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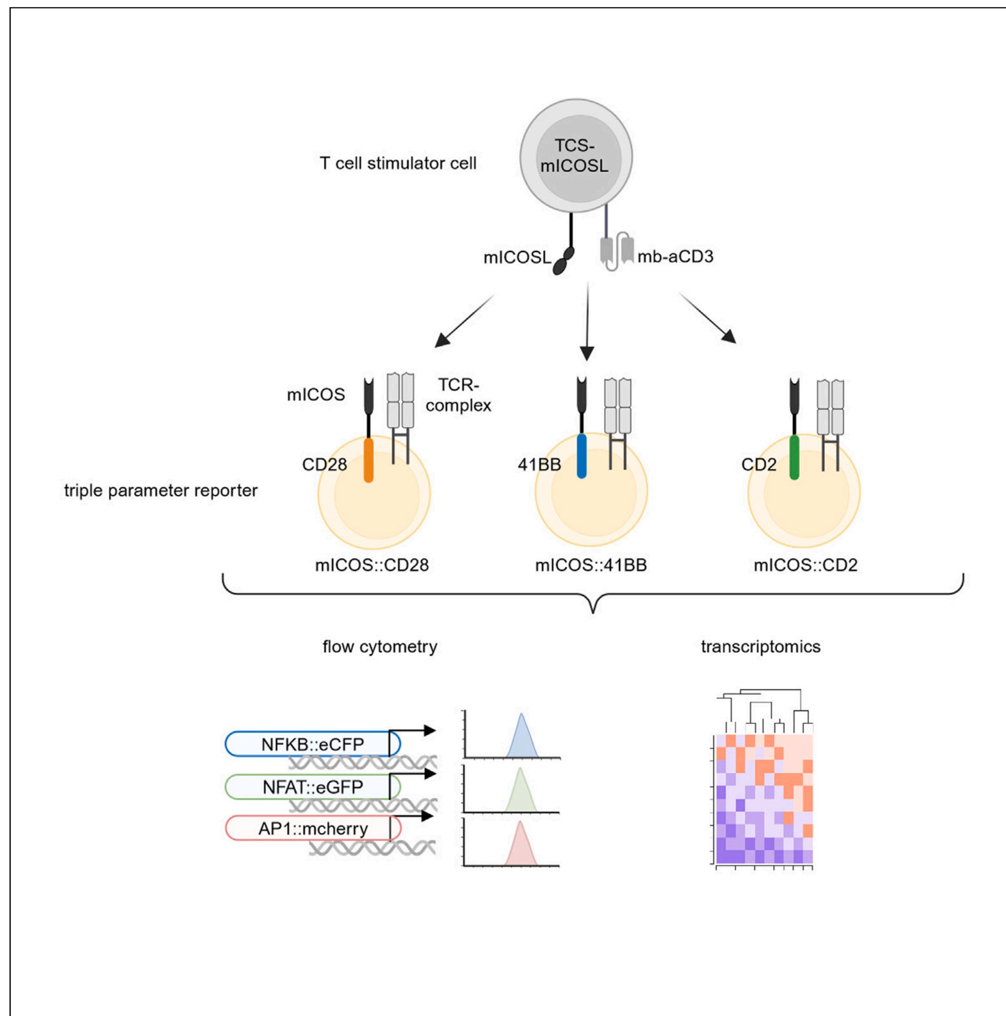

Annika De Sousa Linhares, Sumana Sharma, Peter Steinberger, Judith Leitner

peter.steinberger@meduniwien.ac.at (P.S.)
judith.a.leitner@meduniwien.ac.at (J.L.)

Highlights

Jurkat reporter system to study transcriptional regulation by CD28, 4-1BB, and CD2

CD28 signaling activated NF-κB, NFAT, and AP-1

Strong activation of NFAT by CD2, whereas 4-1BB promoted activation of NF-κB

CD2 and 4-1BB signals induced distinct transcriptomic changes

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Transcriptional reprogramming via signaling domains of CD2, CD28, and 4-1BB

Annika De Sousa Linhares,[1,3] Sumana Sharma,[2] Peter Steinberger,[1,*] and Judith Leitner[1,4,*]

SUMMARY

Costimulatory signals provided to T cells during antigen encounter have a decisive role in the outcome of immune responses. Here, we used chimeric receptors harboring the extracellular domain of mouse inducible T cell costimulator (mICOS) to study transcriptional activation mediated by cytoplasmic sequences of the major T cell costimulatory receptors CD28, 4-1BB, and CD2. The chimeric receptors were introduced in a T cell reporter platform that allows to simultaneously evaluate nuclear factor κB (NF-κB), NFAT, and AP-1 activation. Engagement of the chimeric receptors induced distinct transcriptional profiles. CD28 signaling activated all three transcription factors, whereas 4-1BB strongly promoted NF-κB and AP-1 but downregulated NFAT activity. CD2 signals resulted in the strongest upregulation of NFAT. Transcriptome analysis revealed pronounced and distinct gene expression signatures upon CD2 and 4-1BB signaling. Using the intracellular sequence of CD28, we exemplify that distinct signaling motifs endow chimeric receptors with different costimulatory capacities.

INTRODUCTION

T cell-mediated recognition of antigens critically depends on the presence of additional signals, to initiate productive immune responses. This important fail-safe prevents activation of aberrant T cells that recognize self-antigens in absence of inflammatory stimuli. Such second signals are mainly provided by costimulatory receptors interacting with their cognate ligands, which are upregulated on activated professional antigen-presenting cells (APCs). The engagement of CD28 by its ligands, CD80 and CD86, is generally regarded to constitute the primary T cell costimulatory pathway. CD28 signals promote a plethora of events including cytoskeletal remodeling, production of cytokines and anti-apoptotic molecules, epigenetic modification, differentiation, and proliferation.[1,2] T cell receptor (TCR)/CD3 and CD28 signals are integrated to mediate nuclear translocation of transcription factors like nuclear factor κB (NF-κB), NFAT, and AP-1, which play major roles in the transcriptional re-programming of T cells upon activation.

Many additional receptors promote T cell responses upon TCR/CD3 engagement/activation.[3,4] Such alternative costimulatory receptors may substitute for CD28, but, since they belong to different families, their intracellular signals can differ on qualitative and quantitative levels.

One important costimulatory receptor is 4-1BB (CD137), a member of the tumor-necrosis factor superfamily (TNFR-SF), which is induced on activated T cells and mediates TRAF-dependent signaling processes, resulting in the activation of NF-κB as well as the Jun N-terminal kinase (JNK) and p38.[5–7] Several other members of the TNFR-SF, including OX40 and CD27, also mediate costimulation of T cells and are implicated in engaging similar downstream signaling pathways. Costimulatory receptors of the TNFR-SF are important targets in immunotherapy of cancer.[8] 4-1BB and OX40 are upregulated in activated human T cells and were suggested to have a major role in promoting antigen-experienced T cells. In contrast, CD27, like CD28, is constitutively expressed on naive T cells and downregulated on human CD8^{+} T cells upon repeated exposure to antigen. Recent work in murine models has shown that engagement of CD27 by its natural ligand CD70, or by agonistic antibodies, can potently promote cytotoxic effector CD8^{+} T cell responses.[9,10] Another important costimulatory receptor on human T cells is CD2, a member of the immunoglobulin (Ig) superfamily. Although this receptor is often categorized as an adhesion molecule, it harbors binding sites for several signaling molecules in its long cytoplasmic tail and thus acts as a genuine costimulatory receptor in T cells. In contrast to CD28, 4-1BB, OX40, and CD27, which are not uniformly expressed in T cells, a significant subset of CD2-negative human T cells has not been described. We have previously compared the capacity of costimulatory ligands, to enhance proliferation of human T cells. Our data indicate that CD2 might be the most potent among the alternative costimulatory receptors, and we have furthermore demonstrated that the CD2/CD58 axis constitutes the primary costimulatory pathway for CD8^{+} T cells that lack the CD28 receptor.[11,12] Moreover, CD2 signals counteract the development of an exhaustion signature in human CD8^{+} T cells during persistent stimulation, further highlighting the relevance of CD2 in providing accessory signals to T cells.[13]

[1]Division of Immune Receptors and T Cell Activation, Center for Pathophysiology, Infectiology and Immunology, Institute of Immunology, Medical University of Vienna, Vienna, Austria
[2]MRC Translational Immune Discovery Unit John Radcliffe Hospital, University of Oxford, Oxford OX3 9DS, UK
[3]Loop lab Bio GmbH, Vienna, Austria
[4]Lead contact
*Correspondence: peter.steinberger@meduniwien.ac.at (P.S.), judith.a.leitner@meduniwien.ac.at (J.L.)
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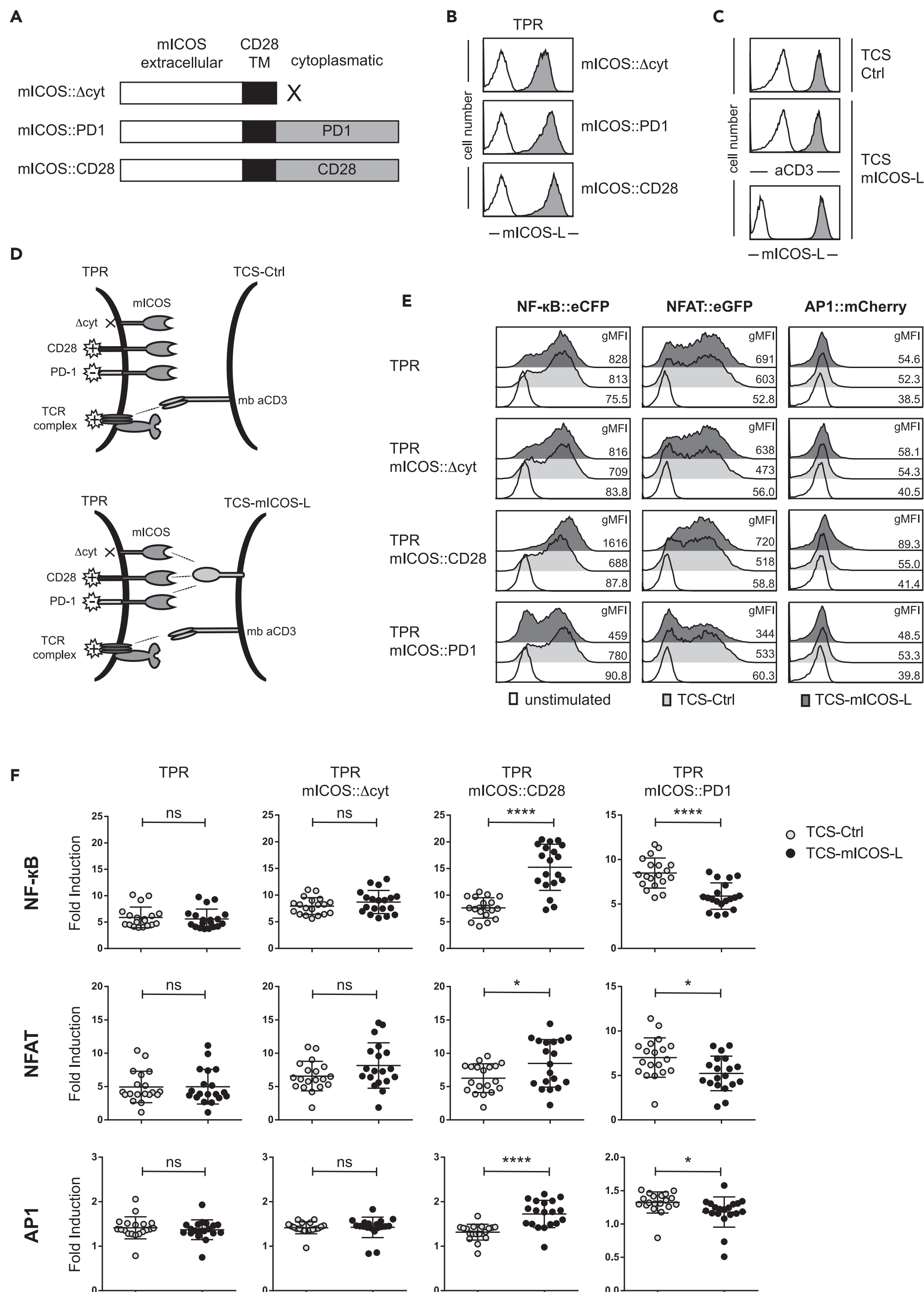

