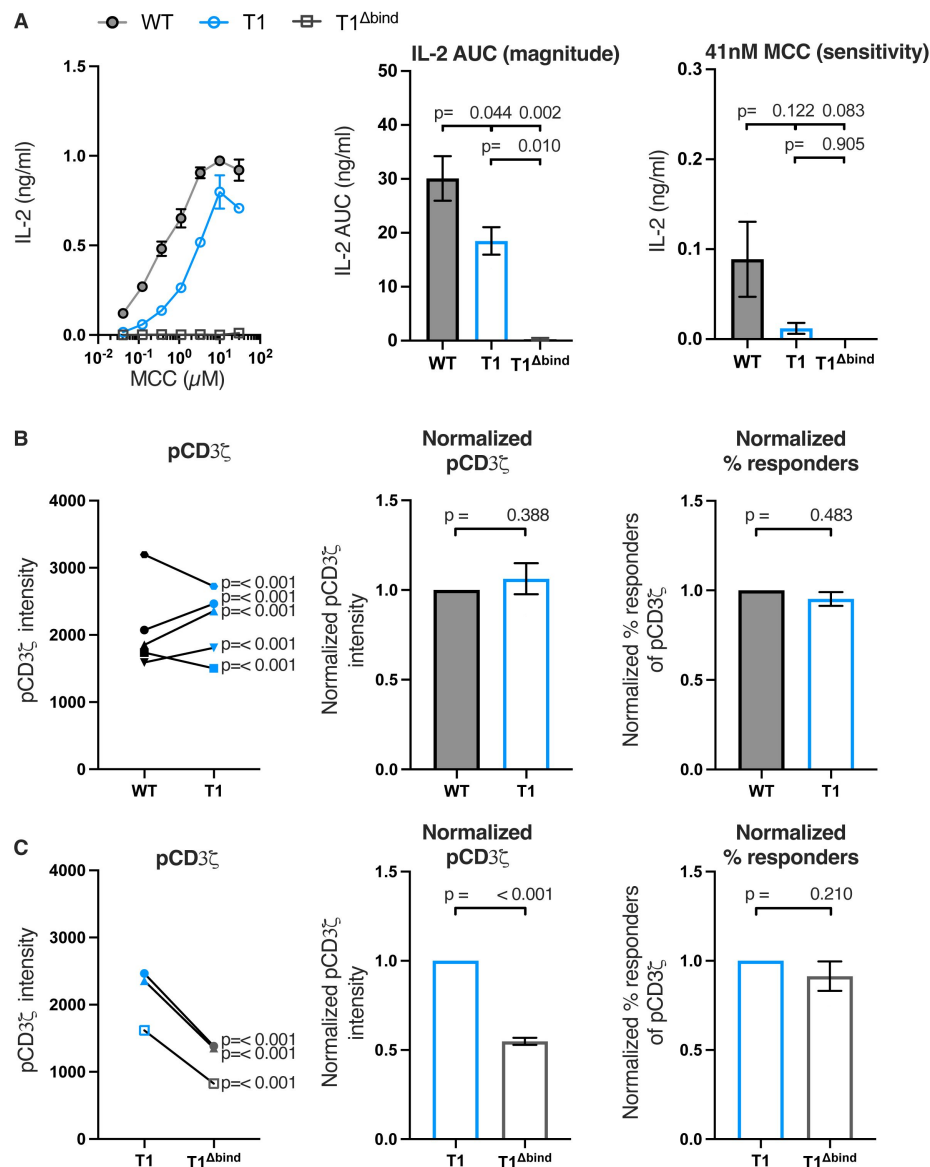


Figure 2.

Truncating CD4 does not reduce CD3 ζ phosphorylation

(A) Representative IL-2 production is shown in response to a titration of MCC peptide from one experiment (left). Experiments were performed in triplicate and each symbol equals the mean $\pm$ SEM at that peptide concentration. AUC analysis for the dose response is shown as a measure of the response magnitude for the average of three independent experiments performed with one independently generated set of cell lines (center). The average response to a low dose (41nM) of peptide is shown as a measure of sensitivity for three independent experiments performed with one independently generated set of cell lines (right). The data are representative of those obtained with 4 (WT vs T1) or 2 (WT vs T1 Δ bind) independently generated sets of cell lines.

(B) Phosphorylation intensity of CD3 ζ (pCD3 ζ) for WT and CD4-T1 (T1) (left), normalized pCD3 ζ intensity for WT and T1 (center), and normalized % responders of pCD3 ζ for WT and T1 (right). Five independently generated cell lines (biological replicates) were tested for WT and T1. For phosphorylation intensity, each pair of lines (connected symbols) was tested in three independent experiments. Data were analyzed and collected as in [Figure 2](#) – [figure supplement 3](#). One-way ANOVA was performed with a Dunnett's posttest using GraphPad Prism9. For normalized intensity and % responders, all individual mutant cell line values were normalized to their paired control values. Bars represent the mean $\pm$ SEM. One-way ANOVA was performed with a Sidak's posttest for specific comparisons of normalized values using GraphPad Prism9.

(C) Phosphorylation intensity of pCD3 ζ for T1 and T1 Δ bind (left), normalized pCD3 ζ intensity for T1 and T1 Δ bind (center), and normalized % responders of pCD3 ζ for T1 and T1 Δ bind (right) were performed and analyzed as in B, with the exception that the open symbols represent data from a single experiment whereas the closed symbols represent aggregate data from three independent experiments. Two-tailed t tests were performed to compare the single T1 vs T1 Δ bind samples as no other samples were collected in parallel.

Mutant Names	IL-2 magnitude	IL-2 sensitivity	Normalized pCD3ζ intensity	Normalized % Responders pCD3ζ	Normalized pZap70 intensity	Normalized % Responders pZap70	Normalized pPcγ1 intensity	Normalized % Responders pPcγ1	Lck DRM AUC	Lck Total AUC	Lck DSM AUC	CD4 expression	CD4 endocytosis
WT	100	100	100	100	100	100	100	100	100	100	100	100	100
T1	61.46	13.52	106.30	95.20	106.10	102.20	104.40	112.60	28.81	19.22	16.08	110.09	14.30

Table 2.

All values presented as percent of WT control (truncation average/WT average x 100) for normalized values

Mutant Names	IL-2 magnitude	IL-2 sensitivity	Normalized pCD3ζ intensity	Normalized % Responders pCD3ζ	Normalized pZap70 intensity	Normalized % Responders pZap70	Normalized pPcγ1 intensity	Normalized % Responders pPcγ1	Lck DRM AUC	Lck Total AUC	Lck DSM AUC	CD4 expression	CD4 endocytosis
T1	100	100	100	100	100	100	100	100	100	100	100	100	100
T1 ^{Δbind}	1.53	0.00	54.86	91.39	71.96	111.9	74.32	106.8	ND	ND	ND	73.93	38.71
TMD	53.54	17.05	76.68	94.23	88.36	98.77	88.24	107.40	62.36	92.97	110.94	131.10	3.63
Palm (2C)	57.99	116.07	76.35	94.09	86.17	93.03	92.09	98.09	274.03	139.40	130.91	80.30	-98.00
TP (2C)	33.11	0.19	62.06	85.61	79.79	75.58	82.57	83.34	121.39	113.03	114.98	96.54	47.50
Palm (3C)	27.84	0.19	71.09	94.76	86.32	76.82	80.37	88.62	137.57	94.34	92.07	87.58	6.99
TP (3C)	31.19	45.53	62.49	84.60	80.16	66.92	70.91	82.81	86.84	101.20	102.74	79.59	-54.69

Table 3.

All values presented as percent of T1 control (mutant average/T1 average x 100) for normalized values

Importantly, we previously noted that position 417 is under purifying selection for all extant CD4 orthologs we analyzed by the fixed effects likelihood (FEL) method, indicating that this residue evolved under purifying selection due to functional importance (Lee et al., 2022). We therefore made T1-Palm (3C) mutants wherein we mutated all three cysteines to serines as well as T1-Palm (2C) mutants where only the core cysteines common to mouse and humans were mutated to serines, as in our prior analysis of CD4 WT. Finally, we made mutants containing both the TMD and Palm mutants that we called T1-TP(3C) or T1-TP(2C).

To confirm that truncating CD4 relieved CD4-Lck association, and evaluate if the mutations impacted any residual CD4-Lck interactions that may occur due to colocalization in particular membrane domains, we performed sucrose gradient analysis of detergent lysates of WT, T1, T1-TMD, T1-Palm(2C), T1-Palm(3C), T1-TP(2C) and T1-TP(3C). We observed significantly reduced CD4-Lck association in detergent resistant membranes (DRMs, aka membrane rafts) and detergent soluble membranes (DSMs) for T1 compared to WT (**Figure 3** – **figure supplement 2**) (Pike, 2006). The mutants did not impact residual CD4-Lck association in the DRMs or DSMs. Furthermore, the total Lck signal associated with CD4 was significantly lower for T1 compared with the WT, and the residual association was not impacted by the mutants (**Table 3** and **Figure 3** – **figure supplement 3**). We therefore conclude that, in our experimental system, any functional differences between the T1-TMD, T1-Palm(2C/3C), or T1-TP(2C/3C) mutants and the T1 control is a function of the motif we are studying in the absence of direct CD4-Lck interactions.

Next, we measured IL-2 production by WT, T1, and CD4 mutant cells to evaluate the impact of the mutations on signaling output in response to MCC. Here again, T1 was slightly lower than WT, while the T1-TMD reduced the IL-2 response magnitude and sensitivity relative to the T1 control (**Figure 3A**). This was expected given our prior work showing that the TMD mutant reduced IL-2 production driven by a shorter CD4 truncation mutant (CD4T, mutated at R420) (Parrish et al., 2015). Mutating the GGXXG or (C/F)CV+C motifs individually or together did not impact pMHCII-induced TCR endocytosis relative to the T1 control, and activation-induced CD4 endocytosis was inhibited for all truncated CD4 molecules (**Figure 3** – **figure supplement 4A** and **4B**). The T1-Palm(2C), T1-TP(2C), T1-Palm(3C), and T1-TP(3C) all reduced IL-2 production relative to T1, indicating that they also influence the Lck-independent contribution of CD4 to pMHCII-specific signaling (**Figure 3B**). Of note, the T1-TP(2C) IL-2 response magnitude was lower than that of T1-Palm(2C) alone, consistent with what we previously observed with the full-length versions of these CD4 mutants (**Figure 3B**) (Lee et al., 2022). Furthermore, the T1-Palm(3C) response magnitude was lower than that of T1-Palm(2C), suggesting that the additional cysteine contributes to signaling. Together, these data indicate that the GGXXG and (C/F)CV+C motifs contribute to the signaling cascade that leads to pMHCII-specific IL-2 production when CD4 does not directly interact with Lck via the intracellular CQC clasp and IKRL motifs. These data do not tell us where in the signaling pathway the contributions are made.

The CD4 GGXXG and (C/F)CV+C motifs are key determinants of proximal signaling

To evaluate if the GGXXG and (C/F)CV+C motifs influence early signaling events, we measured pCD3 ζ , pZap70, and pPlcy1 levels for four independently generated T1-TMD lines, three independently generated T1-Palm(2C) lines, and three independently generated T1-TP(2C) lines relative to their matched T1 controls in response to APCs expressing MCC:I-E^k, as introduced above (**Figure 2** – **figure supplement 3**). For each T1-TMD and T1-TP(2C) mutant line, we observed lower pCD3 ζ , pZap70, and pPlcy1 levels than their paired T1 control. For the T1-Palm(2C) lines, all 3 showed reduced pCD3 ζ levels, but only 2 of 3 showed reduced pZap70, and pPlcy1 levels (**Figure 4A** and **Figure 4** – **Figure supplement 1A** and **2A**). These data indicate that the GGXXG and (C/F)CV+C motifs contribute to CD4's ability to enhance proximal signaling in the absence of CD4-Lck interactions.

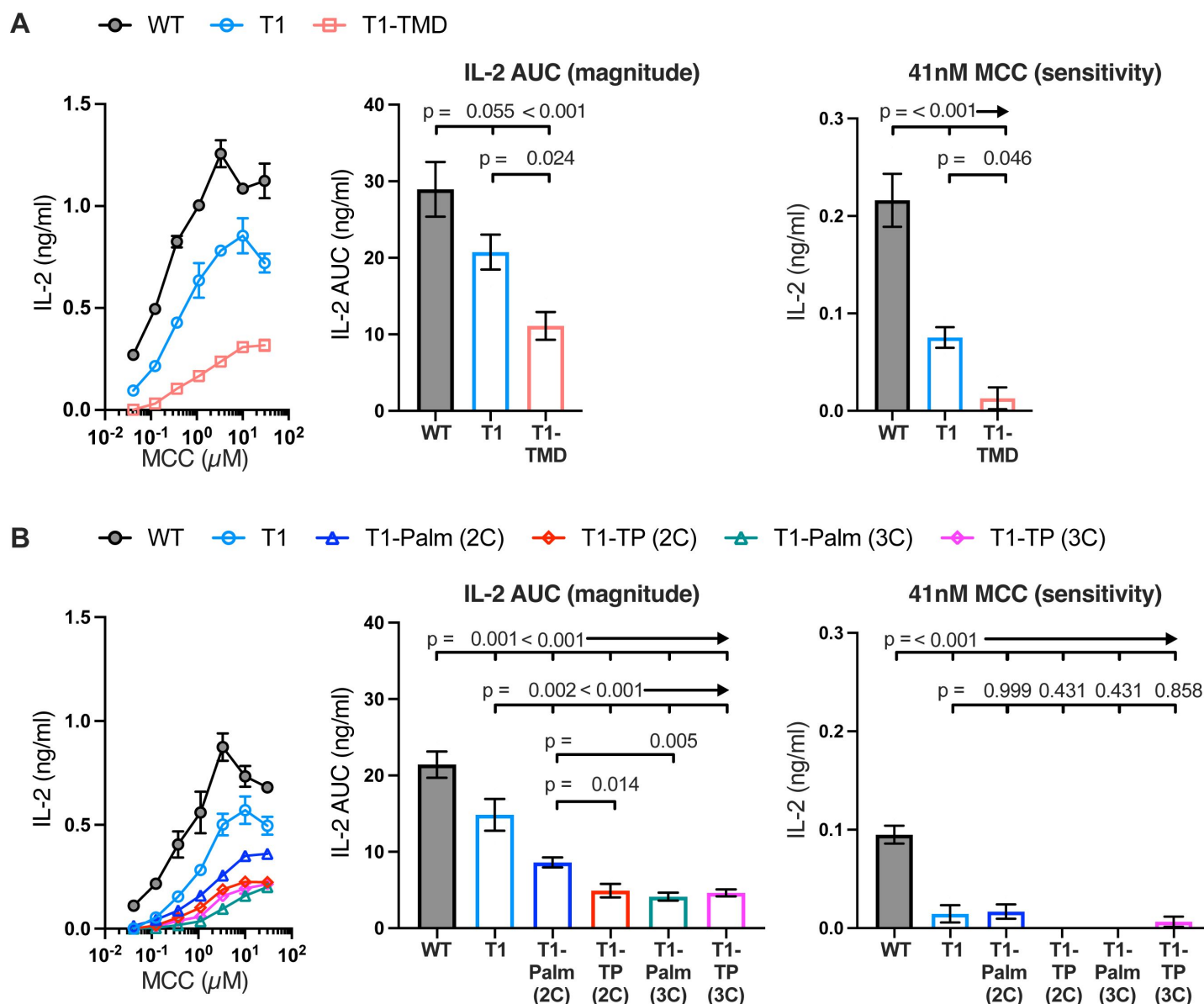


Figure 3.

