Published in final edited form as:

Sci Signal. 2018 May 22; 11(531): . doi:10.1126/scisignal.aan1088.

Tuning ITAM multiplicity on T-cell receptors can control potency and selectivity to ligand density

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Abstract

The T-cell antigen receptor (TCR) recognizes peptides from pathogenic proteins bound in the MHC molecule. To convert this binding event into downstream signaling, the TCR has phosphorylatable intracellular motifs (ITAMs) that act as docking sites for ZAP70, a cytoplasmic tyrosine kinase. Uniquely, the TCR employs 10 ITAMs to transduce pMHC binding to the cell interior. Why this multivalency is required at the mechanistic level remains unclear. Using synthetic, drug-inducible receptor/ligand pairs, we find that greater ITAM multiplicity primarily enhances the efficacy of these engineered receptors, by increasing the efficiency with which ligand binding is converted into an intracellular signal. This manifests as an increase in the fraction of cells that become activated, rather than directly amplifying the intracellular signal and a more synchronous initiation of proximal signaling. Exploiting these findings, we show that the potency and selectivity of chimeric antigen receptors targeted against cancer can be substantially enhanced by modulating the number of encoded ITAMs.

Introduction

T cells are an essential cell-type of our adaptive immune system that are capable of distinguishing between healthy, viable cells and those that are infected by pathogens such as bacteria or viruses. To facilitate the T-cell antigen receptor (TCR) being able to interrogate the intracellular state of potentially abnormal cells, there is a continuous process of peptides derived from both host and pathogen proteins being presented at the cell surface, bound within the MHC protein (pMHC). This allows T cells to efficiently scan host cells for 'foreign' peptides and respond accordingly, by either directly killing the cell, or licensing other cells to do so.

On productive ligand binding, TCR triggering (1) begins with the LCK-mediated tyrosine phosphorylation of signal motifs on the intracellular tails of the TCR, known as ITAMs. These motifs then act as docking sites for ZAP70, an intracellular tyrosine kinase, so it can be recruited to the TCR. Provided that the TCR remains bound by ligand, ZAP70 becomes activated and continues to phosphorylate proteins such as LAT, which is a signaling scaffold that nucleates many canonical downstream pathways.

The TCR is constructed from eight protein chains: the TCR $\alpha\beta$ heterodimer is responsible for ligand binding while the CD3 $\gamma\epsilon$, CD3 $\delta\epsilon$ and CD3 $\zeta\zeta$ dimers are required for intracellular signaling. CD3 ζ comprises 3 ITAMs whereas the remaining CD3 chains have one ITAM each, giving a combined total of 10 ITAMs. A long-standing question in T-cell biology is why the TCR has so many of these binding sites, when almost all other immune receptors function effectively with no more than two (2)?

Previous studies on answering this question have found that decreased ITAM multiplicity has a significant effect in T-cell development, where fewer ITAMs leads to diminished positive selection and impaired thymocyte lineage commitment (2). A similar approach looking at the effect of ITAMs number on peripheral T-cell responses suggested that signaling 'scaled' linearly with ITAM count, but this only held true for activation-induced cell proliferation; cytokine production was almost invariant to changes in ITAM number (3, 4). For all these *in vivo* studies, there was very likely significant adaptation of the signaling network in the mouse during thymocyte development (5), making it difficult to directly isolate the effect of ITAM multiplicity on T-cell signaling *per se*. For example, the absence of CD3 ζ ITAMs does not cause thymic positive selection to fail (4, 6), but rather significantly skews the TCR sequences that undergo selection, suggesting that the genetic perturbation can be well tolerated by thymocyte development, but nonetheless distorts the peripheral T-cell population (7). This perhaps explains why no consensus has been reached from these types of experiments on the mechanistic explanation for high TCR ITAM multiplicity. A more recent biophysical study has suggested that increased ITAM number has an effect on TCR potency through an entropic mechanism (8), but only looked at phosphorylation of the TCR itself and not any downstream signaling output to show if this effect was propagated through the reaction network.

More generally, in previous experiments only the average signaling response of the population has been correlated with the number of TCR ITAMs, making it difficult to elucidate whether modulating number of ITAMs affects the signaling output level homogeneously in all triggered cells, or the proportion of cells that respond. This information is essential to understand the causative mechanism for ITAM multiplicity in the TCR.