Figure 1. Chimeric receptors with costimulatory or coinhibitory signaling domains

(A) Schematic of mICOS chimeric constructs.

(B) Flow cytometric analysis of mICOS chimeric construct expression in triple parameter reporter (TPR) cells (gray histograms); open histograms represent antibody binding to control cells.

(C) Flow cytometric analysis of T cell stimulator cells (TCS), gray histograms: binding of indicated antibodies; open histograms: binding of antibodies to control cells.

(D) Schematic of mICOS chimeric construct expressing TPR, stimulated with TCS control (TCS-Ctrl), or mICOS-L expressing TCS, (mb aCD3, membrane-bound anti-CD3).

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Figure 1. *Continued*

(E) TPR expressing mICOS::Δcyt, mICOS::CD28, and mICOS::PD-1 and non-expressing control TPR were kept unstimulated or stimulated with TCS expressing the membrane-bound aCD3 single-chain fragment (TCS-ctrl), or TCS expressing mICOS-L (TCS-mICOSL). Expression of reporter genes (eGFP, eCFP, and mCherry) was measured via flow cytometry. Histograms of one representative experiment are shown.

(F) Indicated TPRs were stimulated with control TCS or TCS expressing mICOS-L. Normalized data (gMFI of TPR stimulated with the indicated TCS/gMFI of unstimulated TRP) of 19 independently performed experiments with standard deviation are shown, and each dot represents the mean of one experiment performed in triplicates. For statistics, unpaired two-tailed t test was performed (****$p \leq 0.0001$; *$p \leq 0.05$; ns $p > 0.05$).

Costimulatory pathways are major therapeutic targets, and, especially in this context, it is important to identify unique and common downstream signaling pathways induced upon engagement of distinct receptors. Such studies are impaired by the redundancy of costimulatory receptors. In addition, the spatial and temporal regulation and varying expression levels of these molecules as well as different affinities for their ligands impede the comparison of costimulatory processes induced by different receptors. Thus, it might be informative to assess the capability of cytoplasmic domains and motifs of these receptors, to induce costimulatory signaling processes detached from their extracellular moiety. The function of chimeric antigen receptors (CARs) is potently augmented by intracellular motifs derived from costimulatory receptors, demonstrating that costimulatory motifs can function independently of their natural context. Cytoplasmic sequences derived from different costimulatory receptors are used alone or in combination in CAR constructs and can strengthen the capability of adoptively transferred CAR T cells to eradicate tumor cells. Additionally, they can mediate aberrant activation of engineered T cells and potentially unfavorable effects in treated patients.[14–17] Consequently, there are intense efforts to develop optimal cytoplasmic sequences for CAR constructs. For these attempts it is of great interest to compare the potential of cytoplasmic sequences of costimulatory receptors to engage the intracellular signaling machinery of T cells.

In this study, we have explored the potential of a chimeric receptor platform to assess costimulatory signaling. Chimeric mouse ICOS (mICOS) molecules equipped with cytoplasmic tails of the major T cell costimulatory receptors CD28, 4-1BB, and CD2 as well as CD27 were generated and introduced into fluorescence-based transcriptional human T cell reporters, which allow to concomitantly read out the activity of three transcription factors that play major roles in T cell activation processes, NF-κB, NFAT, and AP-1. The chimeric receptors were triggered by engineered stimulator cells co-expressing an anti-CD3 antibody fragment and mouse ICOS ligand (mICOS-L). We have investigated the transcriptomic changes mediated by engagement of chimeric receptors representing CD2, CD28, and 4-1BB. In addition, we have used this approach to assess the contribution of different signaling motifs, contained in the CD28 molecule, to the activity of these transcription factors. Finally, we confirmed some key findings using an alternative chimeric receptor format and demonstrate that our approach can guide the generation of superior signaling modules to enhance responses in engineered T cells.

RESULTS

Evaluation of chimeric receptors harboring costimulatory or coinhibitory signaling domains

For proof-of-principle experiments, chimeric constructs, encoding the cytoplasmic domains of CD28 or PD-1 fused to the CD28 transmembrane domain and the extracellular domain of mICOS, were generated. For control purposes, we also generated a construct expressing the extracellular domain of mICOS without cytoplasmic sequences (Figure 1A). These constructs were introduced into our previously described triple parameter reporter (TPR) cells, which are based on the human Jurkat T cell line (Figure 1B). In these cells the activity of the transcription factors NF-κB, NFAT, and AP-1 induces the expression of the fluorescent proteins eCFP, eGFP, and mCherry, respectively, which can be conveniently measured by flow cytometry.[18] We used T cell stimulator cells (TCS), a previously described cell line, to stimulate the Jurkat reporter lines. TCS are cells engineered to carry a membrane-bound CD3 antibody fragment and can be used to stimulate human T cells and T cell lines via TCR/CD3 complex engagement.[11] For this study, we used TCS-Ctrl, which induced only "signal 1" and TCS-mICOS-L, which by co-engaging TCR/CD3 complex and the chimeric receptors induced "signal 1 and 2." Both TCS expressed similar levels of membrane-bound CD3 antibody fragment, and mICOSL was strongly expressed on TCS-mICOS-L (Figure 1C). TCS-Ctrl or TCS-mICOS-L was used to stimulate TPR expressing different mICOS chimeric constructs (Figure 1D). Stimulation with TCS-Ctrl induced strong upregulation of NF-κB and NFAT reporter gene expression, and a modest upregulation of AP-1 (Figure 1E). Importantly, compared to TCS-Ctrl, TCS-mICOS-L induced significantly higher reporter activity in TPR-mICOS::CD28, whereas in TPR-mICOS::PD-1 expression of all reporter genes was reduced (Figures 1E and 1F). By contrast, both types of TCS (TCS-Ctrl and TCS-mICOS-L) induced similar levels of activation in TPR expressing no mICOS molecules. Compared to TCS-Ctrl, TCS-mICOS-L induced slightly stronger activation of TPR expressing a mICOS chimera lacking a cytoplasmic tail (mICOS::Δcyt; Figures 1E and 1F). A stronger interaction between these cells, mediated by mICOS/mICOS-L binding, is a likely explanation for this effect, which was modest and did not reach statistical significance in our dataset. Taken together, these experiments indicate that our chimeric receptor platform is a well-controlled system to investigate and compare signaling, induced by intracellular sequences derived from different costimulatory receptors.

Intracellular sequences derived from major costimulatory receptors induce distinct transcriptional activation profiles

CD28, 4-1BB, and CD2 are major costimulatory receptors for human T cells. They belong to different superfamilies, and their expression on T cells is differentially regulated. Moreover, their cytoplasmic sequences considerably differ and carry distinct signaling motifs (Figure 2A). To compare the capability of these sequences to induce transcriptional activation, chimeric mICOS constructs, harboring intracellular sequences derived from these molecules, were generated and expressed at high levels on our TPR cells (Figure 2B). A mICOS chimera representing

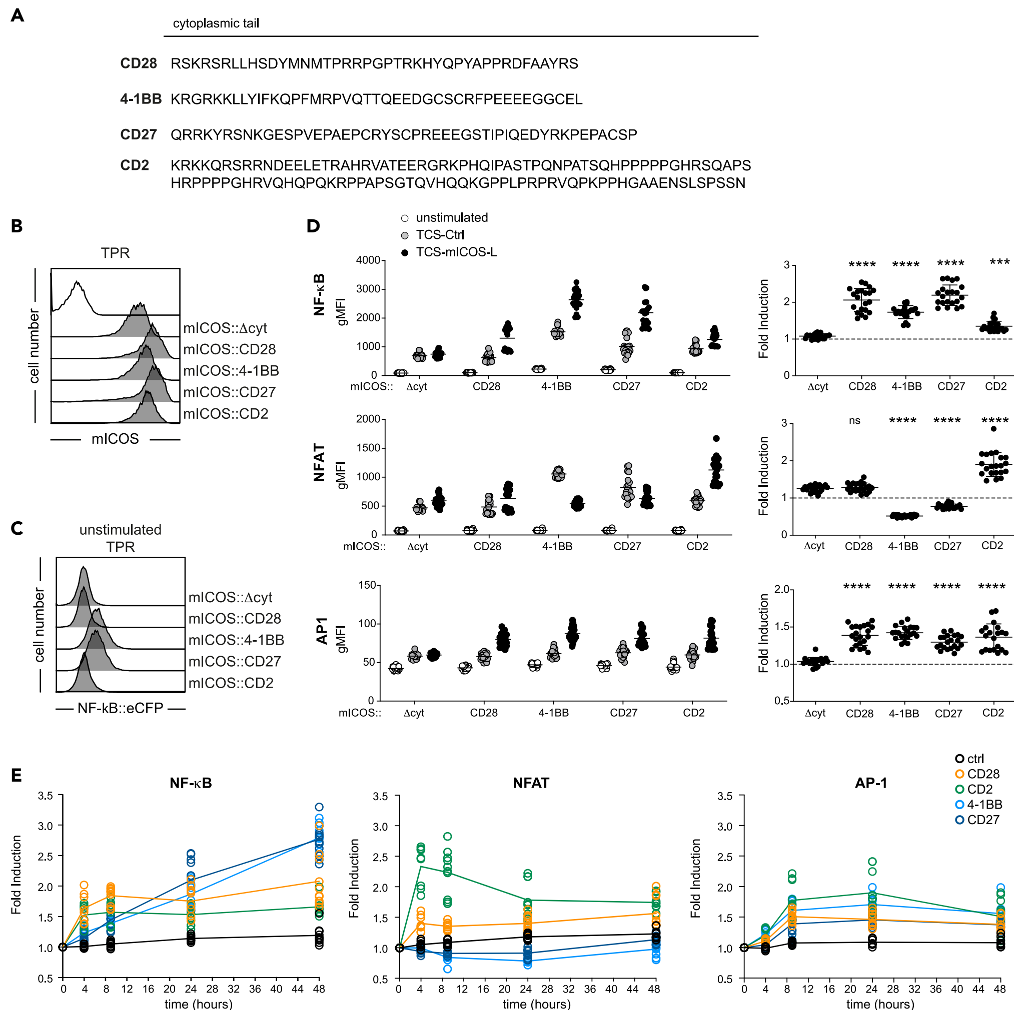

Figure 2. Intracellular sequences derived from major costimulatory receptors induce distinct transcriptional activation profiles

(A) Amino acid sequence of the cytoplasmic tails of costimulatory receptors that were used for mICOS chimeric constructs.