The GGXXG and CV+C motifs are key determinants of CD4 function

(A, B) Representative IL-2 production is shown in response to a titration of MCC peptide from one experiment (left). Experiments were performed in triplicate and each symbol equals the mean $\pm$ SEM at that peptide concentration. AUC analysis for the dose response is shown as a measure of the response magnitude for the average of three independent experiments performed with one independently generated set of cell lines (center). The average response to a low dose (41nM) of peptide is shown as a measure of sensitivity for three independent experiments performed with one independently generated set of cell lines (right). Results are representative of those obtained with at least three independently generated sets of cell lines. One-way ANOVA was performed with a Dunnett's posttest for comparisons with WT and T1 samples, and a Sidak's posttest for comparisons between selected samples.

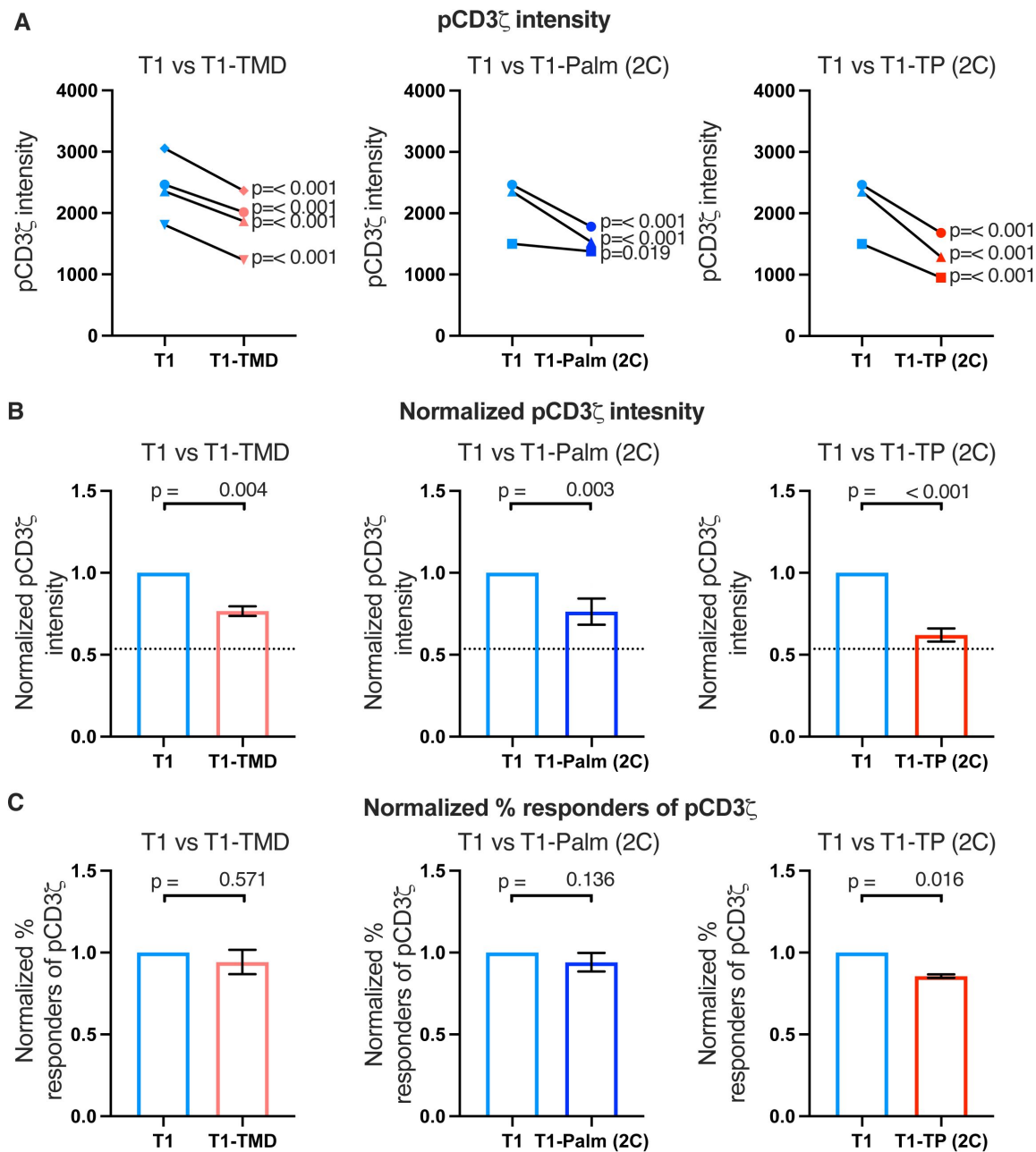


Figure 4.

The GGXXG and CV+C motifs reduce pCD3 ζ levels

(A) Phosphorylation intensity of CD3 ζ for T1 and T1-TMD (left), T1 and T1-Palm (2C) (center), and T1 and T1-TP (2C) (right) are shown for independently generated pairs of (connecting line) T1 and T1-TMD (left), T1 and T1-Palm (2C) (center), and T1 and T1-TP (2C) (right) cell lines. Each pair of lines (connected closed symbols) was tested in three independent experiments (technical replicates). The data from those experiments was aggregated, and the symbols represent the mean intensity of the aggregated pCD3 ζ intensity values. One-way ANOVA was performed with a Dunnett's posttest using GraphPad Prims 9.

(B) Data for each cell line in A are shown as the average pCD3 ζ intensity for all T1-TMD (left), T1-Palm (2C) (center), and T1-TP (2C) (right) cell lines normalized to their paired T1 control. Dotted line is the normalized pCD3 ζ intensity for T1^{Δbind} as a visual reference point for the contributions of CD4-pMHCII interactions. Bars represent the mean $\pm$ SEM. One-way ANOVA was performed with a Sidak's posttest for specific comparisons using GraphPad Prims 9.

(C) The average % responders for phosphorylation of CD3 ζ is shown for T1-TMD (left), T1-Palm (2C) (center), and T1-TP (2C) (right) normalized to their paired T1 control line. Bars represent the mean $\pm$ SEM. One-way ANOVA was performed with a Sidak's posttest for specific comparisons using GraphPad Prims 9.

We also considered the average fold change of the means of the independently generated mutant cell lines relative to their paired T1 controls (**Figure 4B** and **Figure 4C** – **Figure supplement 1B** and **2B**). For this analysis we included the mean of the fold change of the T1^{Δbind} mutant cells from **Figure 2C** relative to their paired T1 controls (dotted line) as a visual to consider the severity of the impact of the mutations on proximal signaling relative to the contributions of T1, which can bind pMHCII, as well as the level of signaling observed with T1^{Δbind} that cannot bind pMHCII. The T1-TMD and T1-Palm(2C) showed reduced pCD3ζ and pZap70 levels midway between T1 and T1^{Δbind}, while the T1-TP(2C) mutant closely approximated the dotted line representing the signaling observed in the T1^{Δbind} cells. Differences in the average pPly1 responses were less pronounced. These data further suggest a role for the GGXXG and (C/F)CV+C motifs in contributing to CD4-mediated pMHCII-specific signaling in the absence of CD4-Lck interactions.

Because we had found that T1^{Δbind} cells failed to produce IL-2 in response to high peptide concentrations in our dose response experiments (**Figure 1A**), whereas the T1-TP(2C) mutant cells made IL-2 in response to high peptide concentrations (**Figure 3B**), we found it interesting that the early signaling responses we measure to APCs expressing high ligand densities of tethered pMHCII were similar between the T1^{Δbind} and T1-TP(2C) cells. We therefore evaluated IL-2 production of the T1, T1^{Δbind}, and T1-TP(2C) in response to the APCs used in these signaling studies to ask if they followed the same pattern of responses to high peptide doses in our prior experiments. As expected, we found that the T1-TP(2C) cells made approximately half as much IL-2 as the T1 controls, whereas IL-2 production by the T1^{Δbind} cells was negligible (**Figure 4** – **Figure supplement 3**). These data indicate that the ability of CD4 to interact with pMHCII can partially overcome the loss of function of the GGXXG and (C/F)CV+C motifs at high ligand densities with respect to a downstream signaling output, such as IL-2 production, even if the early signaling events measured at 2 minutes after pMHCII-engagement were similar.

Finally, we considered the percent of mutant responders normalized to the controls. We observed small decreases in T1-TP(2C) cells with pCD3ζ and pZap70 levels relative to the control, while pPly1 trended lower for this mutant. The single mutants were roughly equivalent to their matched T1 controls. These data indicate that mutating the GGXXG and (C/F)CV+C motifs together in the absence of the intracellular domain can reduce the number of cells that initiate pMHCII-specific signaling (**Figure 4C** and **Figure 4C** – **Figure supplement 1C** and **2C**). We did not observe this phenotype in our prior study when the intracellular domain was present, suggesting this is unique to the absence of the intracellular domain.

We also measured early signaling for three independently generated T1-Palm(3C) and T1-TP(3C) mutant cell lines compared to their paired T1 controls. For all lines, pCD3ζ, pZap70, and pPly1 levels were significantly lower than the controls, which was reflected in the normalized fold change (**Figure 5A** and **5B** and **Figure 5** – **figures supplement 1A**, **1B**, **2A**, **2B**). The fold change for the T1-TP(3C) levels relative to the controls was similar to the T1^{Δbind} cells (**Figure 5B** and **Figure 5** – **figures supplement 1B** and **2B**). These data provide further evidence that the GGXXG and (C/F)CV+C motifs together are key determinants of the Lck-independent contributions that CD4 makes to amplifying pMHCII-specific proximal signaling.

Finally, the percent responders were only lower for pZap70 for both T1-Palm(3C) and T1-TP(3C) relative to WT, while pCD3ζ and pPly1 trended lower (**Figure 5** and **Figure 5** – **figures supplement 1C** and **2C**). These data are consistent with the idea that, in the absence of the intracellular domain, mutating these motifs influences initiation of key signaling events. See **Table 3** for a summary of the data.

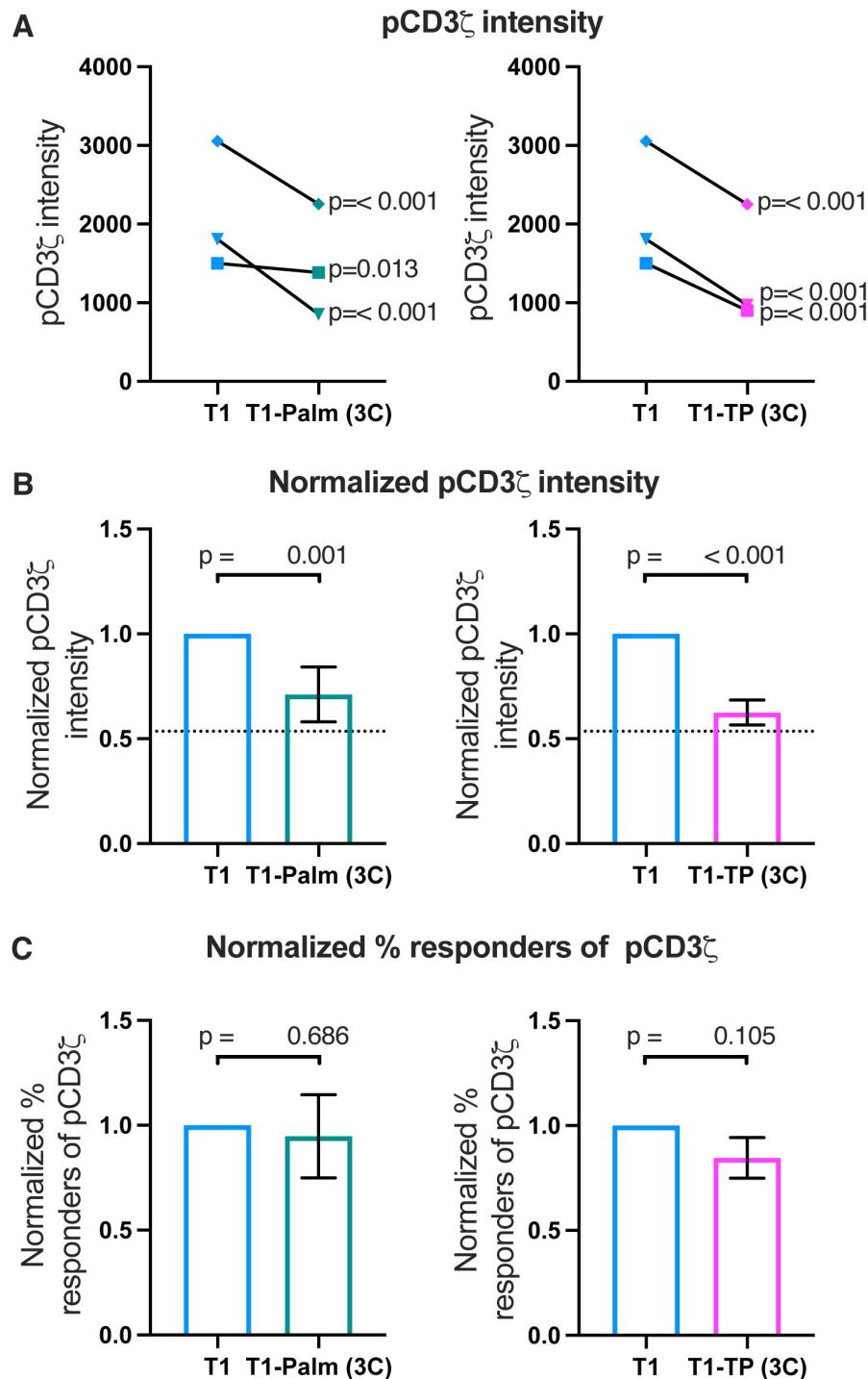


Figure 5.