Because of these points, we wanted to directly address the mechanistic effect of receptor ITAM multiplicity without perturbing the underlying signaling network, and to measure the output response at the single-cell level to identify how ITAM modulation was manifested in downstream T-cell activation. We find that increasing the number of ITAMs robustly enhances the efficacy, or probability of signal transduction by synthetic T-cell receptors, and that this primarily drives an increase in the fraction of activated cells, rather than simply amplifying the downstream response. We then show that these conclusions can be exploited to improve the efficiency of a new class of therapeutics targeted against cancer antigens.

Results

A synthetic, drug-inducible T-cell receptor system

To help elucidate the mechanistic requirement for high receptor ITAM multiplicity on T-cell signaling in a quantitative manner, we needed a means to vary the number of ITAMs in isolation from changes to receptor expression or affinity, and without the overlaid and potentially confounding effects of network adaptation. We therefore designed synthetic receptors that could be expressed in T cells in the presence of the endogenous TCR complex, so that basal signaling in the underlying network would not be disrupted (Fig. 1A). These new receptors utilize an extracellular ligand-binding domain based on the FKBP protein that provides an entirely orthogonal, or independent, T cell input. To create synthetic receptors expressing 0 – 3 ITAMs, we used the extracellular protein domains of CD86, a known monomer (9), as a scaffold to link the FKBP domain to the intracellular signaling region of the CD3 ζ chain (Fig. 1A). CD3 ζ normally encodes 3 ITAMs; to decrease this number, point mutations within the ITAMs of the CD3 ζ chain were used to create equivalent receptors with 0, 1 or 2 functional ITAMs. To construct a synthetic receptor that expressed the full complement of 10 ITAMs, we repurposed the endogenously-expressed complete TCR complex by introducing a modified version of the TCR α chain that encoded the FKBP domain extracellularly into T cells (Fig. 1A). Importantly, the binding affinity of these different synthetic receptors is invariant to ITAM number and equivalent surface expression of the receptors could be quantified and controlled for with flow cytometry through an extracellular HA-epitope present on all constructs.

A key feature of the FKBP domain is that its interaction with its binding partner, FRB (10), is entirely dependent on the presence of a small-molecule (11). Normally this is the drug rapamycin, which can interfere with T-cell activation (12). However, expressing FRB with the T2098L mutation permits use of a rapamycin analog (a ‘rapalog’, known as AP21967 or A/C heterodimerizer) that has negligible binding to the equivalent domain of mTOR endogenously found in T cells. We used Raji B cells that expressed extracellular FRB as the ligand-presenting cell, where engagement between these cells and the synthetic receptor-expressing T cells in the presence of the rapalog can drive receptor triggering and subsequent downstream T-cell activation (Fig. 1B). An important advantage of this drug-inducible receptor system is that it can provide fine temporal control over the initiation of signaling within the physiological context of apposing cell membranes, something that has previously not been possible with other methods for receptor activation. We have also previously shown that a receptor based on FKBP can efficiently recapitulate TCR-mediated triggering (13).

ITAM number enhances receptor potency by increasing its efficacy

We hypothesized that there could be two non-exclusive means by which increased ITAM multiplicity could affect T-cell output. This first is that the primary function of multiple ITAMs is to ‘amplify’ the T-cell input. This would predict that more ITAMs increase the absolute level of a functional readout of activated cells, but would have little effect on the fraction of cells that responded (fig. S1A). An alternative explanation is that having more ITAMs on a receptor enhances its potency, increasing the fraction of bound receptors that are

capable of transducing a signal into the cell. This would not increase the absolute level of a cellular output but rather the number of cells that become activated (fig. S1B). Crucially, it would be very difficult to distinguish these mechanistic explanations for ITAM multiplicity when only the average output response of the population is measured (fig. S1B).