(B) Flow cytometric analysis of indicated mICOS constructs in TPR (gray histograms); open histogram represents mICOS-mAb binding to control TPR.

(C) Representative flow cytometric analysis of NF-κB reporter gene expression in unstimulated TPR expressing mICOS constructs as indicated.

(D) Left panel: TPR expressing indicated mICOS constructs were stimulated with TCS-Ctrl (gray dots) and TCS-mICOS-L (black dots) or kept untreated (unstimulated, open circles). eCFP, eGFP, and mCherry expression was measured via flow cytometry. Geometric means (gMFI) from 7 independently performed experiments in triplicates are shown. Mean is shown. Right panel: normalized data are shown (gMFI of TCS-mICOS-L stimulated cells/gMFI of TCS-ctrl stimulated cells). For statistical analysis one-way ANOVA followed by Tukey's multiple comparison test was performed for comparison with TPR-mICOS::Δcyt (****$p \leq 0.0001$; ***$p \leq 0.001$; ns $p > 0.05$). Control stimulation is shown as a dashed line. Mean and +/−SD are shown.

(E) Reporter activation was assessed following 4, 9, 24, and 48 h of co-culture. Normalized data are shown (gMFI of TCS-mICOS-L stimulated cells/gMFI of TCS-ctrl stimulated cells; n = 3, 3–4 replicates/experiment).

CD27, which like 4-1BB belongs to the TNFR-SF, was also included in these experiments. Interestingly, introduction of the chimeric 4-1BB or CD27 constructs led to a slight, but consistent, upregulation of the NF-κB reporter gene in unstimulated cells. This phenomenon was not observed with CD28 or CD2 (Figure 2C). Compared to reporter cells expressing chimeric receptors representing CD2 or CD28, stimulation of these reporter cells resulted in higher NF-κB reporter gene activation indicating that cytoplasmic domains derived from the TNFR-SF members 4-1BB and CD27 can promote NF-κB activation in a ligand-independent manner (Figure 2D).

Presence of mICOS-L during stimulation further upregulated NF-κB activation in the 4-1BB and CD27 chimeras and resulted in much higher levels of NF-κB reporter gene expression than engagement of mICOS::CD28 and mICOS::CD2 (Figure 2D left panel). Data normalization to reporter expression induced by control TCS revealed that triggering intracellular domains derived from CD28 has similar capacity as the TNFR-SF members to induce NF-κB, whereas engagement of mICOS::CD2 has weaker effects (Figure 2D right panel). Interestingly, reporter cells expressing chimeric receptors containing signaling motifs derived from the TNFR-SF, 4-1BB, and CD27 had significantly higher NFAT reporter activity upon stimulation with TCS-Ctrl compared to other reporters. However, NFAT reporter activity of TPR containing 4-1BB

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or CD27 constructs was strongly reduced, when mICOS-L was present (Figure 2D right). Engagement of mICOS::CD28 resulted in a modest upregulation of NFAT reporter gene expression, whereas mICOS::CD2 introduced the strongest NFAT upregulation, indicating that among the costimulators tested CD2 was the strongest inducer of NFAT activity. All four chimeric constructs induced comparable AP-1 reporter gene expression upon engagement (Figure 2D right).

Costimulatory signals are generally regarded as especially important, when T cells receive weak TCR/CD3 signals, and thus we investigated signaling of mICOS chimeras in presence of weak signal 1. For this, we used a previously described variant of our TCS, which expresses low levels of membrane-bound anti-CD3 (TCS^{low}[11]). Overall, these TCS induced lower reporter gene expression, but engagement of chimeric receptors, representing CD28, 4-1BB, CD27, and CD2 signaling domains, induced reporter cell responses that closely reflected those induced in presence of stronger anti-CD3 signals (Figure S1). We also performed time course stimulation experiments to study induction of reporter expression by costimulatory signals at 4, 9, 24, and 48 h (Figure 2E). These experiments demonstrated a delayed, but sustained, NF-kB reporter gene induction by 4-1BB and CD27 signaling which was highest at 48 h whereas upon CD2 and CD28 signaling NF-kB activation was already high at 9 h but did not increase at later time points. The induction of NFAT reporter gene expression upon CD2 signaling already peaked at 4 h and started to wane after 24 h (Figure 2E).

mICOS is a disulfide-linked homodimer, and this could potentially impact on the signaling of our chimeric receptors. To address this, we have generated and tested chimeric receptors lacking the cysteins mediating homodimerization of the mICOS ectodomains (mICOSmut). The results of these experiments are summarized in Figure S2. The expression of these constructs was rather low, and their costimulatory capacity was weak. Whereas the costimulatory effects of the mutated mICOS chimera harboring signaling domains derived from CD2, 4-1BB, and CD27 resembled those mediated by their dimerization-competent counterparts (strong NFAT activation mediated by $mICOS_{mut}$::CD2 and strong NF-kB activation mediated by mICOSmut:CD27 and mICOSmut:4-1BB), $mICOS_{mut}$::CD28 did not exert costimulatory activity (Figure S2). Like ICOS, CD28 is also a homodimer, and therefore our data may indicate that dimerized ectodomains are required for effective CD28 costimulation.

Taken together, these experiments showed distinct effects of the analyzed costimulatory molecules and furthermore demonstrated ligand-independent NF-κB and NFAT activation by constructs harboring signal motifs derived from 4-1BB and CD27.

Transcriptomic changes mediated by costimulatory signaling

In order to assess transcriptomic changes mediated by signaling via CD2, CD28, or 4-1BB on a genomic scale, we stimulated reporter cells expressing the chimeric receptors mICOS::CD2, mICOS::CD28, and mICOS:4-1BB with control TCS or TCS-expressing mICOSL. Triplicate cultures were set up for each condition. Following 20 h of co-culture, the cells were harvested and subjected to RNA sequencing (RNA-seq). Principal-component analysis (PCA) analysis revealed that reporter cells expressing mICOS:4-1BB stimulated with TCS control and TCS-mICOSL formed clusters that were clearly separated from the other samples indicating that the presence of the cytoplasmic domain was sufficient to mediate transcriptomic changes in the Jurkat T cell line. Furthermore, the engagement of the chimeric receptors containing 4-1BB or CD2 intracellular sequences by TCS expressing mICOSL resulted in a separation from the respective reporter cells stimulated with TCS control, demonstrating that 4-1BB and CD2 costimulation induced a distinct transcriptional program in the T cell reporter cells. By contrast we did not obtain a clear separation of mICOS::CD28-expressing TPR cells stimulated with TCS expressing mICOSL from control-stimulated TPR (Figure 3A). As the Jurkat reporter cells exhibited different background levels of activation without ligand engagement, we opted to perform differential gene expression analysis on matched Jurkat reporter lines that were either stimulated with TCS or left unstimulated. This allowed identification of genes that were differentially regulated because of stimulation rather than through tonic signaling. We observed that engagement of the chimeric receptor with ICOSL in a mICOS:control line did not contain any significantly enriched gene. Similarly, engagement of ICOSL on a mICOS-CD28 chimeric line contained only two differentially expressed genes (*GZMB* and *XCL1*). Engagement of ICOSL on chimeric CD2 and chimeric 41BB however led to significant enrichment of many other genes (depicted as a volcano plot in Figure 3B). This clearly showed that chimeric CD2- and chimeric 41BB-expressing lines were transcriptomically very distinct from control or chimeric CD28-expressing lines when engaged with the ligand. Gene enrichment analysis demonstrated that CD2 and 4-1BB costimulation promoted the expression of genes related to cytokine-cytokine receptor interaction. Among the pathways strongly enriched by 4-1BB costimulation were NF-kB signaling, tumor necrosis factor (TNF) signaling, and cancer and JAK-STAT-signaling (Figure 3C). Next, we checked if there were overlaps between the differentially expressed genes mediated by engagement of mICOS-CD2 and mICOS-41BB. The expressions of genes that significantly differed upon costimulation via 4-1BB (TPR-mICOS:4-1BB stimulated with TCS-mICOSL) and via CD2 (TPR-mICOS::CD2 stimulated with TCS-mICOSL) are displayed as heatmaps for all stimulation conditions (Figure 3D). 4-1BB signaling induced the largest number of differentially expressed genes, and most of these genes were not regulated by engagement of the other chimeric receptors. The number of genes that were upregulated by CD2 signals was considerably lower, and only few genes were significantly upregulated by both costimulators. Interestingly, several of the CD2-regulated genes also tended to be upregulated upon engagement of TPR-mICOS::CD28 and to a lower extent by TPR-mICOS::Δcyt by TCS-mICOSL (Figure 3D).