The (C/F)CVRC motifs reduces proximal TCR-CD3 signaling

(A) Phosphorylation intensity of CD3 ζ for T1 and T1-Palm (3C) (left) and T1 and T1-TP (3C) (right) are shown for independently generated pairs of (connecting lines) T1 and T1-Palm (3C) (left) and T1 and T1-TP (3C) (right) cell lines. Analysis was performed as in [Figure 4A](#).

(B) Data for each mutant cell line in A are shown as the average pCD3 ζ intensity values for T1-Palm (3C) (left) and T1-TP (3C) (right) normalized to their paired T1 controls. The dotted line represents the normalized phosphorylation CD3 ζ intensity for T1^{Abind} as a visual reference for the contributions of CD4-pMHCII interactions. Analysis was performed as in [Figure 4C](#).

(C) Average % responders for phosphorylation of CD3 ζ is shown for T1-Palm (3C) (left) and T1-TP (3C) (right) normalized to their paired T1 control. Analysis was performed as in [Figure 4C](#).

Our computational reconstruction of CD4 evolution provided access to a subset of results from experiments that Nature performed over ~435 million years in a greater variety of jawed vertebrates than could be achieved with mouse or human studies (Lee et al., 2022 [DOI](#)). It allowed us to identify residues that were selected under purifying selection, and thus functionally significant, because acquiring a mutation at these residues affects fitness and results in a failure to propagate the change to future progeny. Our structure-function analysis of these residues in 58 $\alpha\beta^-$ cells, which we continued in this study, has started to provide mechanistic insights into why these residues are functionally important.

The data presented here are inconsistent with the prevailing model in which CD4 recruits Lck to CD3 ITAMs to initiate signaling. The decrease in pMHCII-specific IL-2 production when the CD4 intracellular domain is truncated cannot be attributed to reductions in early signaling events such as pCD3 ζ . Importantly, the pCD3 ζ levels were ~2 fold lower in the absence of CD4-pMHCII interactions (T1 Δ_{bind} mutant) under the experimental conditions tested here. We take these data as evidence that there is a threshold for the number of CD3 ζ molecules phosphorylated, and/or the duration of signaling, that is required to drive IL-2 production in our system. We also conclude that CD4 binding to pMHCII functions to increase the number of TCR-CD3 complexes per cell that experience CD3 ζ phosphorylation to reach that threshold and/or sustain signaling and that it similarly impacts pZap70 levels as well as pPly1 levels, albeit to a lesser extent for the latter.

A logical extension of these conclusions is that there is a density of agonist pMHCII below which CD4 binding to pMHCII is essential for signal initiation. This idea is supported by findings that CD4 is necessary for signal propagation and amplification to the calcium mobilization step in response to fewer than 25 agonist pMHCII (Irvine et al., 2002 [DOI](#)). Attempting to accurately measure the contributions of CD4 to ITAM phosphorylation, with or without mutant motifs, at very low densities of pMHCII would be challenging and is technically beyond the scope of this study. Nevertheless, in this and our prior study we did not observe the expected decreases in pCD3 ζ levels when CD4-Lck interactions were reduced by mutating the CQC clasp motif, the IKRLL motif, or by eliminating the intracellular domain. Furthermore, we found in our prior study that IL-2 production increased significantly when we mutated the full intracellular helix, the helix and CQC clasp together, or the IKRLL motif alone. These direct tests of the TCR signaling paradigm are inconsistent with predictions of the model. As such, they suggest the contributions of CD4 to signal initiation and amplification at the early timepoint we measured are independent of direct CD4-Lck interactions.

Because the combined impact of mutating the GGXXG and (C/F)CV+C motifs on pCD3 ζ levels were roughly equivalent to disrupting CD4-pMHCII engagement, even when direct CD4-Lck interactions were absent, we can conclude that the GGXXG and (C/F)CV+C motifs are key determinants that help CD4 drive signaling above the threshold required for IL-2 production. Our data also provide evidence that mutating the GGXXG and (C/F)CV+C motifs together can reduce the frequency of cells experiencing CD3 ζ and Zap70 phosphorylation in the absence of the intracellular domain. Given that palmitoylation is rapidly reversible, these data suggest that the switch-like function of the palmitoylation motif may influence signal initiation. Interestingly, unlike CD4-pMHCII interactions, these motifs are not essential for signaling output at high ligand densities as we observed low levels of IL-2 produced in cells bearing mutants of these motifs with increasing doses of agonist pMHCII. Reciprocal binding of CD4 and TCR-CD3 to pMHCII can therefore amplify pMHCII-specific signaling without the contributions of the GGXXG and (C/F)CV+C motifs, or motifs in the intracellular domain, albeit at lower levels than if these motifs are intact. This could be due to the contributions of additional unidentified motifs in the transmembrane domain, or to the increase in TCR-CD3 dwell time on pMHCII that is mediated by the CD4 extracellular domain.

(Glassman et al., 2018 [DOI](#)). Given that these motifs were essential for IL-2 production at low ligand densities, a logical extension of these data is that there is an agonist pMHCII density below which these motifs are essential for the initiation of early signaling events.

The data from our current study indicate that the GGXXG and (C/F)CV+C motifs do not influence early signaling events by regulating the ability of CD4 to either recruit Lck to, or sequester it away from, the TCR-CD3 ITAMs as we previously postulated (Lee et al., 2022 [DOI](#)). Instead, they work independently of CD4-Lck interactions to enhance pMHCII-specific signaling. It is tempting to speculate that because these motifs can regulate CD4 membrane domain localization and, in other proteins, palmitate moieties can sandwich cholesterol against the flat surface of a GG patch on a transmembrane domain helix, these motifs may allow CD4 to regulate local concentration of cholesterol or cholesterol sulfate around TCR-CD3, perhaps by taking such molecules away from TCR-CD3 to stabilize allosteric changes associated with signaling (Chen et al., 2022 [DOI](#); Fessler, 2016 [DOI](#); Gil et al., 2002 [DOI](#); Lee et al., 2015 [DOI](#); Song et al., 2014 [DOI](#); Swamy et al., 2016 [DOI](#); Teese and Langosch, 2015 [DOI](#); Wacker et al., 2013 [DOI](#); Wang et al., 2016 [DOI](#)). These CD4 motifs may also impact the accessibility of ITAMs for Lck phosphorylation by changing the local membrane environment around TCR-CD3 upon reciprocal engagement of pMHCII (Aivazian and Stern, 2000 [DOI](#); Xu et al., 2008 [DOI](#)). Finally, it is worth considering that, in our prior study, we found G402 of the GGXXG motif has covaried over evolutionary time with L438 of the intracellular helix IKRL motif that has inhibitory function, suggesting that the function of these motifs are co-evolving (Lee et al., 2022 [DOI](#)). In support of this idea, we reported in that study that the signal enhancing activity of the GGXXG and (C/F)CV+C motifs together counterbalanced the inhibitory activity of the IKRL motif with regards to IL-2 production. It is unclear at this point if this counterbalancing is simply additive of individual motif functions, or if there is a functional interplay of the motifs when the intracellular domain of CD4 is present. Delineating the mechanistic details by which the GGXXG and (C/F)CV+C motifs increase signaling on their own, or when combined, and how their function integrates with those of other motifs will be the subject of future investigations.

Altogether, the data in this and our preceding study provide direct evidence that complement indirect results indicating that pMHCII-specific signaling is not initiated, or dependent on, CD4 recruitment of Lck to the CD3 ITAMs (Glassman et al., 2018 [DOI](#); Horkova et al., 2023 [DOI](#); Killeen and Littman, 1993 [DOI](#); Lee et al., 2022 [DOI](#); Xu and Littman, 1993 [DOI](#)). Recent work on CD8 suggest that this coreceptor also does not recruit Lck to phosphorylate CD3 ITAMs (Casas et al., 2014 [DOI](#); Wei et al., 2020 [DOI](#)). In total, these findings suggest that the TCR signaling paradigm needs revising as it pertains to coreceptor-Lck interactions. For CD4, the existing evidence suggest that reciprocal binding of CD4 and TCR-CD3 enables free Lck and Fyn to initiate signaling by phosphorylating the ITAMs and Zap70 (Glassman et al., 2016 [DOI](#); Glassman et al., 2018 [DOI](#); Horkova et al., 2023 [DOI](#); Lee et al., 2022 [DOI](#); Salmond et al., 2009 [DOI](#); van Oers et al., 1996 [DOI](#)). The contribution of the current study is in showing the GGXXG and (C/F)CV+C motifs work together to enhance these early signaling events independently of direct CD4-Lck interactions.

It is interesting to consider what the primary purpose of CD4-Lck interactions via the CQC clasp and IKRL motifs are if not to recruit Lck to the CD3 ITAMs. Data from two studies suggest that Lck makes a kinase-independent contribution to pMHCII-specific responses; it is therefore plausible that the phenotype reported for CD4 when the CQC clasp is mutated is due to relieving the kinase-independent scaffolding function of Lck (Horkova et al., 2023 [DOI](#); Xu and Littman, 1993 [DOI](#)). In addition, CD4-Lck interaction via the CQC clasp motif and the intracellular helix will prevent the helix from interacting with other partners, thus mutating the CQC clasp would favor helix interactions with other putative partners (Lee et al., 2022 [DOI](#)). Finally, the CQC clasp is thought to play a role in positioning CD4 in proximity to Lat, either through direct or indirect interactions, which could contribute to the phenotype of CQC clasp mutants (Bosselut et al., 1999 [DOI](#); Lo et al., 2018 [DOI](#); Lo et al., 2019 [DOI](#)). If CD4-Lck and CD4-Lat interactions are mutually exclusive, and both

require the CQC clasp, then mutating this motif would have a greater impact than just relieving CD4-Lck interactions (Bosselut et al., 1999 [↗](#)). These questions remain outstanding, as do questions concerning how the intracellular helix regulates pMHCII-specific signaling.

The broader implications of the data presented in our current and prior evolution-structure-function studies provide fertile ground for future directions beyond those mentioned above. Our approach allows us to identify functionally important residues/motif and then interrogate basic principles of their function that should hold true across CD4⁺ T cell subsets since the biochemical and biophysical bases for interactions between CD4 and its interacting partners (i.e. other proteins or membrane components) should be the same in 58αβ⁺ cells as in thymocytes or in different CD4⁺ T cells. However, different CD4⁺ T cell populations will have different levels of expression of relevant proteins (e.g. interacting partners or modifying enzymes), and may even have differences in lipid composition, such that the impact of the motifs we have identified and studied to date may lead to different outcomes in different CD4⁺ T cell subsets (<http://immpres.co.uk/↗>) (Tuosto and Xu, 2018 [↗](#)). For example, differences in expression of enzymes with switch-like functions, such as those that add or remove palmitate to the (C/F)CV+C motif, or those that phosphorylate or dephosphorylate the serines in the intracellular helix that regulate CD4-Lck interactions and inhibitory activity, may vary between naïve CD4⁺ T cells, different Th subsets, or Tregs to differentially tune the activity of these motifs and their impact on pMHCII-specific signaling. Now that we have identified residues and broader motifs that have proven to be functionally important *in vivo* over 435 million years of evolution, while also providing mechanistic insights as to why the residues are important, there is utility in evaluating the impact of mutant CD4 conditional knock-in mice on thymocyte development, naïve CD4⁺ T cell activation and differentiation, and the execution of Th and Treg effector functions. In so doing, we will gain additional insights into why there is a fitness cost if a mutation is acquired in these residues. Such knowledge will increase our fundamental understanding of CD4⁺ T cell biology and will also be critical for biomimetic engineering of synthetic receptors that can redirect CD4⁺ T cell activity for therapeutic purposes.

Materials and methods

The work performed in this manuscript was conducted according to the materials and methods of our previous study, which can be found here: <https://elifesciences.org/articles/79508#s4>.

Funding

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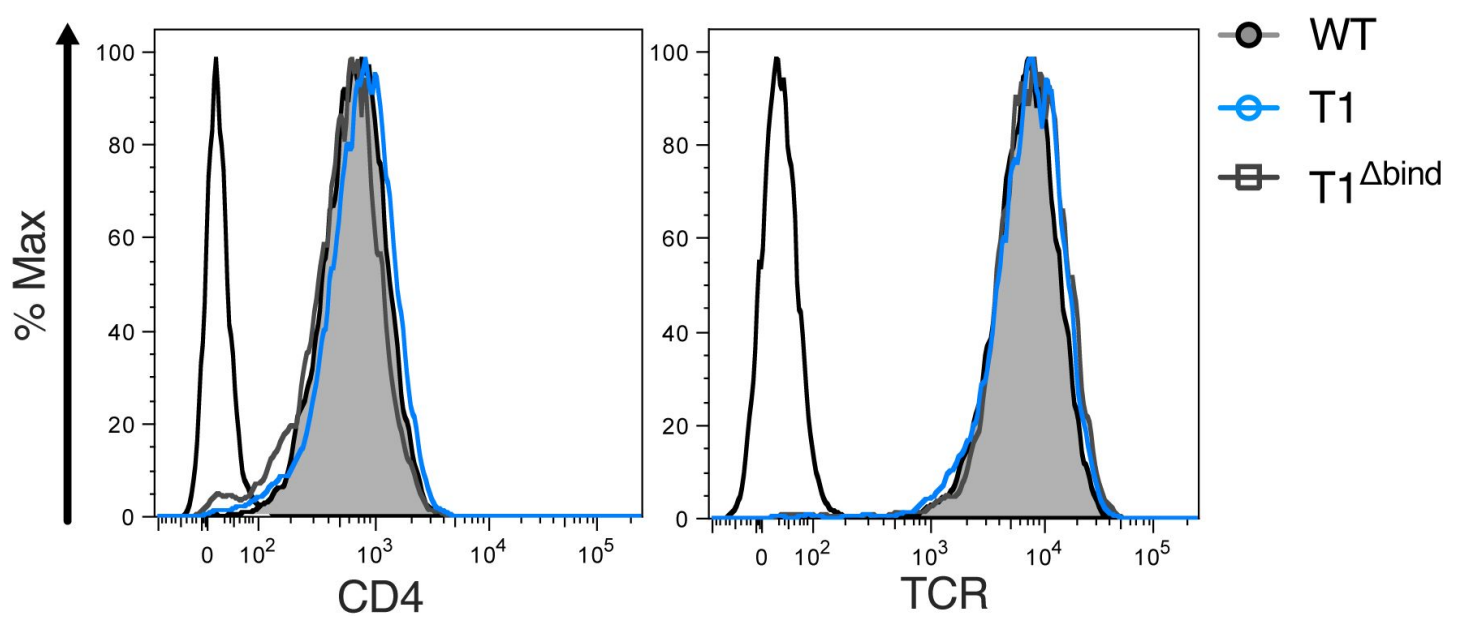


Figure 2- figure supplement 1.