To provide the data required to distinguish between these two models and understand the functional effect of ITAM multiplicity, we utilized the synthetic receptors described above to measure downstream T-cell signaling at single-cell resolution with defined levels of signaling input. Jurkat T cells expressing the synthetic receptors with 0 – 3 or 10 ITAMs were conjugated with Raji B cells expressing the cognate FRB domain in the presence of the dimerizing rapalog drug. We used two different concentrations of the rapalog to explore the effect of ITAM multiplicity at different cell signaling input ‘strengths’. After stimulation of the cells for 16 hours, we measured *de novo* gene expression on activation mediated by the NFAT transcription factor, in a Jurkat T-cell clone that expresses the fluorophore GFP under the control of NFAT-responsive elements. We could therefore measure GFP intensity as a readout of downstream signaling output at the single-cell level (Fig. 1, C and D), and used histogram unmixing to recover the distribution of activated cells from the GFP output histograms (fig. S1C). We found that the number of ITAMs had a substantial impact on the fraction of cells that responded to stimulation (Fig. 1E) but did not greatly affect the overall magnitude of the output response, especially when more than one ITAM was present (Fig. 1F). This held true at both ‘low’ (Fig. 1C) and ‘high’ (Fig. 1D) levels of receptor input mediated by the different rapalog concentrations. We also measured the effect of ITAM multiplicity on IL-2 cytokine secretion using an equivalent assay and found that IL-2 production correlated well with the fraction of activated cells (fig. S2).

As an alternative downstream functional output, we measured activation-induced CD69 expression, which is driven by the AP-1 transcription factor (14). We observed the same effects of ITAM multiplicity, with a substantial increase in the fraction of activated cells with essentially no amplification of the absolute levels of CD69 (fig. S1D-G). A recent study has suggested that T cells show increased upregulation of CD69 when presented with increasing ligand density (15), something we also observed (fig. S1G), suggesting CD69 is not an entirely digital response. This data also showed that the conjugation efficiency with the B cells was sufficient to activate essentially the entire population of T cells, implying that the signaling threshold for CD69 upregulation was lower than that for the NFAT-GFP reporter where complete activation was not always observed.

By pooling T cells that had been transduced with synthetic receptors driven by promoters of different efficiencies, we could express a wide range of the receptors at the cell surface within a single experiment. This allowed us to quantitatively determine the relationship between the cellular input and output to the signaling network whilst varying the number of ITAMs (Fig. 1G and fig. S3). For the subsequent analysis, the absolute levels of receptor expression were quantified using beads with known amounts of fluorophores to calibrate the flow cytometer prior to analyzing the samples. Receptor expression at the cell surface was used as an independent variable to modulate the signal ‘strength’ for each ITAM variant so that we could directly compare NFAT-mediated GFP expression at equivalent input levels. We plotted the fraction of responding cells as a function of receptor expression at two

different rapalog concentrations. For both levels of total input signal controlled by rapalog concentration, it was evident that increasing ITAM multiplicity decreased the number of receptors required to cause an equivalent fraction of activated cells (Fig. 1, H and I). By fitting these datasets, we could quantify this effect on receptor potency directly by calculating the EC_{50} , the cell input required for a half-maximal response (Fig. 1, J and K).

The potency of a signaling response is defined as the combination of the affinity and efficacy of ligand binding to a receptor. Given that all the synthetic receptors bear an identical ligand-binding domain, and so have equivalent affinity, the increased potency with more ITAMs must be the result of increased efficacy. This suggested that the primary effect of the increased number of ITAMs is to improve the likelihood, or efficiency, of productive signal transduction on ligand binding and was not found to substantially amplify the input signal *per se*. It was nonetheless possible for a decreased number of ITAMs to elicit the same response as the complete TCR construct but this required a greater number of receptors to be engaged to drive an equivalent response. This showed that low potency could be overcome by increasing the total ligand input to the cell, at least for the outputs we measured.

Increased receptor ITAM number synchronizes initiation of T-cell signaling

The previous data demonstrated that the number of ITAMs within the receptor affected the efficiency with which the receptor transduced ligand binding to the proximal signaling network. If this were the case, then it should have a significant effect on the rate at which signaling was initiated at the receptor, with a higher number of ITAMs predicated to drive more efficient, or synchronous signaling. We thought that this effect should be evident in the kinetics of the early events of receptor signaling in the T cells. To test this hypothesis, we used one of the earliest readouts of receptor stimulation in T-cell activation, which is the increased concentration of intracellular Ca^{2+} ions. Importantly, this signaling flux can be measured at single-cell resolution by flow cytometry using the Indo-1 ratiometric Ca^{2+} indicator (16).