Engagement of chimeric receptors in absence of signal 1

To investigate the capability of chimeric receptors to transduce signals in absence of the TCR/CD3 signal, the reporter cells were co-cultured with BW cells expressing high levels of mICOS-L, but no membrane-bound anti-CD3 (Figure 4A). High levels of NF-κB reporter activation were induced in presence of 4-1BB and CD27, but also CD2 chimeras, whereas engagement of constructs containing CD28 domains induced much lower activity (Figure 4B). CD3-independent NFAT reporter activation was induced by CD2 chimeras, and surprisingly also by the TNFR-SF

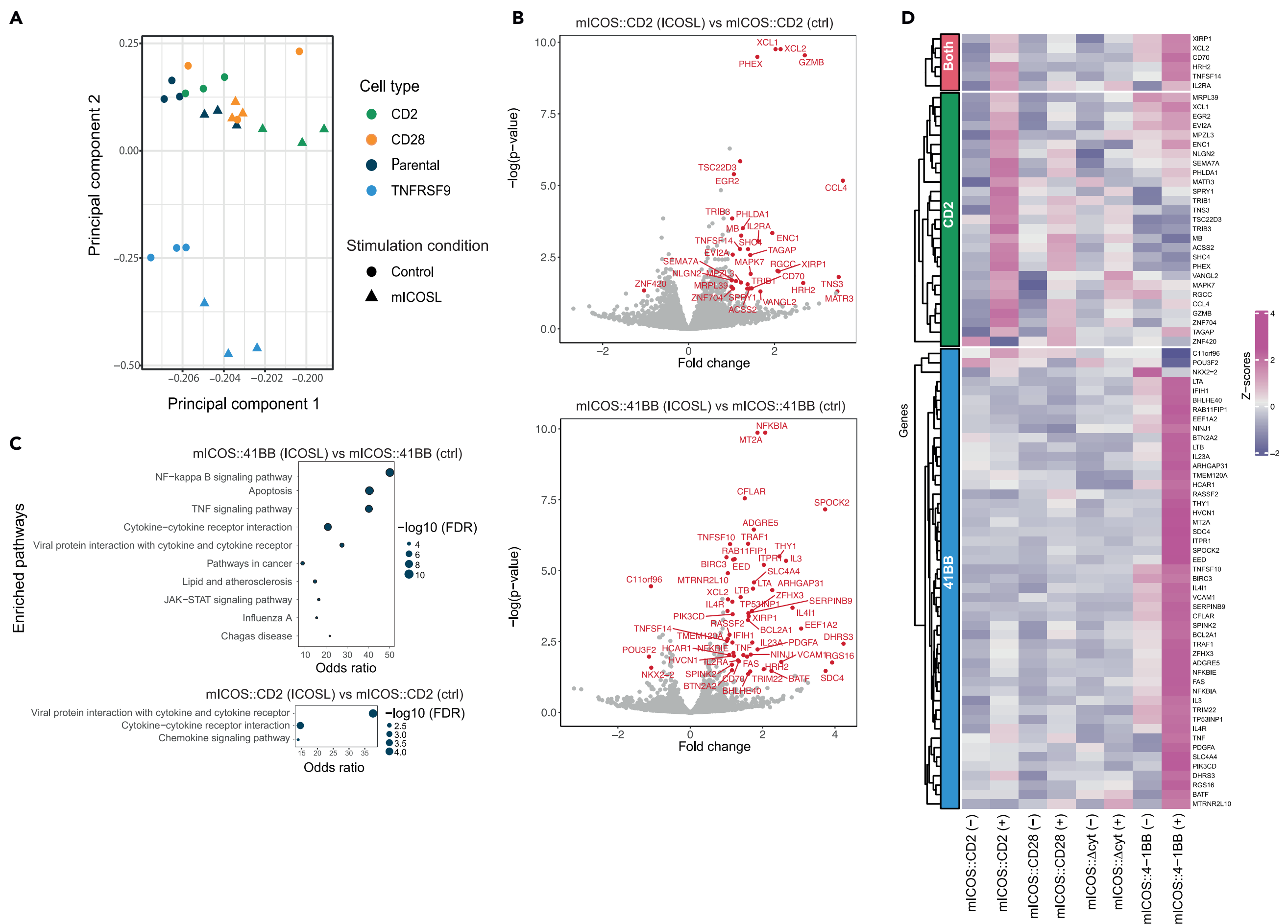

Figure 3. Transcriptomic changes mediated by engagement of chimeric receptors

(A) PCA plots of all samples.

(B) Volcano plots depicting the differentially expressed genes when comparing indicated stimulated Jurkat cells vs. matched control stimulation.

(C) Bubble plots of gene set enrichment analysis using the Hallmark database to identify pathways enriched upon 4-1BB (upper panel) or CD2 (lower panel) signaling.

(D) Heatmap showing the *Z* scores based on normalized expression of differentially expressed genes (FDR<0.05, abs (LFC) > 1) upon engagement of chimeric receptors harboring the intracellular domain of CD2 or 4-1BB for all conditions.

members 4-1BB and CD27, but not by CD28. All constructs induced a modest upregulation of the AP-1 reporter gene expression, but this effect was only significant in reporter cells expressing the CD2 chimera (Figure 4B).

Taken together, our results indicate that cytoplasmic sequences of 4-1BB, CD27, and CD2 are more prone to induce signaling in absence of CD3 engagement, than CD28.

Response to costimulatory signals provided by separate cells

The classical scenario of T cell activation proposes that T cells receive costimulatory signals from APCs, which present their antigen. However, there are several studies demonstrating that costimulatory signals provided by adjacent cells are able to enhance the response to antigens, presented by APCs in absence of costimulation.[19–21] We used our reporter platform to address, whether i) distinct costimulatory domains differ in their capability to mediate costimulation when provided from separate cells and ii) activation of transcription factors is differentially affected by a spatial separation of “signal 1” and “signal 2.” For this, we assessed reporter activation by costimulation provided in combination with “signal 1” (TCS-mICOS-L) or segregated from “signal 1” (TCS-control and BW-mICOS-L; Figure 5A).

NF-κB activation was strongly enhanced under both conditions, but segregation of signal 1 and 2 did not appear to affect CD28 and 4-1BB, whereas costimulation of CD27 and CD2 was slightly attenuated (Figure 5B). This was in sharp contrast to costimulation of NFAT activity, which was strictly dependent on co-localization of ligands for CD3 and mICOS on the same cell. Engagement of chimeric receptors containing cytoplasmic domains derived from TNFR-SF reduced NFAT reporter gene expression, and surprisingly this inhibitory effect was maintained upon segregation of signal 1 and 2. Provision of costimulatory signals from separate cells resulted in a greatly reduced AP-1 reporter activation by

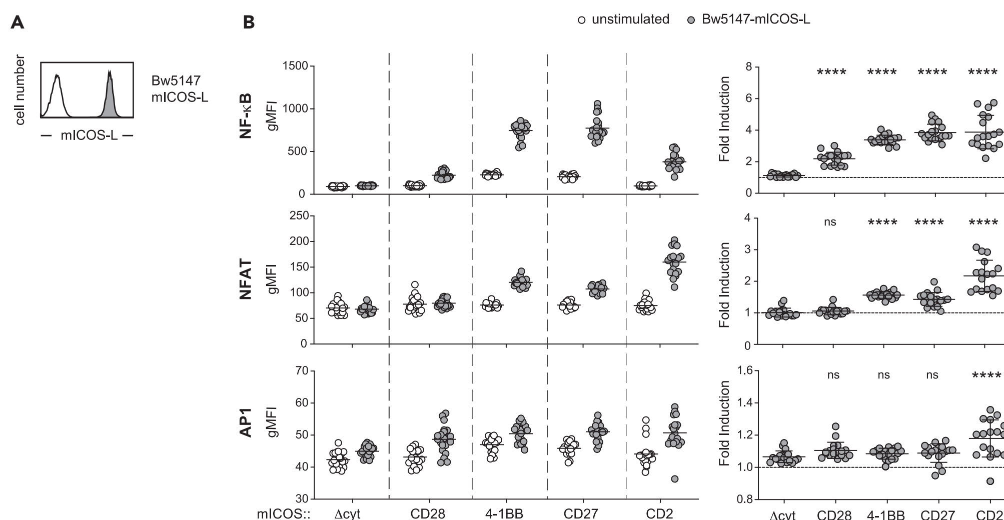

Figure 4. Engagement of chimeric receptors in absence of signal 1

(A) mICOS-L expression on BW-mICOS-L cells (gray histogram); open histogram shows reactivity of mICOS-L-mAb to BW cells.

(B) Left panel: TPR expressing indicated mICOS constructs were stimulated with BW-mICOS-L (gray dots) or left unstimulated (empty dots). eCFP, eGFP, and mCherry expression was measured via flow cytometry. Geometric means (gMFI) from 7 independently performed experiments in triplicates are shown. Mean is shown. Right panel: data on the left were normalized (gMFI of BW-mICOS-L stimulated reporter cells/gMFI of unstimulated reporter cells). For statistical analysis one-way ANOVA followed by Tukey's multiple comparison test was performed for comparison with TPR-mICOS::Δcyt (****$p \leq 0.0001$; ns $p > 0.05$). Mean and +/−SD are shown. Unstimulated cells are indicated as a dashed line.

CD28 and 4-1BB and completely abolished costimulation by CD27 and CD2 (Figure 5B). In conclusion, segregated provision of costimulatory signals appears to have differential effects on major transcription factors involved in T cell activation. Whereas NF-κB activity was only marginally affected, costimulation of NFAT and AP-1 reporter gene expression was largely abolished. By contrast, inhibition of NFAT activity, by engagement of chimeric receptors representing TNFR-SF members, was even more pronounced, when these receptors were engaged by separate cells.

The role of CD28 signaling motifs in the activation of T cell reporter cells

Three signaling motifs in the primary costimulatory receptor CD28 have been identified, but their individual contribution to signal transduction is not fully understood. Jurkat E6.1, and therefore also TPR cells, endogenously express CD28, which hampers studies on mutated CD28 molecules. Here, we used the mICOS construct platform and designed a panel of mutated mICOS::CD28 chimeras (Figure 6A). Three mutated versions of the YMNM motif (FMNM, YMAM, YMNL) were designed; the putative ITK binding motif PRRP was mutated to ARRA, and the membrane-distal PYAP motif was mutated to AYAA or PFAP. The mutations were designed to interfere with the binding of signaling molecules like PI3K, VAV1, PP2A, Grb2 and GADS (YMNM), Itk, Tec (PRRP) and Lck, Grb2, GADS, and Filamin A (PYAP).[22–25] These mICOS chimeras, along with a chimera harboring the wild-type CD28 cytoplasmic tail, were expressed in the TPR, and high expression of all CD28 chimeras was confirmed by flow cytometry (Figure 6B). The resultant TPRs were stimulated, and the response to TCS-mICOS-L, normalized to reporter gene expression induced by TCS-Ctrl, is shown (Figure 6C). Interestingly, mutation of the tyrosine residue in the YMNM motif to phenylalanine (FMNM) significantly increased activation of NF-κB and AP-1, compared to wild-type CD28. By contrast, the YMAM mutation slightly reduced NF-κB activation and completely abrogated NFAT activation. A mICOS::CD28 construct, where the YMNM motif was mutated to YMNL, was similarly effective in enhancing NF-κB, NFAT, and AP-1 reporter gene expression as the wild-type mICOS::CD28 construct. Changes in the PRRP motif did not affect activation of the three transcription factors. By contrast, mutation of the PYAP motif to PFAP strongly impaired reporter activation, and a mICOS::CD28 variant lacking the proline residues in this motif (AYAA) was completely ineffective in activating NF-κB, NFAT, and AP-1 expression (Figure 6C). Triggering mutated CD28 signaling motifs in the context of low CD3 signal yielded responses that closely reflected those obtained in the context of strong CD3 signals (Figure 6D). Taken together, these results suggest the membrane-distal PYAP motif to be the most important mediator of CD28 signaling. Unexpectedly, mutating the tyrosine residue of the ITTM motif (YMNM) enhanced, rather than reduced, NF-κB and AP-1 activation.