Flow cytometry analysis of CD4 (left) and TCR (right) expression on 58 $\alpha\beta^{-}$ hybridoma cells. Parental 58 $\alpha\beta^{-}$ hybridoma cells served as negative control for surface expression (open black histogram trace).

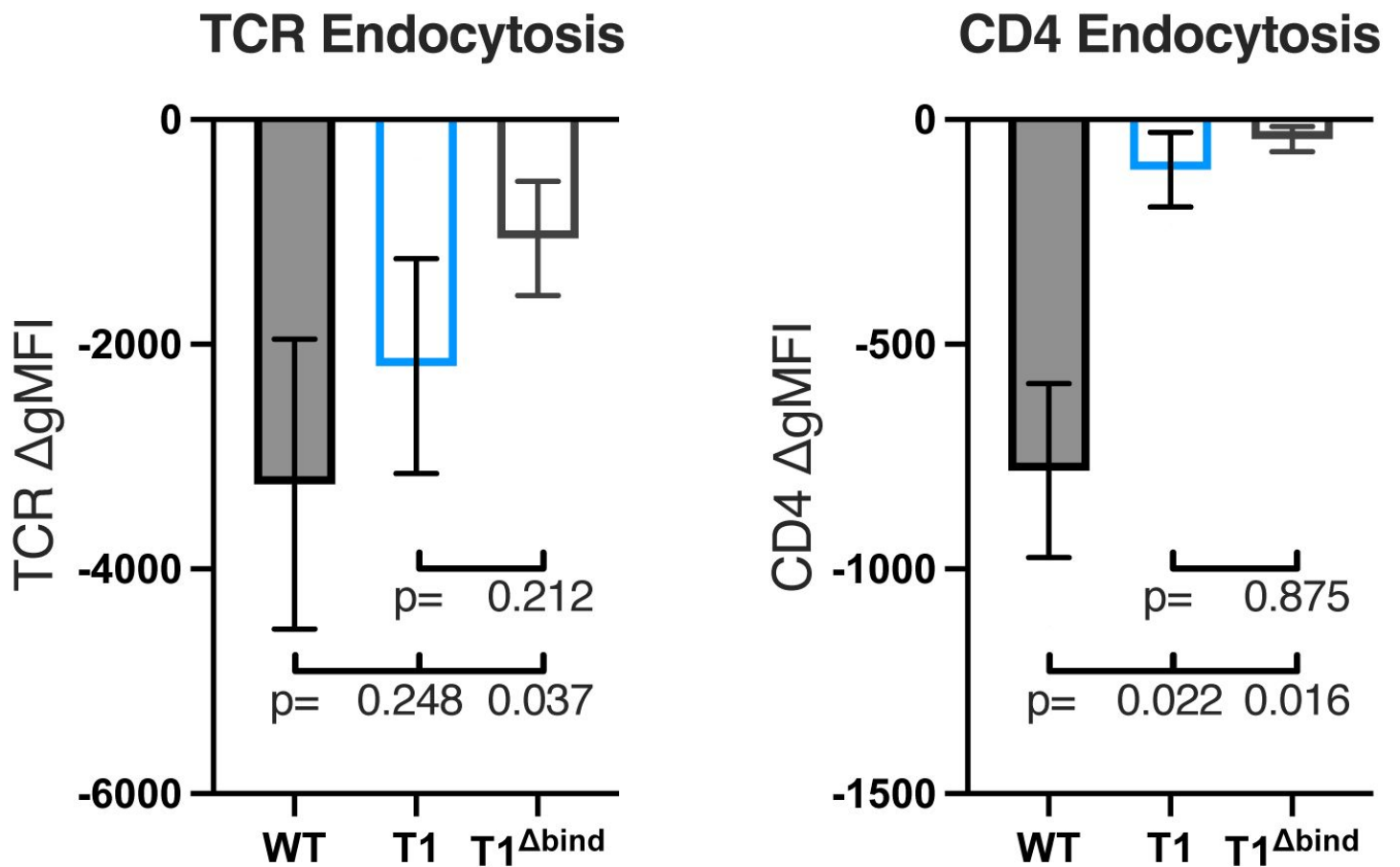


Figure 2- figure supplement 2.

TCR (left) and CD4 (right) endocytosis after pMHCII engagement is shown for the indicated cell lines after 16 hours coculture with APCs in the presence of 10 μ M MCC peptide. The change in TCR and CD4 gMFI, as measured by flow cytometry, is shown for each cell line relative to an equivalent sample cultured with APCs in the absence of MCC peptide. Each data point represents the mean $\pm$ SEM for three independent experiments. For endocytosis measurements were performed in triplicate for each experiment. One-way ANOVA was performed with a Dunnett's posttest.

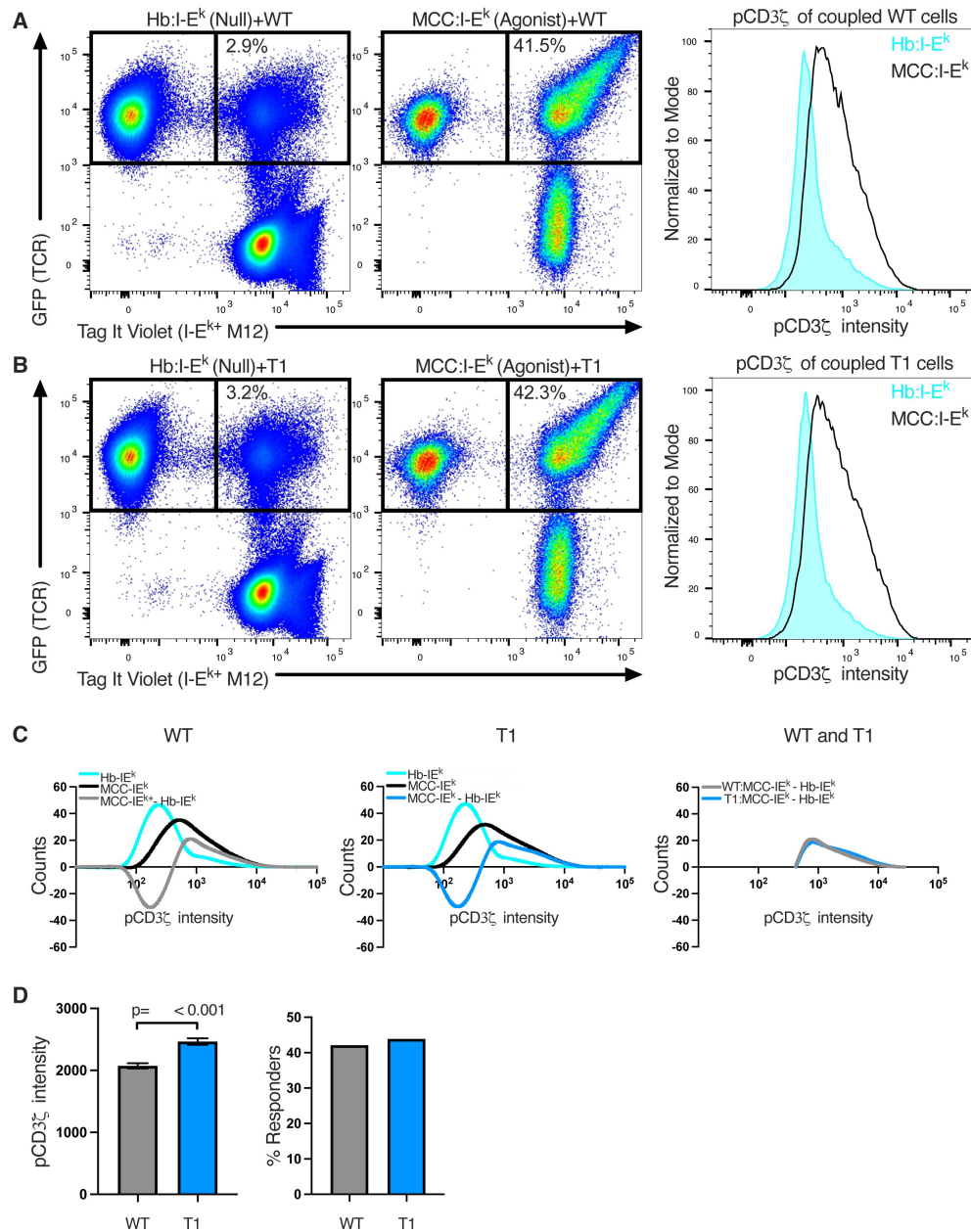


Figure 2- figure supplement 3.

Example of intracellular signaling analysis workflow.

(A) Flow cytometry analysis of WT 58αβ⁺ hybridoma:M12 cell couples. Representative dot plots are shown for TCRβ^{GFP+} CD4⁺ 58αβ⁺ hybridoma cells coupled to Tag-it Violet- labeled M12s expressing the indicated tethered pMHCII Hb:I-E^k (left) and MCC:I-E^k (center). Representative histograms of WT 58αβ⁺ hybridoma cells coupled to M12 cells transduced to express the indicated tethered pMHCII are shown for pCD3ζ intensity (right). 10,000 coupled cells were collected per individual experiment.

(B) Flow cytometry analysis of T1 58αβ⁺ hybridoma-M12 cell couples. Representative dot plots are shown for TCRβ^{GFP+} CD4⁺ 58αβ⁺ hybridoma cells coupled to Tag-it Violet- labeled Hb:I-E^k (left) and MCC:I-E^k (center) M12 cells. Representative histograms of T1 58αβ⁺ hybridoma cells coupled to M12 cells expressing the indicated tethered pMHCII are shown for pCD3ζ intensity (right). 10,000 coupled cells were collected per individual experiment.

(C) A representative smoothed overlapping histogram of pCD3ζ intensity is shown for 58αβ⁺ cells coupled to Hb:I-E^k (cyan) or MCC:I-E^k (black) M12 cells. Histogram of pCD3ζ intensity for 58αβ⁺ cells coupled to MCC:I-E^k M12 cells subtracted from Hb:I-E^k M12 cell couples show the difference in pCD3ζ intensity on a bin-by-bin basis after stimulation with agonist MCC:I-E^k compared with null Hb:I-E^k for WT (left) and T1 (center) cells. Overlapping pCD3ζ histogram (right) of cells responding to MCC:I-E^k after Hb:I-E^k subtraction shows the responding populations for the WT and T1 cell lines. Data represent the aggregate from three individual experiments (30,000 couples analyzed). One-way ANOVA was performed with a Dunnett's posttest for comparison with the WT sample because other mutants were simultaneously analyzed in this experiment (not shown).

(D) Concatenated pCD3ζ average intensity ± SEM of WT and T1 cells (left) and the percent of responding WT and T1 cells (right).

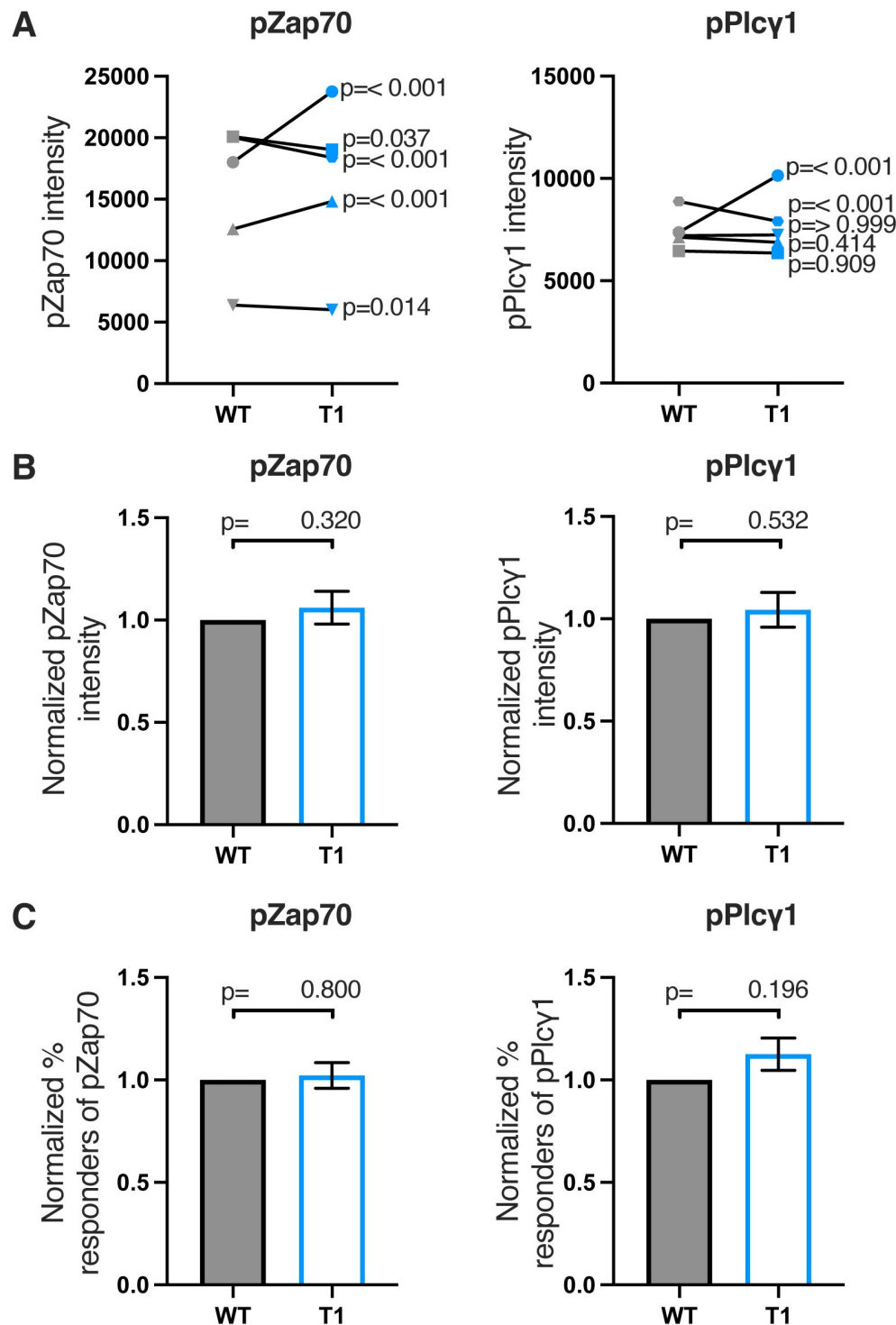


Figure 2- figure supplement 4.