Inhibitory signaling via the YMNM motif of CD28

Enhanced reporter activation via CD28 sequences lacking the tyrosine residue in the ITTM motif (YMNM) suggested that this motif may be able to recruit inhibitory signaling proteins. To test this hypothesis, we generated truncated mICOS::CD28 chimera lacking the membrane-distal activating PYAP motif ($CD28_{(1-22)}$) and the same chimera, but with a mutated YMNM motif ($CD28_{(1-22)}$_FMNM). These constructs and corresponding chimeras, harboring full-length cytoplasmic sequences of CD28, were expressed on the TPR (Figures 7A and 7B). Indeed,

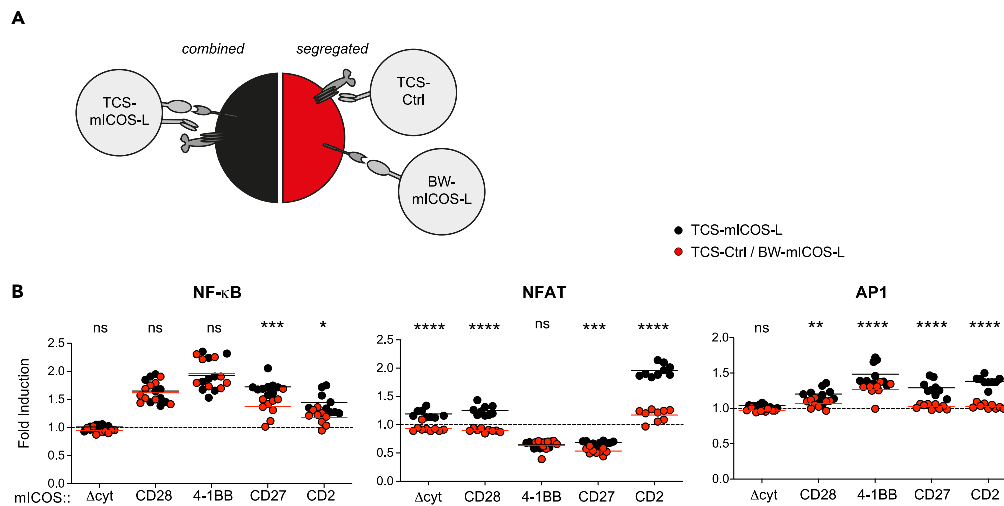

Figure 5. Response to segregated costimulatory signals

(A) Schematic of T cell reporter cells receiving signal 1 and 2 from one cell or from two different cells.

(B) TPR expressing indicated mICOS chimeric molecules receiving signal 1 and 2 from the same cell (black dots) or from two different cells (red dots). eCFP, eGFP, and mCherry activation was measured via flow cytometry. Results are shown from 3 independent experiments performed in triplicates. Normalized reporter activation is shown (gMFI of TCS-ctrl stimulated reporter cells/gMFI of reporter cells receiving signal 1 and 2 from the same cell (via TCS-mICOSL) or from separate cells (via TCS-ctrl + BW-mICOSL)). Control stimulation is shown as a dashed line. For statistical analysis two-way ANOVA followed by Sidak's multiple comparison test was performed to compare reporter cells receiving signal 1 and 2 from one cell with reporter cells receiving signal 1 and 2 from separate cells (****$p \leq 0.0001$; ***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$; ns $p > 0.05$).

we observed that the full-length cytoplasmic domain of CD28 strongly enhanced NF-κB reporter gene activation, whereas engagement of the $CD28_{(1-22)}$ chimera led to a slight inhibition of NF-κB (Figures 7C and 7D). The full-length cytoplasmic domain of CD28 harboring the mutated ITTM motif, FMNM, strongly enhanced the expression of the NF-κB and AP-1 reporter genes, whereas the truncated FMNM chimera did not show any effect (Figures 7C and 7D).

These results indicate that the ITTM motif (YMNM) indeed inhibits NF-κB activation in our T cell reporter platform and confirmed that the PRRP motif, which is also contained in the truncated mICOS::$CD28_{(1-22)}$ chimera, does not mediate significant reporter gene activation.

Membrane-distal sequences are sufficient to mediate CD28 costimulation

In a next set of experiments, we addressed, whether membrane-distal sequences of CD28 harboring the PYAP motif ($CD28_{(23-41)}$) are sufficient to mediate CD28 costimulation in our T cell reporter platform. In addition, we investigated how spacing, i.e., the distance from the transmembrane region, impacts on the function of this motif. We reasoned that the membrane-proximal sequence of the inhibitory receptor PD-1 ($PD\text{-}1_{(1-17)}$) could be used as an inert spacer sequence, since it does not contain inhibitory motifs. We performed a set of experiments with mICOS chimera harboring length variants and mutants of the cytoplasmic tail of human PD-1, to confirm this hypothesis. Furthermore, these experiments corroborated previous reports in showing that the ITSM motif of this receptor mediates its inhibitory effects (Figure S3). We generated mICOS chimera harboring the $CD28_{(23-41)}$ sequence fused to the transmembrane domain either directly or via a shorter or longer PD-1 spacer sequence (Figure 8A) and expressed these constructs and control constructs in our TPR cells (Figure 8B). Stimulation experiments with the resultant TPR revealed that the $CD28_{(23-41)}$ sequence is sufficient to promote strong NF-κB and AP-1 activation (Figure 8C). Furthermore, our results indicate that a certain distance from the transmembrane region is required to confer full functionality to this motif, since a chimera, where the $CD28_{(23-41)}$ sequence was fused directly to the transmembrane domain, was less effective than constructs harboring a five or 17 amino acid spacer. Finally, we generated TPR expressing a chimera containing a tandem repeat of the $CD28_{(23-41)}$ motif to analyze whether duplication of the $CD28_{(23-41)}$ sequence would result in a more potent costimulation (Figure 8D). We observed that duplication of the $CD28_{(23-41)}$ sequence did indeed endow our mICOS chimeric receptor with a superior capacity to costimulate NF-κB and AP-1 activation (Figure 8E). We also tested cytoplasmic CD28 sequences in a αCD19-CAR format (Figure S4A). Upon expression in TPR, these receptors were triggered with TCS co-expressing membrane-bound anti-CD3 and high levels of human CD19 (Figure S4B). Results with αCD19-CARs corroborated our findings with mICOS chimeras, demonstrating a strongly enhanced costimulatory capacity of tandem $CD28_{(23-41)}$ sequences (Figure S4E).

DISCUSSION

Activation of T cells by professional APCs presenting their cognate antigen is an intricate multilayered process, where, in addition to the TCR-CD3 complex, a plethora of activating and inhibitory receptors participate. Upon engagement by APC-expressed ligands, they transduce intracellular signals. Additionally, ligand engagement enhances T cell activation by promoting the interaction between APCs and T cells.

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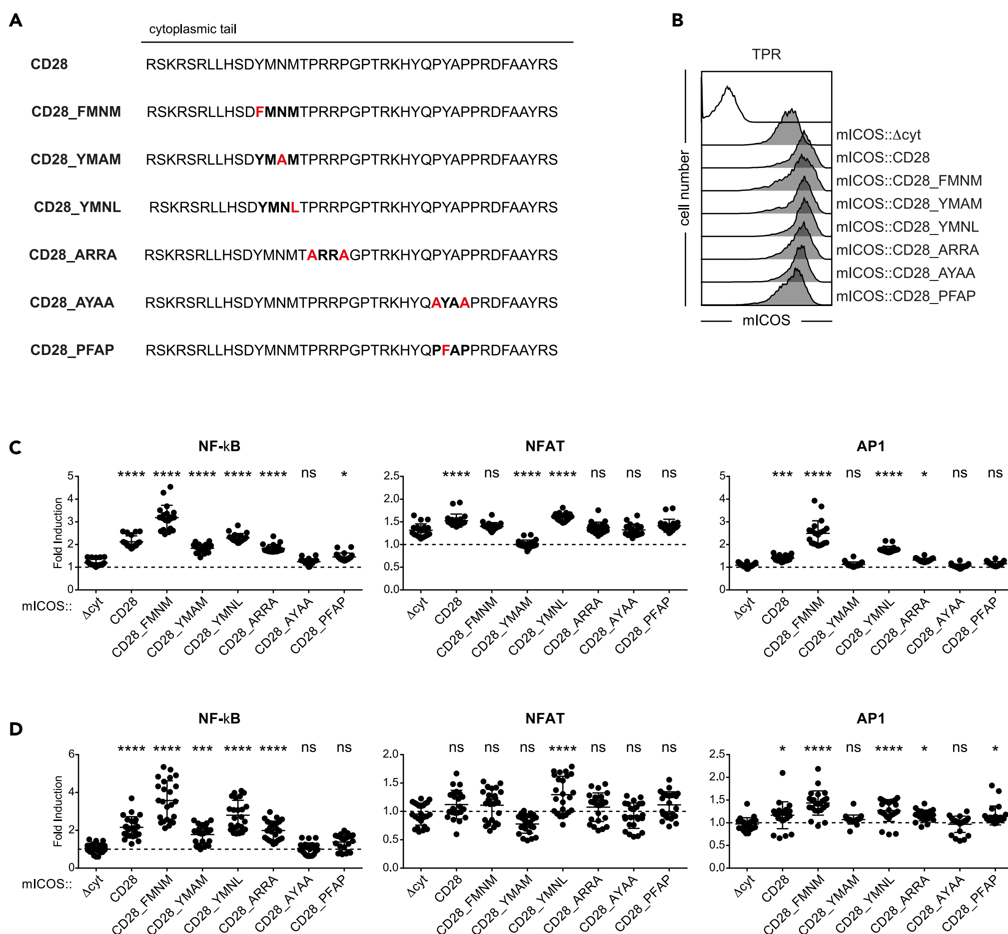

Figure 6. The role of CD28 signaling motifs in the activation of T cell reporter cells

(A) Amino acid sequences of the cytoplasmic tail of wild-type CD28 and mutated CD28 variants that were used for mICOS chimeric constructs.

(B) Flow cytometric analysis of indicated mICOS constructs in TPR (gray histograms); open histogram represents antibody binding to control TPR.

(C) TPR expressing indicated mICOS constructs were stimulated with TCS-mICOS-L. eCFP, eGFP, and mCherry expression was measured via flow cytometry. Reporter activation in response to TCS-mICOSL was normalized to reporter activation induced by control TCS (gMFI of TCS-mICOS-L stimulated cells/gMFI of TCS-ctrl). Results from 7 independently performed experiments in triplicates are shown.

(D) TPR expressing indicated mICOS constructs were stimulated with TCS expressing low levels of membrane-bound anti-CD3 antibodies (TCS^{low}-ctrl and TCS^{low}-mICOS-L (Figure S1). Data were normalized as in (C). eCFP, eGFP, and mCherry expression was measured via flow cytometry. Results from 8 independently performed experiments in triplicates are shown.

(C and D) For statistical analysis one-way ANOVA followed by Tukey's multiple comparison test was performed for comparison with TPR-mICOS::Δcyt (****$p \leq 0.0001$; ***$p \leq 0.001$; *$p \leq 0.05$; ns $p > 0.05$). Mean and +/−SD are shown. Control stimulations are shown as a dashed line.