(A) Zap70 (left), and Plcy1 (right) phosphorylation intensity for WT and T1 are shown for paired (connecting line) WT and T1 cell lines. Five independently generated cell lines were tested (biological replicates). For phosphorylation intensity, each pair of lines (connected symbols) was tested in three independent experiments (technical replicates). The data from those experiments was aggregated, and the symbols represent the mean intensity of the aggregated pCD3ζ intensity values. One-way ANOVA was performed with a Dunnett's posttest.

(B) Average T1 phosphorylation for Zap70 (left) and Plcy1 (right) from A are shown normalized to the paired WT controls. Bars represent the mean +/- SEM. One-way ANOVA was performed with a Sidak's posttest for specific comparisons.

(C) Average T1 % responders for pZap70 (left), and pPlcy1 (right) are shown normalized to the paired WT controls for the cell lines shown in A. Bars represent the mean +/- SEM. One-way ANOVA was performed with a Sidak's posttest for specific comparisons.

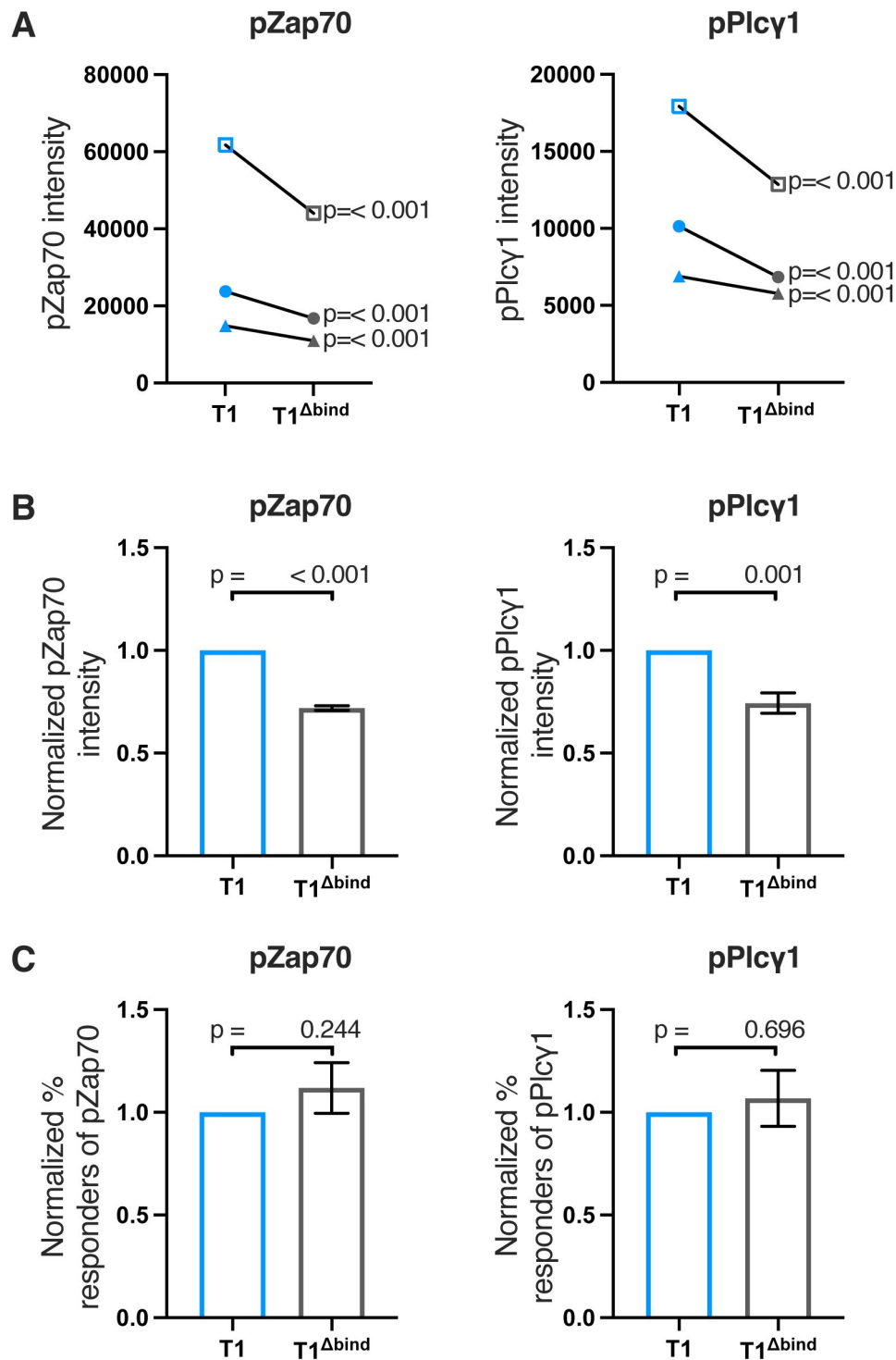


Figure 2- figure supplement 5.

(A) Phosphorylation intensity for T1 and T1^{Δbind} Zap70 (left), and Plcy1 (right) are shown for paired (connecting line) T1 and T1^{Δbind} cell lines. Three independently generated cell lines were tested (biological replicates). Analysis was performed as in **Figure 2** — figure supplement 4 with the exception that the open symbol represent data from a single experiment whereas the closed symbols represent the average of aggregated data from three independent experiments. For the open symbol comparisons we performed a two- tailed t test as no other samples were collected in parallel. (B) Average T1^{Δbind} cell line phosphorylation intensity for Zap70 (left), and Plcy1 (right) are shown normalized to the average intensity of the paired T1 control cells shown in A. Analysis was performed as in **Figure 2** — figure supplement 4. (C) Average T1^{Δbind} cell line % responders for pZap70 (left) and pPlcy1 (right) are shown normalized to their paired T1 control. Analysis was performed as in **Figure 2** — figure supplement 4.

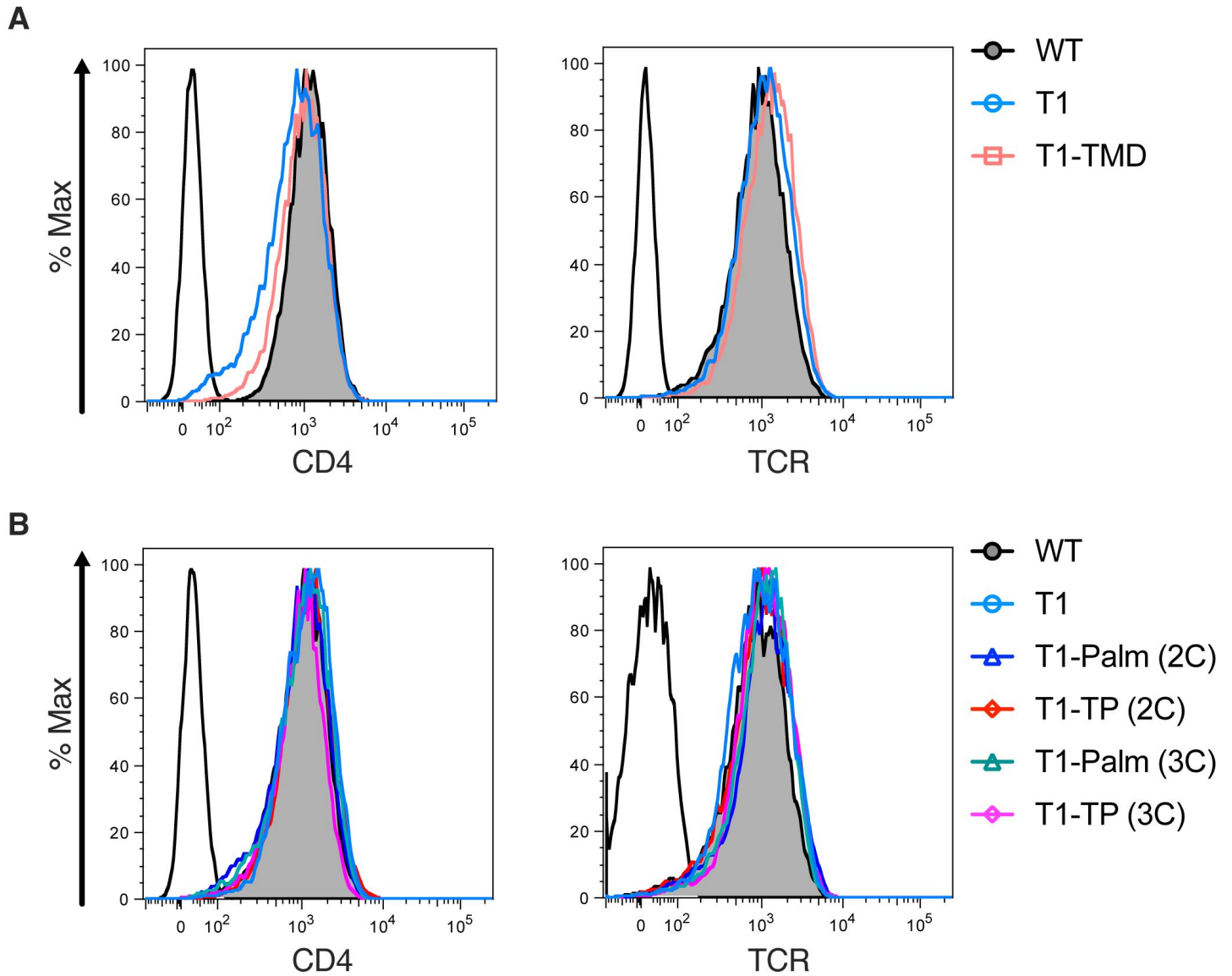


Figure 3- figure supplement 1.

(A, B) Flow cytometry analysis of CD4 (left) and TCR (right) expression on 58αβ⁺ hybridoma cells. Parental 58αβ⁺ hybridoma cells served as negative control for surface expression (open black histogram trace).

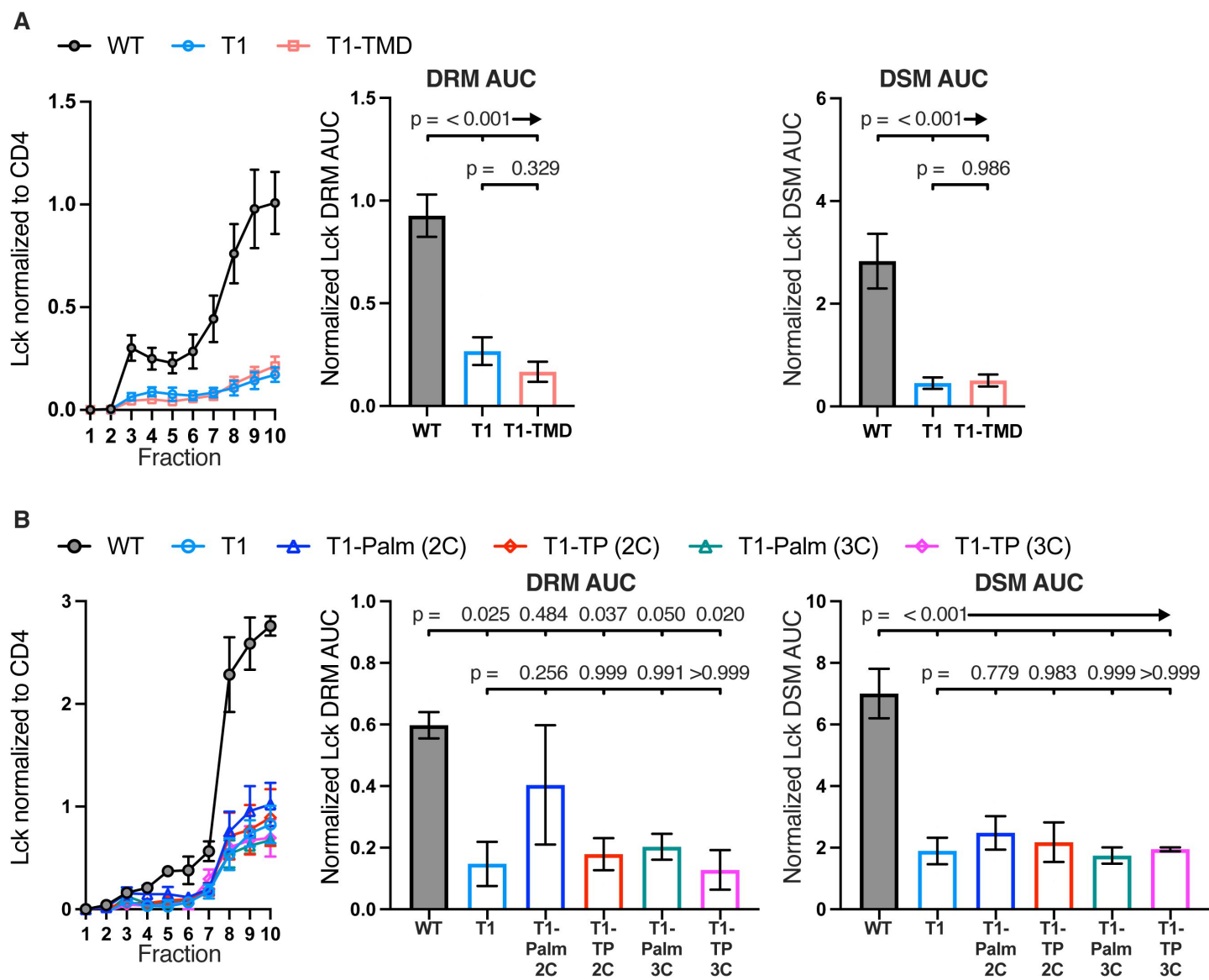


Figure 3- figure supplement 2.