The individual contribution of a given receptor to T cell activation processes is difficult to study on primary T cells. This is greatly affected by expression levels, availability, and concentrations of the ligands, but also by affinity and adhesive properties of ligand interactions. Additionally, due to redundant functions of different activating or inhibitory receptors expressed on T cells, discriminating intracellular signals, transduced by the receptor of interest, is challenging.

Here, we have used chimeric receptors harboring the extracellular domain of mICOS to study signaling processes mediated by cytoplasmic sequences of the major T cell costimulatory receptors CD28, 4-1BB, CD27, and CD2. The extracellular part of mICOS was chosen because it is a classical receptor, which transduces intracellular signals upon interaction with its ligand, mICOS-L (B7-H2). Moreover, ICOS is not expressed on the human Jurkat TPR line that was used to assess the capability of these chimeric receptors to induce NF-κB, NFAT, and AP-1 activity. In contrast to human ICOS-L, which interacts with human CD28, mICOS-L does not bind to CD28,[26,27] and thus there was no interference from interaction of mICOS or mICOS-L with other molecules in our system. We have expressed mICOS-L on TCS and used the resultant TCS-mICOS-L cells to trigger the chimeric receptors on the reporter cells in the context of TCR/CD3. TCS were generated by engineering BW5147 cells, a murine thymoma line, to express a membrane-bound anti-human CD3 antibody fragment. We have previously established and extensively employed these cells to stimulate and expand human T cells and human T cell lines.[11,28] More recently,

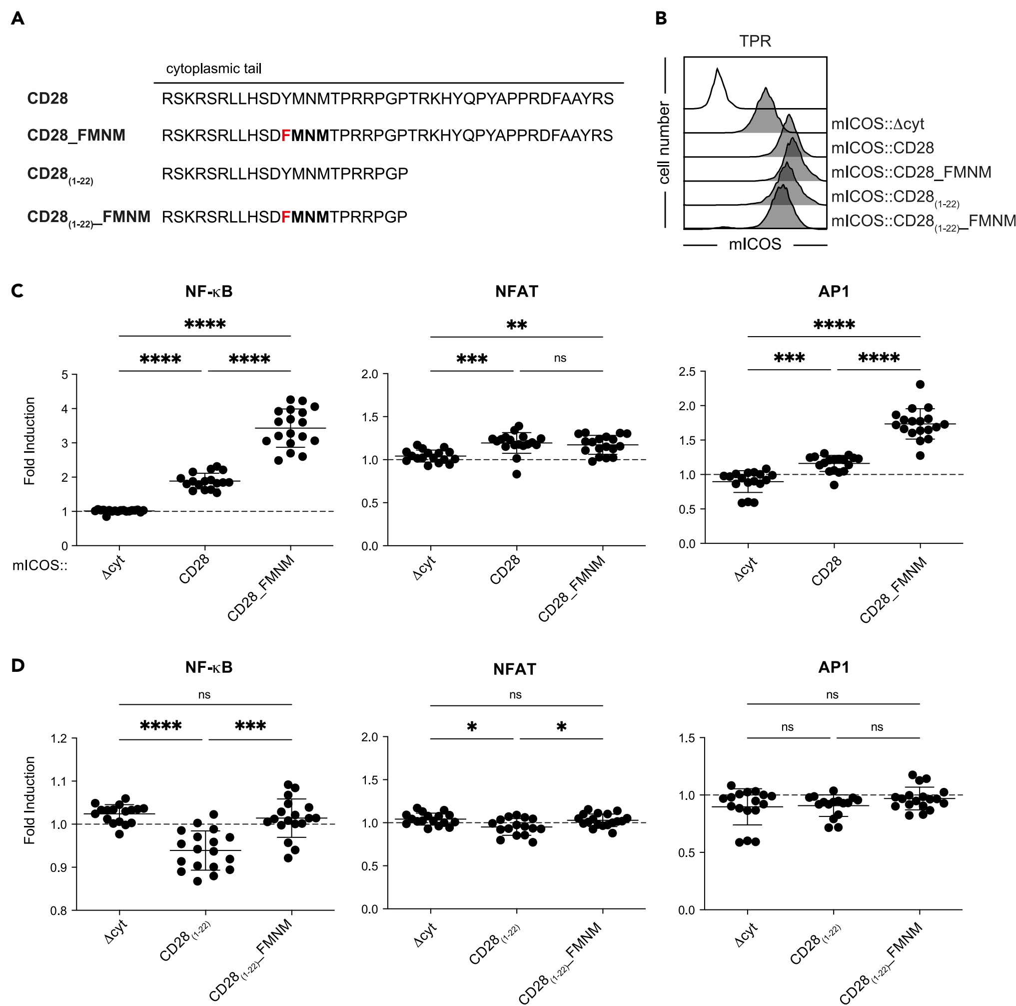

Figure 7. Inhibitory signaling via the YMNM motif of CD28

(A) Amino acid sequence of cytoplasmic tails of mICOS::CD28 chimeric constructs.

(B) Flow cytometric analysis of indicated mICOS constructs in TPR (gray histograms); open histogram represents antibody binding to control TPR.

(C and D) TPR expressing indicated mICOS constructs were stimulated with TCS-mICOS-L and reporter gene expression normalized to reporter gene expression induced by control TCS is shown (gMFI of TCS-mICOS-L stimulated cells/gMFI of TCS-ctrl stimulated cells). eCFP, eGFP, and mCherry expression was measured via flow cytometry. Results from 6 independently performed experiments in triplicates are shown. For statistical analysis one-way ANOVA followed by Tukey's multiple comparison test was performed (****$p \leq 0.0001$; ***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$; ns $p > 0.05$). Mean and +/−SD are shown. Control stimulation is shown as a dashed line.

other laboratories have also used cells expressing membranous CD3 antibodies and reported that they are superior in expanding human CD8^{+} T cells compared to CD3/CD28 antibody-coated beads.[29,30] TCS also offer the advantage that the membrane-bound ligands engage the chimeric mICOS receptors in a more physiological manner, compared to immobilized ligands or agonistic antibodies. Engagement of chimeric receptors with membrane-bound ligands can be expected to closely reflect the kinetics of natural signaling events, and time course stimulation experiments revealed a distinct kinetic profile of the costimulatory receptors used in our study. NF-kB activation via 4-1BB and CD27 was slow and peaked at 48 h, whereas the kinetic of NF-kB activation enhancement by CD2 and CD28 signaling was fast and did not significantly increase after the 9 h time point. The induction of NFAT signaling via CD2 signaling had the fastest kinetic and was highest already at 4 h.

Two of the costimulatory molecules studied here belong to the Ig superfamily like ICOS, whereas 4-1BB and CD27 belong to the TNFR-SF. mICOS is a disulfate-linked homodimer, and this could potentially induce aberrant signaling in mICOS chimera. Therefore, a potential concern with our approach was that the interaction of mICOSL with chimeric mICOS receptors harboring the intracellular domains of the TNFR-SF members might not faithfully reflect the intracellular signals induced upon engagement of these 4-1BB and CD27 with their natural ligands. We have recently investigated 4-1BB and CD27 in our TPR system, and the transcriptional regulation mediated by mICOS:41BB and mICOS::CD27 closely matched the results obtained in this study. Specifically, we found that engagement of 4-1BB and CD27 by their natural

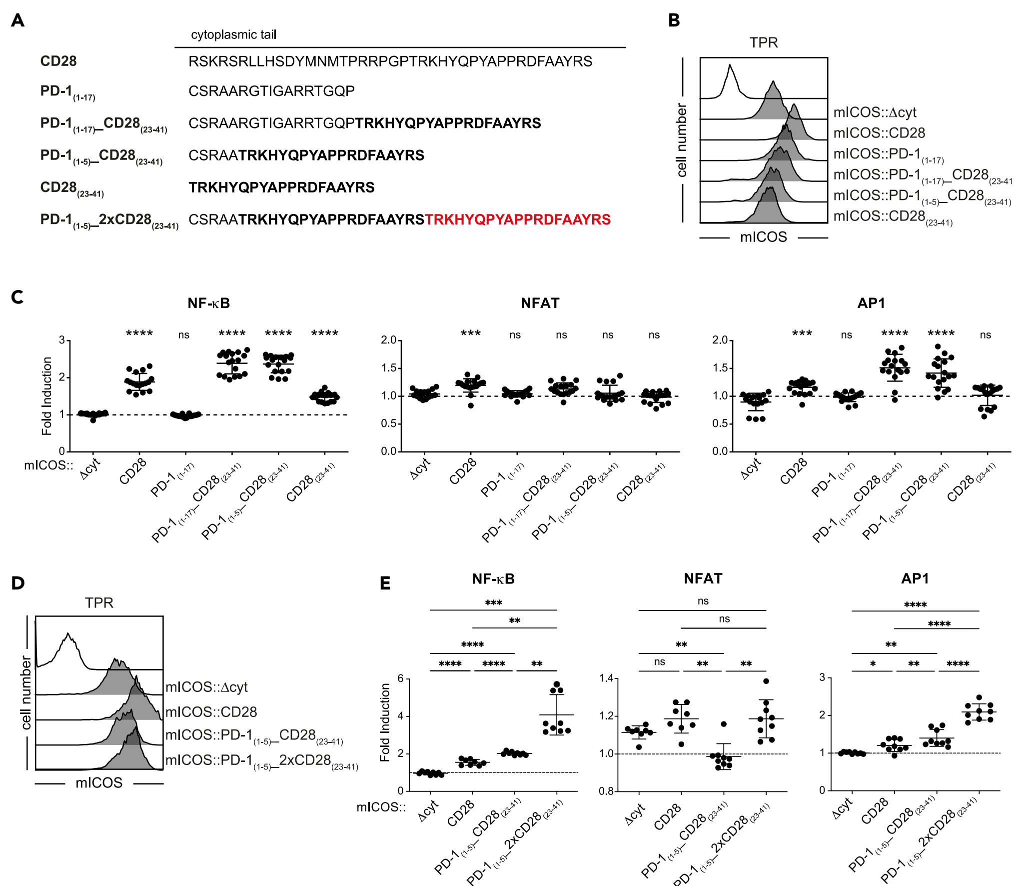

Figure 8. Membrane-distal sequences are sufficient to mediate CD28 costimulation

(A) Amino acid sequences of the cytoplasmic tails of mICOS chimera.

(B) Flow cytometric analysis of TPR expressing the indicated mICOS constructs (gray histograms); open histogram represents antibody binding to control TPR.

(C) TPR expressing indicated mICOS constructs were stimulated with TCS-mICOS-L. eCFP, eGFP, and mCherry expression was measured via flow cytometry. Results from 6 independently performed experiments in triplicates are shown.

(D) Flow cytometric analysis of indicated mICOS constructs in TPR (gray histograms); open histogram represents antibody binding to control TPR.