(A, B) Lck signal is shown for each sucrose fraction normalized to the CD4 signal detected in the corresponding fraction (left). The AUC is shown for the normalized Lck signal in the DRM (center) and DSM (right) fractions. Each data point represents the mean $\pm$ SEM for three independent experiments with the same cell line. Data are representative of experiments performed with three independently generated sets of lines for TMD, Palm(3C), and TP(3C) mutants. Analysis was performed with two set of lines for the Palm(2C) and TP(2C) mutants. One-way ANOVA was performed with a Dunnett's posttest for comparisons with WT and T1 samples, and a Sidak's posttest for comparisons between selected samples.

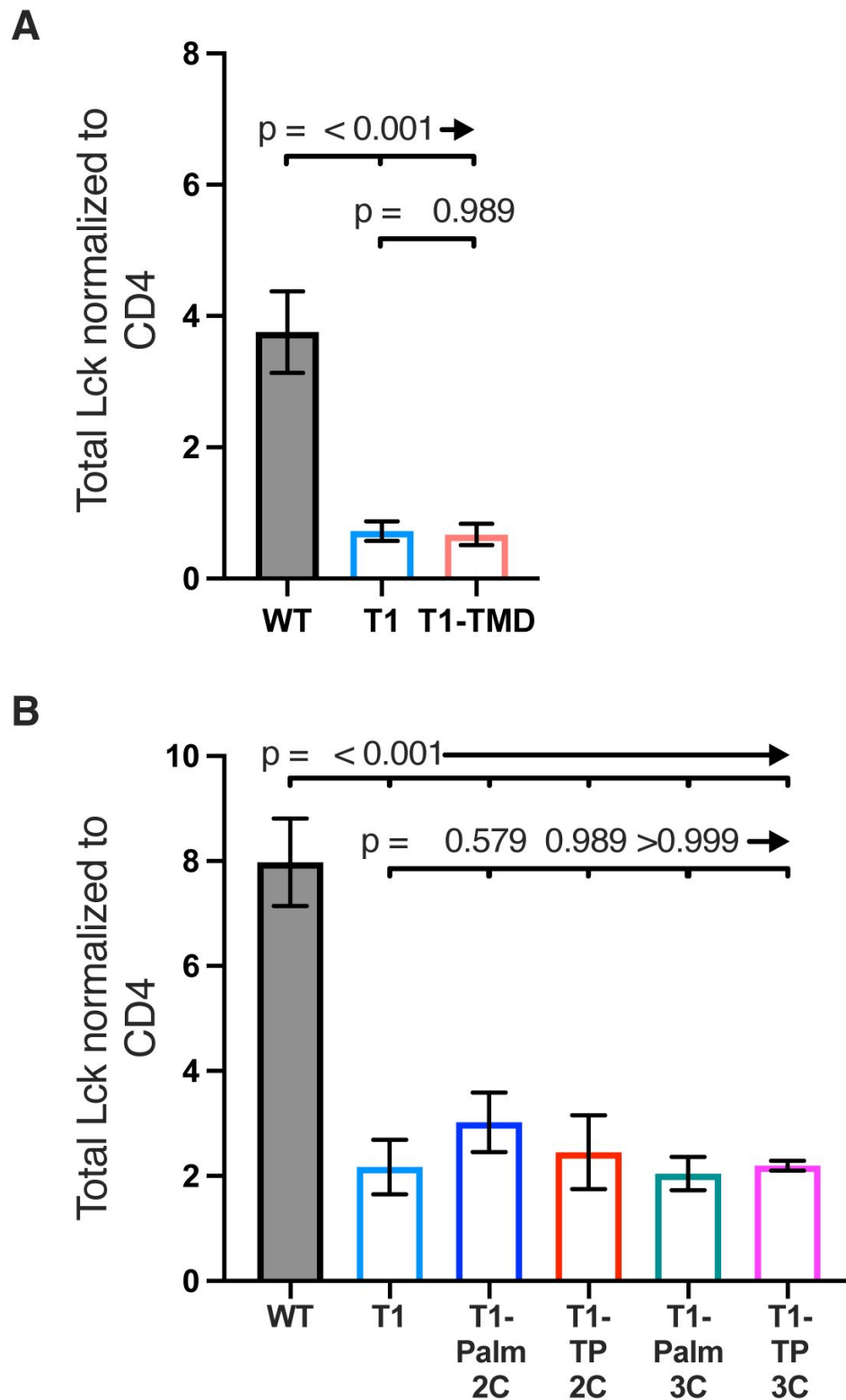


Figure 3- figure supplement 3.

(A, B) Total Lck signal (total AUC for sucrose gradient) normalized to CD4 signal is shown for the indicated cell lines. Each data point represents the mean $\pm$ SEM for three independent experiments with the same cell line (experimental replicates). Data are representative of experiments performed with three independently generated sets of lines for TMD, Palm(3C), and TP(3C) mutants (biological replicates). Analysis was performed with two set of lines for the Palm(2C) and TP(2C) mutants. One-way ANOVA was performed with a Dunnett's posttest for comparisons with WT and T1 samples, and a Sidak's posttest for comparisons between selected samples.

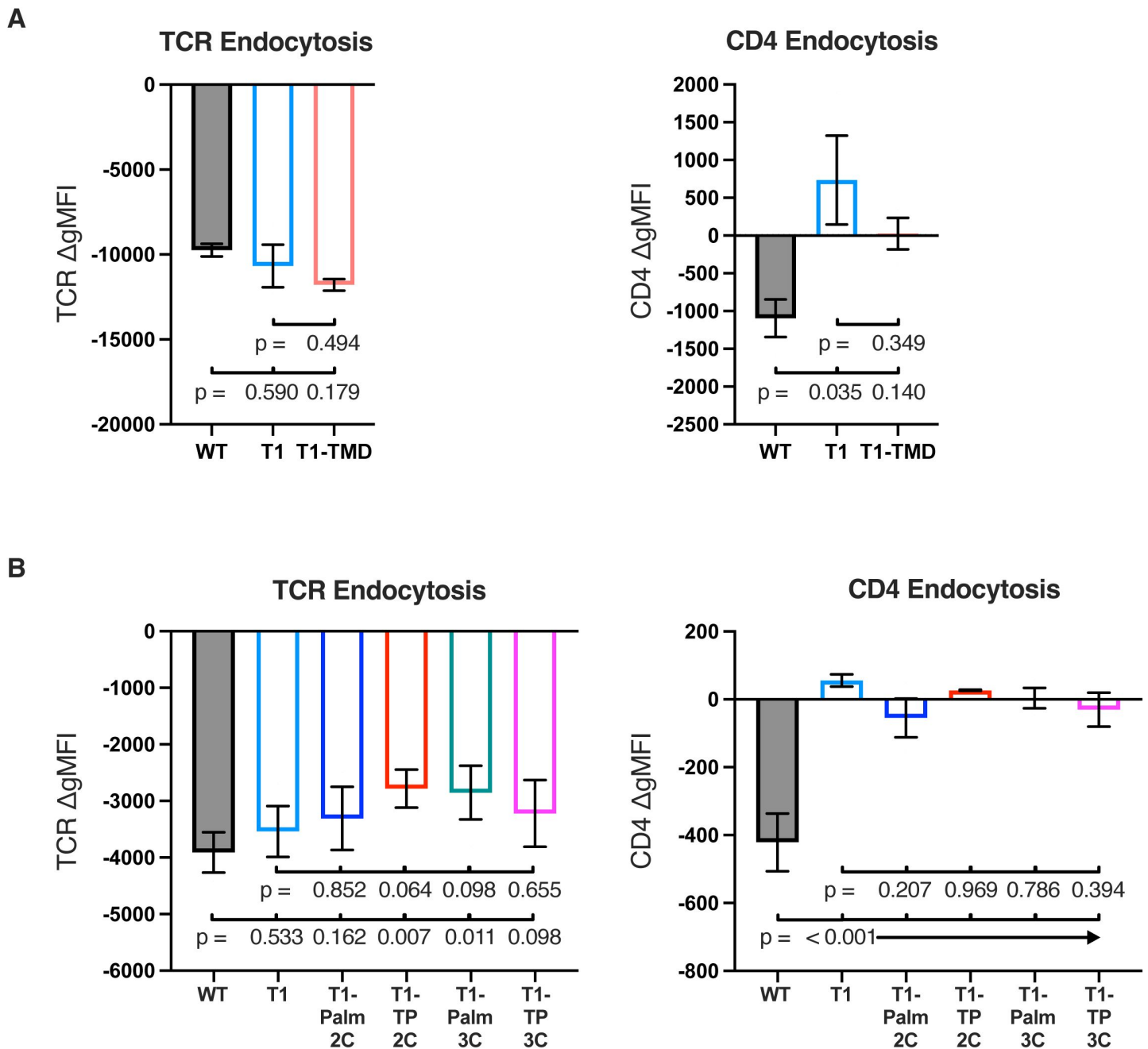


Figure 3- figure supplement 4.

(A, B) TCR (left) and CD4 (right) endocytosis after pMHCII engagement is shown for the indicated cell lines after 16 hours coculture with APCs in the presence of 10 μ M MCC peptide. The change in TCR and CD4 gMFI, as measured by flow cytometry, is shown for each cell line relative to an equivalent sample cultured with APCs in the absence of MCC peptide. Each data point represents the mean $\pm$ SEM for three independent experiments (experimental replicates). Data are representative of those acquired with at least two independently generated sets of cell lines (biological replicates). Endocytosis measurements were performed in triplicate (technical replicates) for each experiment. One-way ANOVA was performed with a Dunnett's posttest for comparisons with WT and T1 samples.

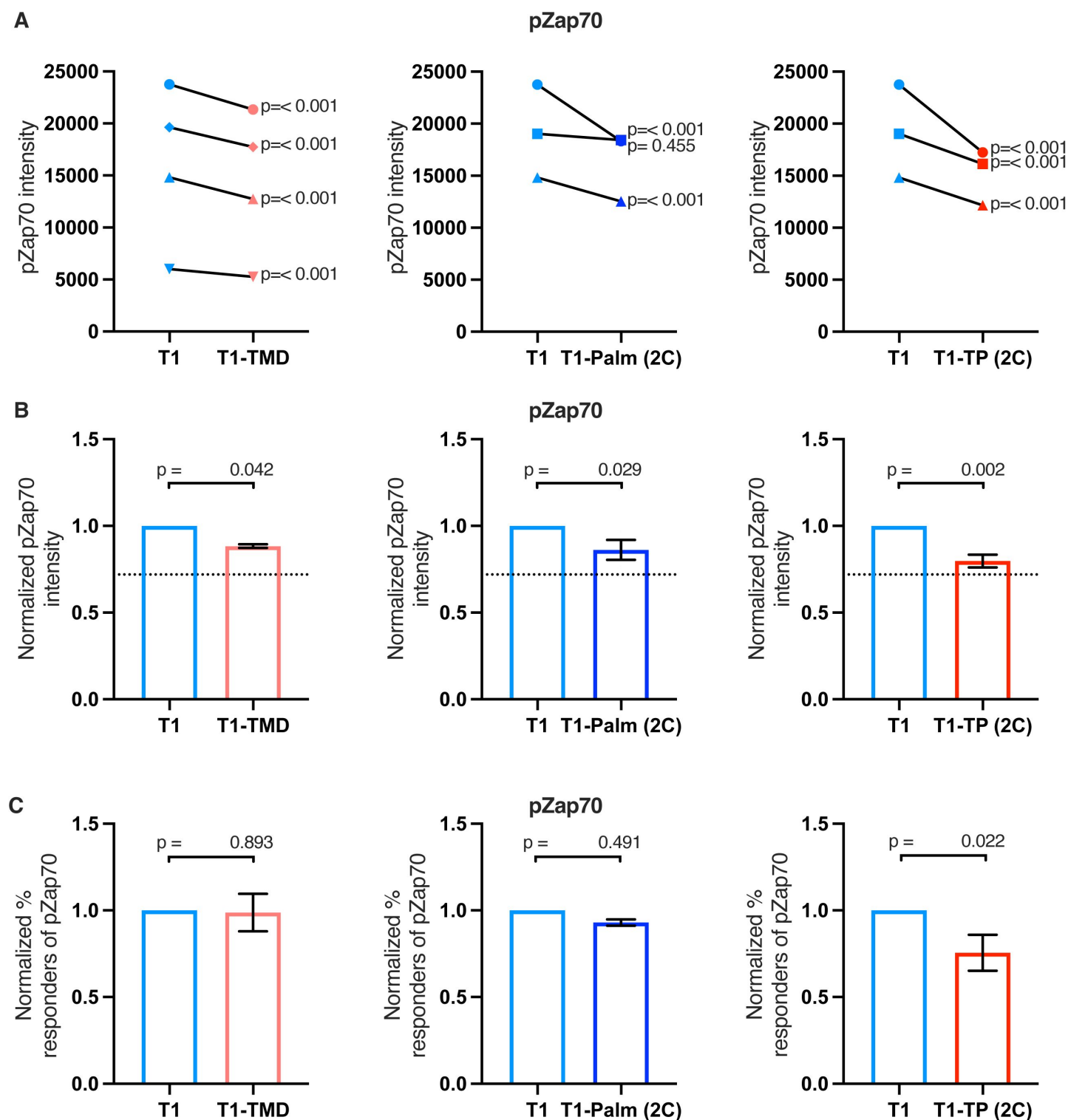


Figure 4- figure supplement 1.

(A) Phosphorylation intensity of Zap70 for T1 and T1-TMD (left), T1 and T1-Palm (2C) (center), and T1 and T1-TP (2C) (right) are shown for paired (connecting line) cell lines. Analysis was performed as in [Figure 4A](#).

(B) Normalized phosphorylation intensity of Zap70 for T1-TMD (left), T1-Palm (2C) (center), and T1-TP (2C) (right) are shown as bars. Analysis was performed as in [Figure 4B](#).

(C) Normalized % responders of the phosphorylation Zap70 for T1-TMD (left), T1-Palm (2C) (center), and T1-TP (2C) (right) are shown as bars for T1 and T1^{Abind} cell lines. Analysis was performed as in [Figure 4C](#).

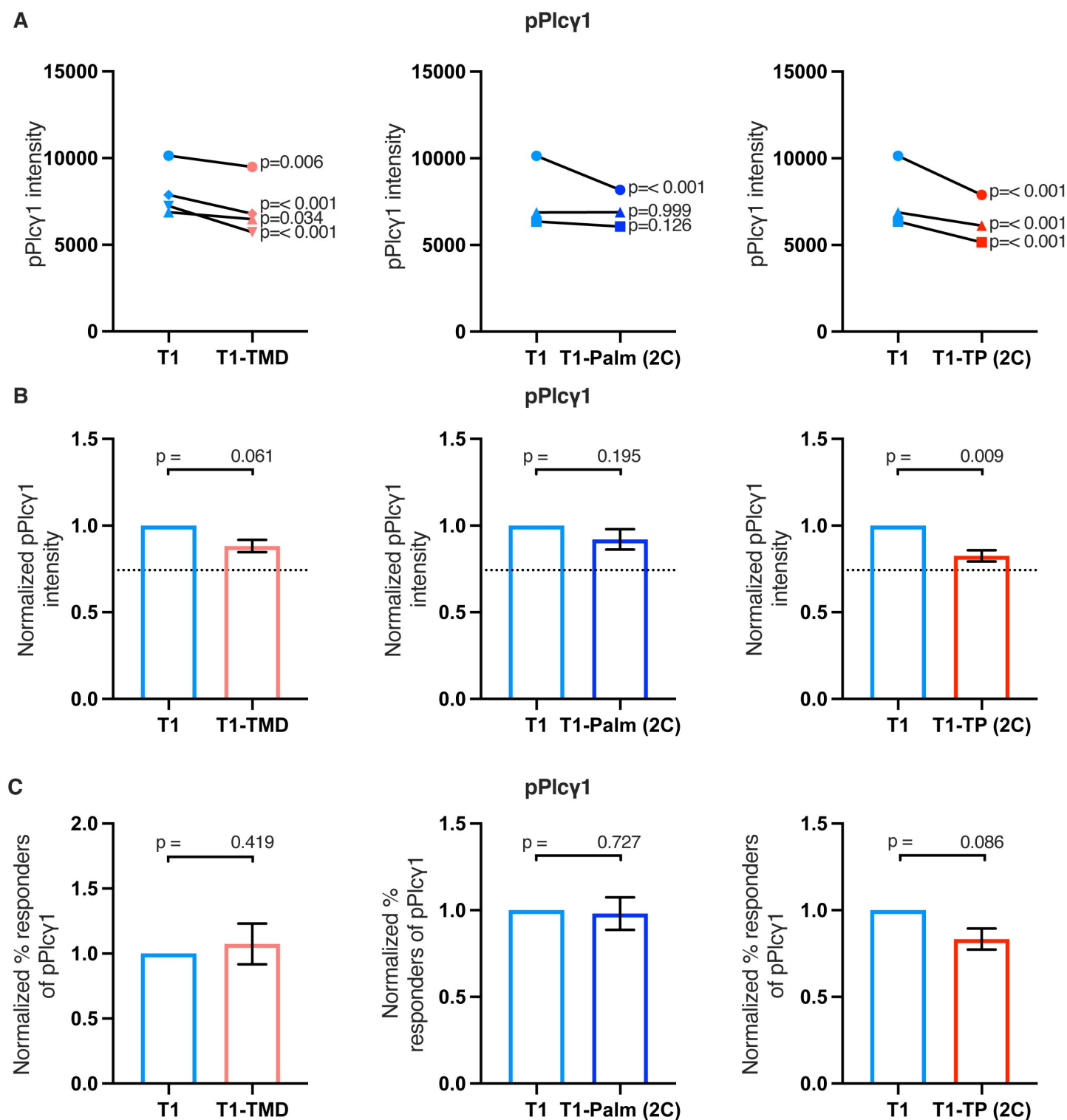


Figure 4- figure supplement 2.

(A) Phosphorylation intensity of Plcy1 for T1 and T1-TMD (left), T1 and T1-Palm (2C) (center), and T1 and T1-TP (2C) (right) are shown for paired (connecting line) cell lines. Analysis was performed as in [Figure 4A](#).

(B) Normalized phosphorylation intensity of Plcy1 for T1-TMD (left), T1-Palm (2C) (center), and T1-TP (2C) (right) are shown as bars. Analysis was performed as in [Figure 4B](#).

(C) Normalized % responders of phosphorylated pPlcy1 for T1-TMD (left), T1-Palm (2C) (center), and T1-TP (2C) (right) are shown as bars for T1 and T1^{Abind} cell lines. Analysis was performed as in [Figure 4C](#).

MCC:I-E^k co-culture

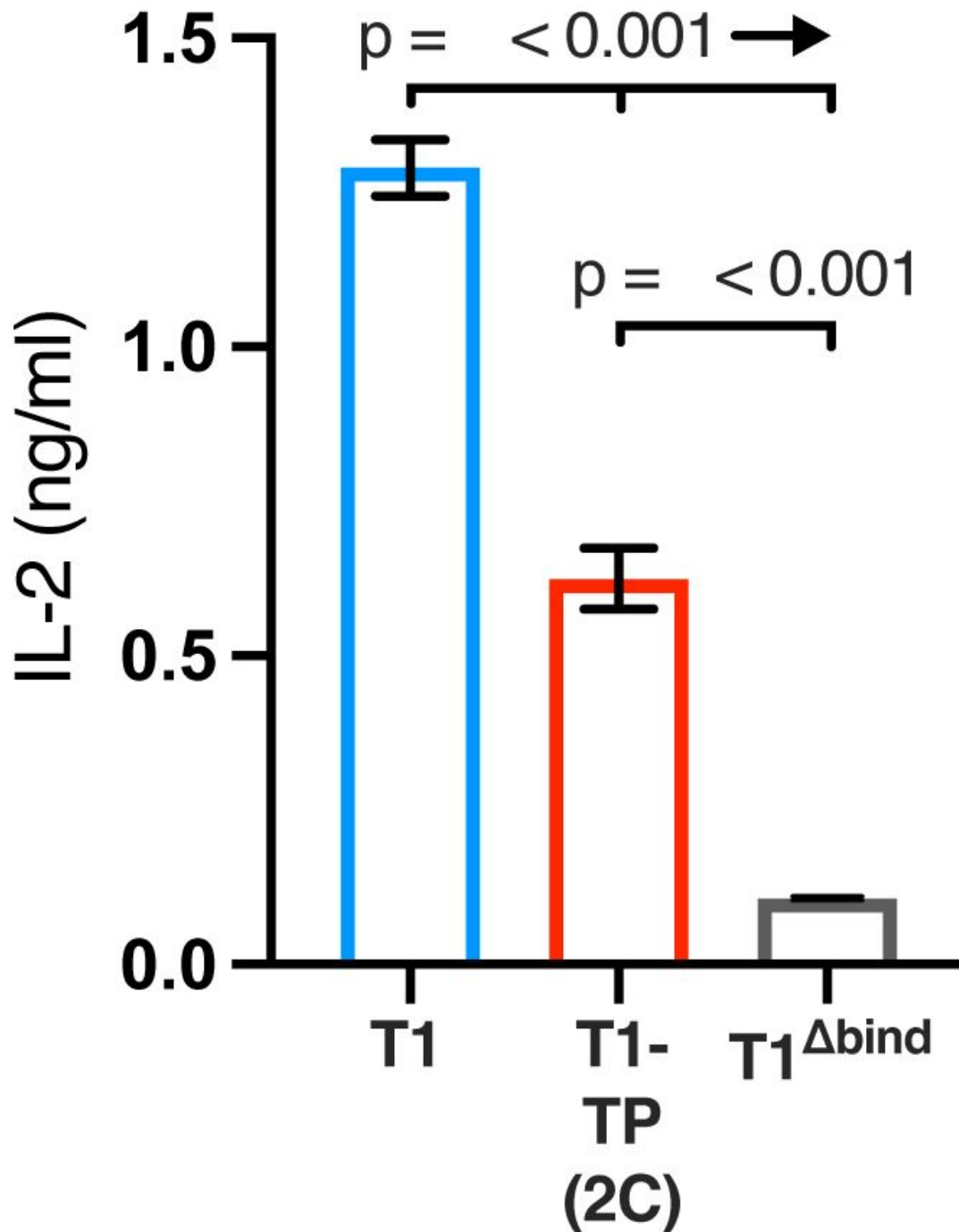


Figure 4- figure supplement 3.

Representative IL-2 production is shown in response to M12 cells transduced to express tethered MCC:I-E^k constructs as used in phosphorylation analysis. Experiments were performed in triplicate and each bar equals the mean $\pm$ SEM at that peptide concentration. Results are representative of those obtained with two independently generated matched sets of cell lines. One-way ANOVA was performed with a Dunnett's posttest for comparisons with WT and T1 samples, and a Sidak's posttest for comparisons between selected samples.

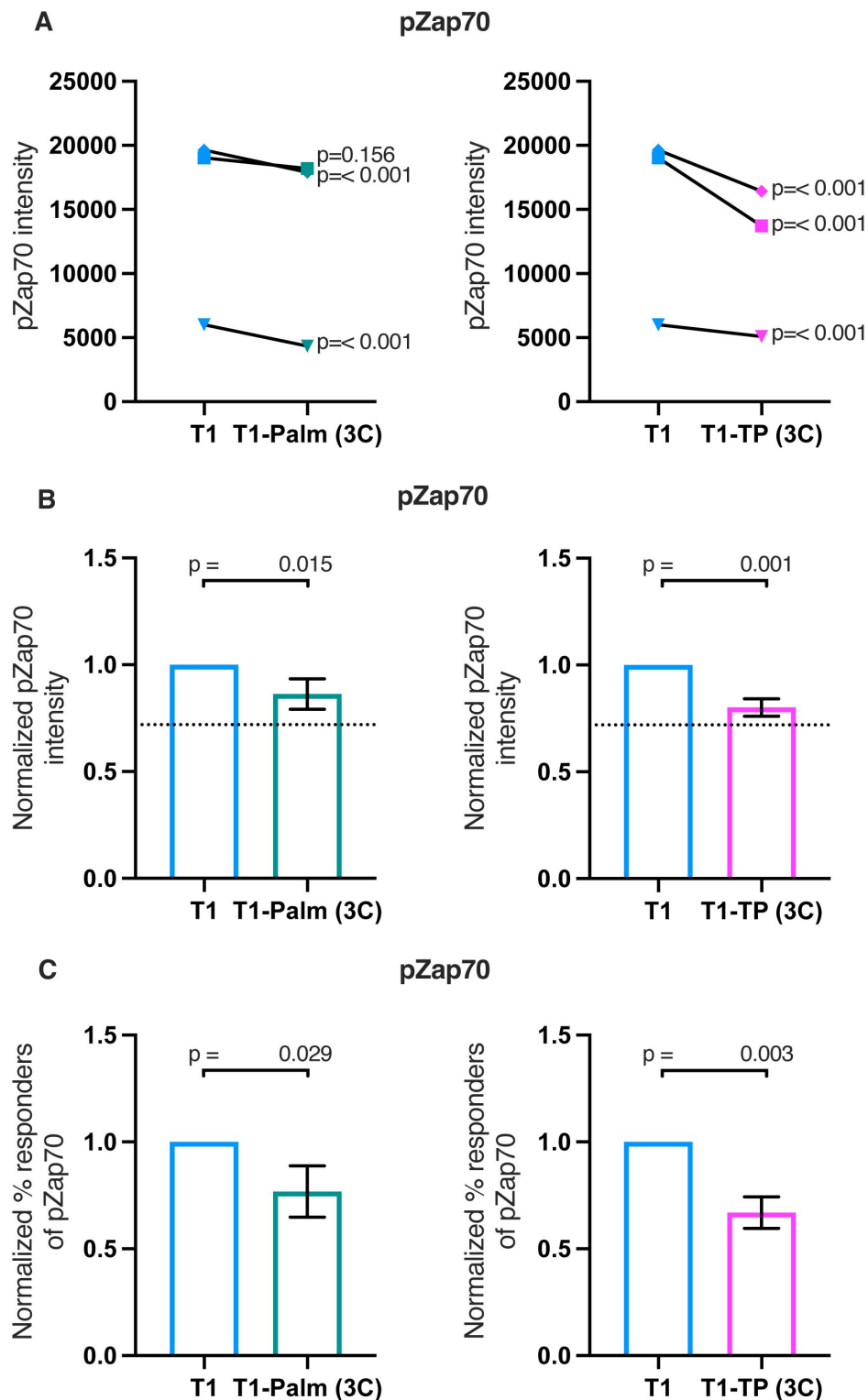


Figure 5- figure supplement 1.

(A) Phosphorylation intensity of Zap70 for T1 and T1-Palm (3C) (left) and T1 and T1-TP (3C) (right) are shown for paired (connecting line) cell lines. Analysis was performed as in [Figure 5A](#).

(B) Normalized phosphorylation intensity of Zap70 for T1-Palm (3C) (left) and T1-TP (3C) (right) are shown as bars. Analysis was performed as in [Figure 5B](#).

(C) Normalized % responders of phosphorylated Zap70 for T1-Palm (3C) (left) and T1-TP (3C) (right) are shown as bars. Analysis was performed as in [Figure 5C](#).