(E) TPR expressing indicated mICOS constructs were stimulated with TCS-mICOS-L. eCFP, eGFP, and mCherry expression was measured via flow cytometry. Results from 3 independently performed experiments in triplicates are shown.

(C and E) Normalized data are shown (gMFI of TCS-mICOS-L stimulated cells/gMFI of TCS-ctrl stimulated cells). For statistical analysis one-way ANOVA followed by Tukey's multiple comparison test was performed. In (C) compared to TPR-mICOS::Δcyt (****$p \leq 0.0001$; ***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$; ns $p > 0.05$). Mean and +/−SD are shown. Control stimulation is shown as a dashed line.

ligands also induced potent upregulation of NF-kB and AP-1 reporter gene expression and a modest downregulation of NFAT activity.[31] We also performed some experiments with chimera harboring mICOS ectodomains lacking the dimerization motif, but due to a poor expression of these chimeric receptors their costimulatory capacity was weak. Furthermore, downregulation of NFAT activation via 4-1BB and CD27 signaling, which also occurs upon engagement of 4-1BB and CD27 with their natural ligands, was not observed with mICOS chimera lacking the dimerization motif. CD28 is also expressed as a homodimer, and interestingly mICOS-CD28 chimera lacking the dimerization motif failed to costimulate reporter activation. Altogether these data indicate that chimera based on unmutated mICOS ectodomains have utility to study signaling induced by different costimulatory signaling domains.

Our results highlight that costimulatory signals transduced from major costimulatory receptors have distinct qualities. They confirmed activation of NF-κB, NFAT, and AP-1 by CD28 signaling, and strong upregulation of NF-κB and AP-1 reporter gene expression upon engagement of chimera harboring cytoplasmic domains derived from the TNFR-SF-members 4-1BB and CD27. However, TPRs expressing chimeric 4-1BB and CD27 receptors also reveal unexpected ligand-independent effects of these two costimulators, i.e., significantly higher NF-κB and NFAT activation upon stimulation via the TCR/CD3 complex. Whereas engagement of these receptors during activation further upregulated NF-κB activation, it strongly reduced the expression of the NFAT reporter gene.

CD2, which is mainly regarded as an adhesion molecule, recruits CD2 adaptor protein (CD2AP) upon activation via its SH3 binding domain. CD2AP subsequently mediates CD2 clustering, cytoskeletal polarization, and protein segregation.[32] Our results show that CD2 is a genuine

signaling receptor that induces the activation of major transcription factors involved in T cell activation. Importantly, the cytoplasmic tail of CD2 was the most potent activator of NFAT among the costimulatory molecules analyzed. The intracellular part of CD2 is very long, but currently little is known regarding the role of motifs, contained in its cytoplasmic tail, in transcriptional regulation.

We also performed RNA-seq analysis to investigate transcriptomic changes induced by CD28, CD2, and 4-1BB signals. Previous studies have addressed the effects of CD28 and ICOS signals on gene expression, but to our knowledge the major costimulatory receptors have not been compared in this respect.[33,34] Our experiments revealed that CD2 and 4-1BB signaling induces significant upregulation of a number of genes. 4-1BB costimulation induced the largest set of differentially expressed genes many of which did not show a tendency for upregulation in the other conditions indicating that 4-1BB costimulation mediates a transcriptional program in T cells that is distinct from CD2 and CD28 costimulation. The introduction of mICOS:4-1BB (and mICOS::CD27) led to a slight increase of NF-kB:eCFP expression in unstimulated reporter cells. In line with this we observed a clear separation of control-stimulated reporter cells harboring mICOS:4-1BB from the other reporter cells indicating that the presence of receptors harboring cytoplasmic 4-1BB sequences (and potentially of sequences derived from other TNFR-SF) mediates transcriptomic changes that are independent of receptor engagement. Pathway analysis highlighted the prominent role of 4-1BB in NF-kB signaling but also in apoptosis, which is in line with reports that implicated 4-1BB overactivation with cell death/ACID. In line with a distinct transcriptional program induced by 4-1BB, the overlap between genes regulated by 4-1BB and CD2 signals was limited. Pathway analysis indicated that both costimulatory pathways promoted the expression of genes related to cytokine and chemokine signaling. Although in our reporter cells CD28 signals strongly upregulated the activity of NF-kB and AP-1 and showed a tendency of enhanced NFAT activity, they did not induce a significant regulation of genes in our dataset. There is evidence that CD28 signals mainly act as amplifier of TCR signals rather than triggering distinct downstream signaling pathways.[2,33] In line with that we found that genes such as IL2RA, CD69, EGR1, or EGR2, which are strongly induced during TCR activation were further induced upon engagement of mICOS::CD28 although this augmentation did not reach statistical significance in our dataset. Moreover, many genes that were significantly regulated by CD2 signals also showed a tendency to be upregulated by CD28 signals indicating an overlap between CD2 and CD28 costimulation. Interactions that do not result in the generation of intracellular signals might enhance T cell activation by promoting the adhesion between T cells and APCs. As a control for signaling-independent T cell reporter activation in our system, we expressed a truncated mICOS molecule (mICOS::Δcyt) and observed that activation of the resultant reporter cells in presence of mICOSL only marginally increased reporter activation. Engagement of mICOS::Δcyt did not induce significant changes in the gene expression, but several of the genes significantly upregulated by CD2 costimulation also showed a tendency of higher expression upon mICOS::Δcyt-mICOSL interaction which indicates that the transcriptional regulation mediated by mICOS::CD2 engagement integrates CD2 signals and activating signals mediated by enhanced cell-cell interaction via the interaction of mICOSL and the ectodomain of the chimeric receptor.

The main role of costimulatory molecules is to support the activation of T cells that recognize antigens, and engagement of costimulatory receptors in absence of TCR signals should not promote significant T cell activation. We found that all chimeric receptors mediated some degree of T cell reporter gene expression in absence of TCR/CD3 signals. However, the TCR complex-independent NF-κB and NFAT activation induced by mICOS::CD28 was considerably weaker than those induced by chimera representing 4-1BB, CD27, or CD2. These receptors are more broadly expressed in non-T cells and thus must be capable to signal independently of the TCR. By contrast, the expression of CD28 is almost exclusively restricted to T cells, and thus this receptor may have evolved to preferentially function as amplifier of TCR/CD3 complex signaling.

Under physiological conditions, the costimulatory signal is preferentially provided by APCs, which present antigens to T cells. This will ensure that self-antigens are mainly presented with weak costimulatory signals, whereas antigens derived from pathogens will be presented in the context of strong costimulatory signals since pathogen-associated molecular patterns will induce a strong upregulation of costimulatory ligands on APC.[35] However, *trans*-costimulation has been demonstrated *in vitro* and *in vivo*.[36,37] Here, we have observed that costimulation of NF-κB activation is largely maintained upon segregation of signal 1 and 2, whereas costimulation of NFAT and AP-1 activation requires both signals to be provided by the same cells. As a consequence, costimulatory signals might have different qualities when provided by cells that are distinct from cells that present the antigen.

CD28 harbors three signaling motifs, and the contributions of individual motifs to the costimulatory properties of this receptor are not completely clarified. We generated six mutated mICOS::CD28 chimera to address the role of these motifs in the transcriptional activation of T cells. Our results indicate that the costimulatory effect of CD28 in our T cell reporter model is almost exclusively mediated by the membrane-distal PYAP motif. By contrast, the membrane-proximal YMNM motif does not mediate reporter activation. Moreover, eliminating the tyrosine residue from this motif (FMNM) even enhanced costimulation via CD28. The YMNM motif promotes PI3K signaling, since it recruits the p85 subunit of this kinase. The importance of this pathway for the costimulatory function of CD28 is highly controversial. It has been argued that Jurkat T cells may not be a good model to study the role in PI3K signaling, since it lacks PTEN and SHIP-1, the counter-players of this pathway.[38,39] However, there is accumulating evidence that the PYAP motif mediates CD28 costimulation *in vivo*, whereas mutating the YMNM motif has no discernable effect.[40–43] Importantly, the cytoplasmic tail of the coinhibitory receptor CTLA-4 also contains a similar motif, also mediating p85 binding,[44] and further ruling out a prominent role of this motif in costimulatory signaling. A likely explanation for the enhanced costimulatory activity observed in the FMNM variant is abrogation of recruitment of the serine/threonine phosphatase PP2A. PP2A was shown to bind the YMNM motif of CD28 (and also to the corresponding motif [YVKM[in CTLA-4) and to inhibit T cell activation.[45] To further confirm a major role of the PYAP motif in CD28 costimulation, we tested chimeric receptors harboring only membrane-distal sequences of CD28 containing the PYAP motif. It was found that they were sufficient to mediate strong costimulation, and moreover we could demonstrate mICOS chimera harboring a duplication of this motif-mediated superior costimulatory capacities. The PYAP motif is bound by GRB2 and GADS through their SH3 domains, and especially GADS is implicated in the formation of CARMA1-Bcl-10-Malt1 complex, which

contributes to the activation of IKKs that regulate NF-κB activation.[46,47] Furthermore, the phosphorylation of the kinases PDK1 and PKC-theta as well as binding and activation of Lck is also mediated by the PYAP motif.[1]

In conclusion, we have obtained novel insights on the capability of intracellular sequences of costimulatory receptors to induce signaling and activation of relevant transcription factors. Our results could be harnessed for the design of improved strategies to combat infections or tumors by adoptively transferring genetically engineered autologous T cells into patients. In CAR constructs intracellular sequences derived from costimulatory receptors are inserted upstream of the CD3ζ sequence to improve the capability of CAR T cells to persist and expand *in vivo*, and to efficiently eradicated target cells. CARs, where intracellular sequences of two costimulatory receptors are inserted, so-called 3rd generation CARs, are being tested, but potentially signal 1 might be impaired in such constructs due to an unfavorable membrane-distal location of the CD3ζ sequences. Using the extended PYAP motif derived from the primary costimulatory CD28, we exemplify that isolated signaling motifs can be sufficient to confer costimulatory properties to chimeric receptors. Thus, it might be possible to combine intracellular motifs derived from selected costimulatory receptors to obtain optimal CAR constructs. We demonstrate that the long cytoplasmic CD2 can transduce strong costimulatory signals and is the most potent mediator of NFAT activation. NFAT plays a central role in T cell activation, and thus CD2-derived motifs might have utility in generating improved CAR signaling domains.

Limitation of the study

While our study provides insight on the capability of intracellular domains derived from different costimulatory receptors to mediate T cell activation, it has some limitations. Since different fluorescent proteins are used to read out the activity of NF-kB, NFAT, and AP-1, the results could be affected by the stability of the reporter genes. Furthermore, the human Jurkat T cell line was used, and, although this cell line has been instrumental for studying T cell signaling,[48] key findings of this study should be confirmed using primary human T cells. The Jurkat T cell line is a CD4 T cell line, but, since we have previously observed that the effects of different costimulatory signals on the proliferation of CD4 and CD8 T cells were similar, we expect that our results are relevant for both T cell subsets.[49] Nevertheless, it will be especially important to verify the differential effects of costimulatory pathways observed in our model in primary human CD8 T cells. By introducing chimeric receptors harboring cytoplasmic sequences into primary T cells, the contribution of individual costimulatory signaling domains to T cell activation processes could be studied without interference from varying expression levels and affinities that hamper the comparison of signaling pathways when using costimulatory receptors naturally expressed on T cells.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109267.

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AUTHOR CONTRIBUTIONS

A.D.S.L. performed experiments and wrote the manuscript. S.S. performed bioinformatic analysis. J.L. performed experiments, supervised the study, and wrote the manuscript. P.S. supervised the study and wrote the manuscript. All authors critically revised the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
h/mICOS-APC	Biolegend	Cat#313510; RRID: AB_416334
biotinylated Strep-tag II (NWSHPQFEK Tag ab biotin)	GenScript	Cat#A01737
mICOS-L-PE (HK5.3)	Biolegend	Cat#107405; RRID: AB_2248797
CD19-APC (HIB19)	Biolegend	Cat#302212; RRID: AB_314242
mCD45.2-APC (104)	Biolegend	Cat#109814; RRID: AB_389211
DyLight-649-labeled goat-anti-mouse IgG (H+L)	Jackson ImmunoResearch	Cat#115-475-003; RRID: AB_2338786
Chemicals, peptides, and recombinant proteins		
Streptavidin-PE	BD Pharmingen	Cat#554061; RRID: AB_10053328
Critical commercial assays		
RNeasy	Qiagen	Cat#74004
Qubit dsDNA HS Assay	Invitrogen/Thermo Fisher	Cat#Q32851
Deposited data		
Sequencing data have been submitted to the NCBI Sequence Read Archive	BioProject	PRJNA991135
Experimental models: Cell lines		
Jurkat cell line (JE6.1)	in house stock	
BW5147	in house stock	
Jurkat TPR	Jutz et al.[18]	
Recombinant DNA		
pCJK2 retroviral expression vector	Leitner et al.[11]	N/A
pHR-SIN-BX-IRES-Emerald (lentiviral expression vector)	Paster et al.[50]	N/A
Software and algorithms		
FlowJo software version 10.4.1	Tree Star	
Graphpad Prism software version 9	GraphPad Software, Inc	
Illumina bcl2fastq command line tool (v2.19.1.403)		
STAR aligner version 2.6.1a	Dobin et al.[51]	

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources, reagents and source data should be directed to and will be fulfilled by the lead contact Judith Leitner (Judith.a.leitner@meduniwien.ac.at) and Peter Steinberger (peter.steinberger@meduniwien.ac.at).

Material availability

Plasmids used for generation of the reporter lines are available from Addgene or by the lead contact upon request. Plasmids for chimeric receptors are provided by the lead contact upon request.

There are restrictions to the availability of the Jurkat-reporter and Stimulator cell lines due to a material transfer agreement (MTA).

Data and code availability

- Any additional information required to reanalyze the data reported in this study is available from lead contact upon request.
- No original code was generated in this study.
- Sequencing data have been submitted to the NCBI Sequence Read Archive and are available under the BioProject accession number PRJNA991135.

EXPERIMENTAL MODEL AND STUDY PARTICIPANTS

Cell lines

The Jurkat cell line (JE6.1) and the mouse thymoma cell line BW5147 (short designation: BW) were derived from in house stocks. The Triple parameter T cell reporter line (short: TPR) are based on the JE6.1 cells and have been described previously.[18] For TPR stimulation, T cell stimulator cells (short designation: TCS) were used. The TCS are based on the BW cell line and express a membrane-bound human CD3 antibody single chain fragment (short: mb aCD3).[11]

METHOD DETAILS

Cell culture, antibodies and flow cytometry

In this study cell lines were cultured in RPMI1640 at 37°C and 5% CO_2. Staining of the cell lines with a panel of antibodies provided authentication. Mycoplasma testing was performed for all cell lines using an THP-1 reporter assay developed in our lab.[52] For surface expression of mICOS constructs a h/mICOS-APC (C398.4A, Biolegend, San Diego, CA) antibody was used. αCD19 construct expression was validated with a biotinylated Strep-tag II mAb (GenScript, NJ) followed by Streptavidin-PE staining (BD Pharmingen, San Diego, CA). Membrane bound αCD3 expression on TCS was detected with a DyLight-649-labeled goat-anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch, West Grove, PA). mICOS-L and CD19 expression were verified using mICOS-L-PE (HK5.3) and CD19-APC (HIB19) from Biolegend. For TCS exclusion in reporter assays a mCD45.2-APC (104) from Biolegend was used. LSRFortessa™ or FACSCalibur™ (BD Bioscience, Franklin Lakes, NJ) flow cytometers were used for analysis, followed by a data analysis on the FlowJo software (Tree Star, Ashland, OR).

Retroviral and lentiviral transduction

Mouse ICOS-L (UniProt: Q9JHJ8-1, short: mICOSL) and CD19 (UniProt: P15391) molecules were cloned into the pCJK2 retroviral expression vector[11] and expressed in TCS via retroviral transduction. Single cell clones with high expression of the molecules were established. Mouse ICOS (short: mICOS) and αCD19 constructs were cloned into the lentiviral vector pHR-SIN-BX-IRES-Emerald,[50] which contains the puromycin N-acetyl transferase. High expressing TPR were obtained via puromycin selection (2 μg/ml, Sigma Aldrich) after transduction.

Generation of chimeric constructs

mICOS chimeric constructs contain the extracellular part of mICOS (UniProt: Q9WVS0-1; aa 1-144). For the extracellular part of the αCD19 constructs a CD19 CAR construct, that was previously described,[53] was modified. The extracellular domain contains a GM-CSF signal sequence followed by a CD19 scFv (single chain variable fragment), a StrepTag II and a human CD8 hinge sequence. Both, mICOS and αCD19 constructs, harbor a codon optimized CD28 transmembrane domain. The intracellular parts vary and were generated as followed: CD28 (UniProt: P10747; aa 180-220), PD-1 (UniProt: Q15116; aa 192-288), 4-1BB (UniProt: Q07011; aa 214-255), CD27 (UniProt: P26842; aa 213-260), CD2 (UniProt: P06729; aa 236-351), CD28_FMNM (Y191F), CD28_YMAM (N193A), CD28_YMNL (M194L), CD28_ARRA (P196A; P199A), CD28_AYAA (P208A; P211A), CD28_PFAP (Y209F), $CD28_{(1-22)}$ (aa 180-201), $CD28_{(1-22)}$_FMNM (aa 180-201; Y191F), PD-1_Y32F (Y223F), PD-1_Y47F (Y248F), PD-1_Y32F/ Y47F (Y223F; Y248F), $PD\text{-}1_{(1-17)}$ (aa 192-208), $PD\text{-}1_{(1-62)}$ (aa 192-253), $PD\text{-}1_{(1-17)}$_$CD28_{(23-41)}$ (aa 192-208_aa 202-220), $PD\text{-}1_{(1-5)}$_$CD28_{(23-41)}$ (aa 192-196_aa 202-220), $CD28_{(23-41)}$ (aa 202-220), $PD\text{-}1_{(1-5)}$_2x$CD28_{(23-41)}$ (aa 192-196_(aa 202-220)x2). Additionally, as a control, mICOS and αCD19 constructs lacking a cytoplasmic domain were generated (Δcyt). Further, constructs where the mICOS dimerization domain was mutated (C136A and C137A; designation within this work mICOSmut) and fused to the CD28 transmembrane domain and the cytoplasmic part of CD28, CD2, CD27 or 4-1BB were gene synthesized at TWIST (TWIST Bioscience, CA, USA).

Reporter assays and RNA isolation

Reporter cells ($5x10^4$ cells/well) were co-cultured with TCS as indicated ($2x10^4$ cells/well) in a 96-well plate (total volume 100 μl). After 24 hours, cells were harvested and, for TCS exclusion, stained with a mCD45.2-APC antibody. For time course experiments co-culture was done for 4h, 9h and 48h. Using a LSRFortessa™ flow cytometer, expression of reporter genes (eGFP, eCFP, mCherry) was measured. For further analysis APC negative cells (viable reporter cells) were used and displayed using the geometric mean of fluorescence intensity (gMFI). If appropriate, reporter gene expression was normalized to unstimulated or control stimulated cells and then indicated and expressed as fold induction. For RNA sequencing experiments, RNA was isolated, following 24h of reporter cell – TCS co-culture, using RNeasy according to the manufacturers' instructions (Qiagen, Germany).

RNA sequencing, data analysis and data availability

Sequencing libraries from total RNA were prepared at the Core Facility Genomics, Medical University of Vienna, using the QuantSeq FWD protocol (Lexogen). 15 PCR cycles were used for library prep, as determined by qPCR according to the library prep manual. Libraries were QC-checked on a Bioanalyzer 2100 (Agilent) using a High Sensitivity DNA Kit for correct insert size and quantified using Qubit dsDNA HS Assay (Invitrogen). Pooled libraries were sequenced on a NextSeq500 instrument (Illumina) in 1x75bp single-end sequencing mode. On average, 8 million reads per sample were generated. Reads in fastq format were generated using the Illumina bcl2fastq command line tool (v2.19.1.403). Reads were trimmed and filtered using cutadapt version 2.8 to trim polyA tails, remove reads with N's and trim bases with a quality of less than 30 from the 3' ends of the reads. On average, 5 million reads were left after this procedure. Trimmed reads in fastq format were aligned to the

human reference genome version GRCh38 with Gencode 29 annotations using STAR aligner version 2.6.1a in 2- pass mode.[51] Raw reads per gene were counted by STAR. Raw reads were processed as follows: Genes with less than 1 count-per-million reads (cpm) in half the samples were removed using the cpm function in the edgeR library. Normalised read counts were generated using the Voom function in limma package. A linear model was applied to the normalised data to identify the differentially expressed genes. Genes with false discovery rate (FDR) corrected p value < 0.05 and absolute fold change of > 1 were considered as differentially expressed genes. ggplot2 in R was used for data visualization. Pathway map enrichments were performed using the enrichR package and the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. Sequencing data have been submitted to the NCBI Sequence Read Archive and are available under the BioProject accession number PRJNA991135 (NCBI Sequence Read Archive: PRJNA991135).

QUANTIFICATION AND STATISTICAL ANALYSIS

For proof of principle experiments in Figure 1, unpaired two-tailed t-test was performed. For comparison of reporter cells receiving signal 1 and 2 from one cell with reporter cells receiving signal 1 and 2 from separate cells (Figure 5), a two-way ANOVA followed by Sidak's multiple comparison test was used. Every other statistical analysis in this work was performed using one-way ANOVA followed by Tukey's a multiple comparison test. Additional statistical analyses are summarized in Table S1. Statistical analysis was calculated using Graphpad Prism software (La Jolla, CA). Levels of significance: $p > 0.05$ ns, not significant; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.