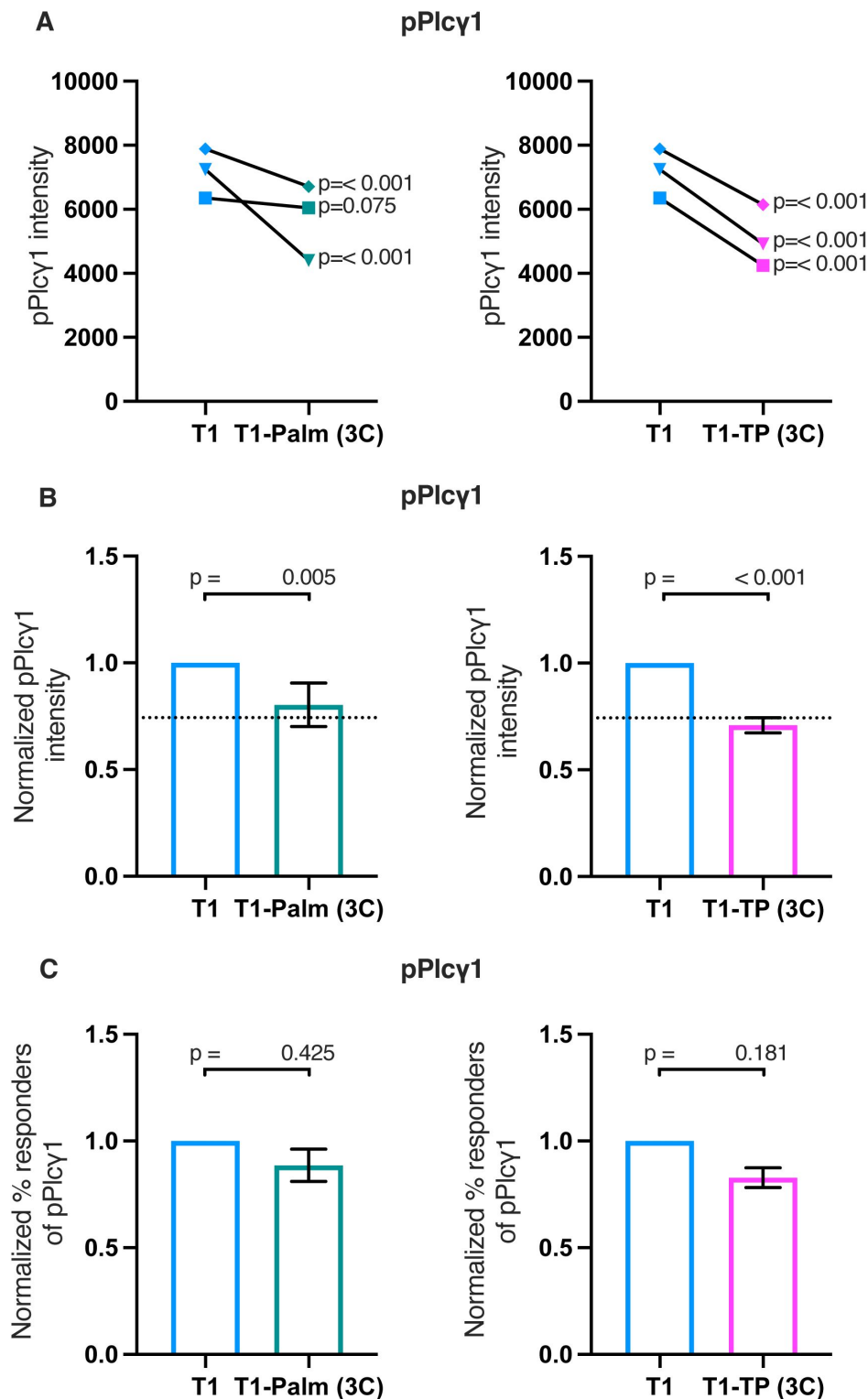


Figure 5- figure supplement 2.

(A) Phosphorylation intensity of Plcy1 for T1 and T1-Palm (3C) (left) and T1 and T1-TP (3C) (right) are shown for paired (connecting line) cell lines. Analysis was performed as in [Figure 5A](#).

(B) Normalized phosphorylation intensity of Plcy1 for T1-Palm (3C) (left) and T1-TP (3C) (right) are shown as bars. Analysis was performed as in [Figure 5B](#).

(C) Normalized % responders of phosphorylated Plcy1 for T1-Palm (3C) (left) and T1-TP (3C) (right) are shown as bars. Analysis was performed as in [Figure 5C](#).

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Reviewer #1 (Public Review):

Summary:

This study by Lee et al. is a direct follow-up on their previous study that described an evolutionary conservancy among placental mammals of two motifs (a transmembrane motif and a juxtamembrane palmitoylation site) in CD4, an antigen co-receptor, and showed their relevance for T-cell antigen signaling. In this study, they describe the contribution of these two motifs to the CD4-mediated antigen signaling in the absence of CD4-LCK binding. Their approach was the comparison of antigen-induced proximal TCR signaling and distal IL-2 production in 58/- T-cell hybridoma expressing exogenous truncated version of CD4 (without

the interaction with LCK), called T1 with T1 version with the mutations in either or both of the conserved motifs. They show that the T1 CD4 can support signaling to the extent similar to WT CD4, but the mutation of the conserved motifs substantially reduced the signaling. The authors conclude that the role of these motifs is independent of the LCK-binding.

Strengths:

The authors convincingly show that T1 CD4, lacking the interaction with LCK supports the TCR signaling and also that the two studied motifs have a significant contribution to it.

Weaknesses:

The study has several weaknesses.

1. The whole study is based on a single experimental system, genetically modified 58/- hybridoma. It is unclear at this moment, how the molecular motifs studied here contribute to the signaling in a real T cell. The evolutionary conservancy suggests that these motifs are important for T cell biology. However, the LCK-binding motif is conserved as well (perhaps even more) and it plays a very minor role in their model. Without verifying their results in primary cells, the quantitative, but even qualitative, importance of these motifs for T-cell signaling and biology is unclear. Although the authors discuss this issue in the Discussion, it should be noted in all important parts of the manuscript, where conclusions are made (abstract, end of introduction, perhaps also in the title) that the results are coming from the hybridoma cells.
2. Many of the experiments lack the negative control. I believe that two types of negative controls should be included in all experiments. First, hybridoma cells without CD4 (or with CD4 mutant unable to bind MHCII). Second, no peptide control, i.e., activation of the hybridoma cells with the APC not loaded with the cognate peptide. These controls are required to distinguish the basal levels of phosphorylation and CD4-independent antigen-induced phosphorylation to quantify, what is the contribution of the particular motifs to the CD4-mediated support. Although these controls are included in some of the experiments, they are missing in other ones. The binding mutant appears in some FC results as a horizontal bar (without any error bar/variability), showing that CD4 does not give a huge advantage in these readouts. Why don't the authors show no peptide controls here as well? Why the primary FC data (histograms) are not shown? Why neither of these two controls is shown for the % of responders plots? Although the IL-2 production is a very robust and convincing readout, the phosphoflow is much less sensitive. It seems that the signaling is elevated only marginally. Without the mentioned controls and showing the raw data, the precise interpretation is not possible.
3. The processing of the data is not clear. Some of the figures seem to be overprocessed. For instance, I am not sure what "Normalized % responders of pCD3zeta" means (e.g., Fig. 1C and elsewhere)? Why do not the authors show the actual % of pCD3zeta+ cells including the gating strategy? Why do the authors subtract the two histograms in Fig. 2- Fig.S3? It is very unusual.
4. The manuscript lacks Materials and Methods. It only refers to the previous paper, which is very unusual. Although most of the methods are the same, they still should be mentioned here. Moreover, some of the mutants presented here were not generated in the previous study, as far as I understand. Perhaps the authors plan to include Materials and Methods during the revision...
5. Membrane rafts are a very controversial topic. I recommend the authors stick to the more consensual term "detergent resistant microdomains" in all cases/occurrences.
6. Last, but not least, the mechanistic explanation (beyond the independence of LCK binding) of the role of these motifs is very unclear at the moment.

Reviewer #2 (Public Review):

Summary:

The paper by Kuhn and colleagues follows upon a 2022 paper in which they identified residues in CD4 constrained by evolutionary purifying selection in placental mammals and then performed functional analyses of these conserved sequences. They showed that sequences distinct from the CXC "clamp" involved in recruitment of Lck have critical roles in TCR signaling, and these include a glycine-rich motif in the transmembrane (TM) domain and the cys-containing juxtamembrane (JM) motif that undergoes palmitoylation, both of which promote TCR signaling, and a cytoplasmic domain helical motif, also involved in Lck binding, that constrains signaling. Mutations in the transmembrane and juxtamembrane sequences led to reduced proximal signaling and IL-2 production in a hybridoma's response to antigen presentation, despite retention of abundant CD4 association with Lck in the detergent-soluble membrane fraction, presumably mislocalized outside of lipid rafts and distal to the TCR. A major conclusion of that study was that CD4 sequences required for Lck association, including the CXC "clamp" motif, are not as consequential for CD4 co-receptor function in TCR signaling as the conserved TM and JM motifs. However, the experiments did not determine whether the functions of the TM and JM motifs are dependent on the Lck-binding properties of CD4 - the mutations in those motifs could result in free Lck redistributing to associate with CD4 in signaling-incompetent membrane domains or could function independently of CD4-Lck association. The current study addresses this specific question.

Using the same model system as in the earlier paper (the entire methods section is a citation to the earlier paper), the authors show that truncation of the Lck-binding intracellular domain resulted in a moderate reduction in IL-2 response, as previously shown, but there was no apparent effect on proximal phosphorylation events (CD3z, Lck, ZAP70, PLCg1). They then evaluated a series of TM and JM motif mutations in the context of the truncated Lck-nonbinding molecule, and showed that these had substantially impaired co-receptor function in the IL-2 assay and reduced proximal signaling. The proximal signaling could be observed at high ligand density even with a MHC non-binding mutation in CD4, although there was still impaired IL-2 production. This result additionally illustrates that phosphorylation of the proximal signaling molecules is not sufficient to activate IL-2 expression in the context of antigen presentation.

Strengths:

The strength of the paper is the further clear demonstration that the classical model of CD4 co-receptor function (MHCII-binding CD4 bringing Lck to the TCR complex, for phosphorylation of the CD3 chain ITAMs and of the ZAP70 kinase) is not sufficient to explain TCR activation. The data, combined with the earlier paper, further implicate the gly-rich TM sequence and the palmitoylation targets in the JM region as having critical roles in productive co-receptor-dependent TCR activation.

Weaknesses:

The major weakness of the paper is the lack of mechanistic insight into how the TM and JM motifs function. The new results are largely incremental in light of the earlier paper from this group as well as other literature, cited by the authors, that implicates "free" Lck, not associated with co-receptors, as having the major role in TCR activation. It is clear that the two motifs are important for CD4 function at low pMHCII ligand density. The proposal that they modulate interactions of TCR complex with cholesterol or other membrane lipids is an interesting one, and it would be worth further exploring by employing approaches that alter membrane lipid composition. The JM sequence presumably dictates localization within the membrane, by way of palmitoylation, which may be critical to regulate avidity of the TCR:CD4 complex for pMHCII or TCR complex allosteric effects that influence the activation threshold. Experiments that explore the basis of the mutant phenotype could substantially enhance the impact of this study.

Author Response

We thank the Editors and the Reviewers for the time spent on our manuscript entitled “The CD4 transmembrane GGXXG and juxtamembrane (C/F)CV+C motifs mediate pMHCII-specific signaling independently of CD4-Lck interactions”. We appreciate the helpful feedback and the opportunity to participate in eLife’s new model for publishing.

We are writing to provide the following provisional author responses for posting with the first version of the reviewed preprint:

1. To address comments about the limited scope of this study and referencing of the Methods section to our prior study, we would like to note that we submitted the current study via the Research Advance mechanism. Our goal was to build upon the conclusions of our 2022 eLife publication (PMID: 35861317) and address an unresolved question from that study (as nicely summarized by Reviewer #2). In the current manuscript we present data from reductionist experiments that were designed specifically for this purpose and, as noted by the reviewers, we provide answers to the question being asked. We think that the Research Advance mechanism is an ideal opportunity to make these results available to the field given the stated purpose of such articles (for reference: “A Research Advance might use a new technique or a different experimental design to generate results that build upon the conclusions of the original research by, for example, providing new mechanistic insights or extend the pathway under investigation...”).

- a. The Methods were not duplicated in this manuscript because we referenced our prior study as per instructions for the Research Advance mechanism.

1. The constituent residues of the motifs analyzed in this and our prior study were determined to be functionally significant *in vivo* through the computational reconstruction of CD4’s evolutionary history, which provided us with data from ~435 million years of natural experiments with CD4 in numerous jawed vertebrate species. We agree that having conditional knock-in mice of these CD4 mutants, and those characterized in our last study, would be useful for determining how these mutations impact T cell development, activation, differentiation, and effector function. Given the costs involved with making genetically engineered mouse model systems, the computational and experimental data we have generated in the current and prior study will help us prioritize next steps to dig deeper into the details of why the residues we are studying are under purifying selection (fail to propagate to progeny if mutated, meaning terminal). In short, only now, with the data in hand, can we prioritize mouse studies. We think it is important for the advancement of the field that we make these results available in a timely manner rather than waiting to report them together with the results of mouse models once generated and analyzed.

2. The reductionist experimental data presented here provide us with mechanistic insights into why the residues we are studying are functionally important. We therefore think it is of value to note that 58a-b- T cell hybridomas were used in seminal work that established a link between CD4Lck association, via motifs in the CD4 intracellular domain, and signaling output as measured by IL-2 production (Glaichenhaus, et al., 1991). Importantly, the impact of disrupting CD4-Lck interactions on proximal signaling were not interrogated until the work we describe here and in our preceding study, wherein we establish that CD4-Lck association does not regulate proximal signaling in 58a-b- T cell hybridomas. Given that this experimental system was used to help establish the dominant paradigm (i.e. the widely held view that CD4 recruits Lck to TCR-CD3 to initiate pMHCII-specific signaling), we think it is a

legitimate system to directly test this model and further test core questions of CD4 function by employing more modern experimental techniques.