Jurkat T cells expressing equivalent levels of the synthetic receptors (fig. S4A) were first conjugated with FRB-expressing B cells in the absence of the rapalog drug. These cell conjugates could then be gated on by flow cytometry, a process that was independent of the particular receptor expressed (fig. S4B). The Indo-1 ratio in these conjugates, which reports the intracellular Ca^{2+} concentration, was measured for an initial period to define a baseline ratio before the rapalog drug was added, which synchronously initiated receptor signaling (Fig. 2A). From this data, we quantified the temporal evolution of the activated fraction of cells (Fig. 2, B and C) and the mean Indo-1 ratio of the activated population (Fig. 2, D and E). The clear result was that the number of ITAMs present on the receptor had a potent effect on the rate at which T cells responded to stimulation, at both the 'low' (Fig. 2, B and D) and 'high' (Fig. 2, C and E) rapalog concentrations, with a strong correlation between the increased ITAMs per receptor and the synchrony of the activation response. This can be better illustrated by taking the differential of these plots to derive the rate of activation, showing that increased ITAMs leads to a sharpened or more 'normal' temporal distribution of receptor triggering (Fig. 2, B to E). As for the previous experiments, this effect can be

solely ascribed to the number of ITAMs present on the receptor, as both receptor expression and ligand affinity was consistent between all experiments.

We could not collect the Ca^{2+} flux data beyond ~8 min due to technical limitations. This precluded integrating over the time-varying data to extract the overall output response to know whether the increase in the instantaneous Indo-1 ratio with greater ITAM multiplicity (Fig. 2, D and E) was simply the result of the more synchronized response. A model dataset where an invariant individual cellular response is combined with varying temporal distributions for activation could replicate the datasets well (fig. S4C), suggesting that the absolute Ca^{2+} flux of individual cells could be constant despite altered ITAM multiplicity. Nonetheless, we cannot exclude the possibility that for the Ca^{2+} flux during T-cell activation, there is some ITAM-mediated amplification of receptor signaling, perhaps driven by the positive feedback loop of Ca^{2+} -induced Ca^{2+} entry into the cell through CRAC channels.

Effect of ITAM multiplicity on signaling through the MAPK pathway

The previous experiments demonstrated that increased ITAM multiplicity on the synthetic receptors caused an increased fraction of cells to be activated, with signaling initiated in a more synchronous fashion. We next wanted to investigate whether these responses could be observed as receptor activation was propagated through the intracellular signaling networks. To measure this, we used phosphorylation of ERK (a MAP kinase) as an output for intracellular T-cell signaling that is distinct from both the Ca^{2+} flux and NFAT signaling pathways.

We followed a similar strategy as for the Ca^{2+} flux experiments, where synthetic receptor-expressing T cells were conjugated with FRB-presenting B cells in the absence of the rapalog drug. Subsequent addition of the rapalog initiated synchronous activation of receptor signaling that is expected to drive ERK phosphorylation, a functional output that can be measured at the single-cell level using intracellular cytometry staining. We found that after 5 min of signaling, ERK phosphorylation was readily detectable for both rapalog concentrations used previously (Fig. 3, A and B). Through an equivalent analysis as used for the NFAT-mediated signaling output (fig. S1C), we could again show that the effect of higher receptor ITAM multiplicity was to increase the fraction of activated cells (Fig. 3C), with almost no effect on the mean phosphorylation of ERK (Fig. 3D). This result agrees with a previous study that found ERK phosphorylation shows a 'digital' response to graded T-cell activation (17).

Because of the rapid kinetics of ERK phosphorylation, we wanted to know whether we could also observe the more synchronous signaling observed in the Ca^{2+} flux data when more ITAMs were encoded by the receptor. We therefore measured ERK phosphorylation at earlier time points to follow its kinetics. In agreement with our previous results, we found that greater ITAM multiplicity drove a more rapid response, which was evident especially at 2 min after rapalog addition (Fig. 3E and fig. S5) and the mean ERK phosphorylation became essentially equivalent once the dynamics reached equilibrium after ~3 min (Fig. 3F).

Overall, our data measuring ERK phosphorylation confirmed our previous findings on the effect of ITAM multiplicity on both the increased synchrony of signaling and the fraction of cells that become activated.

ZAP70 kinase activation primarily depends on autophosphorylation

The preceding experiments showed that the number of ITAMs present on the receptor was predominantly affecting the efficiency of transducing the ligand binding event into a downstream signal. What enhancement could high ITAM multiplicity then have on receptor potency, if not through downstream signal amplification? One explanation could be that multiple binding sites within a single antigen receptor are required to bring multiple ZAP70 kinases into close physical proximity. This could be important for ZAP70 because full activation of its kinase activity is known to require phosphorylation of Y493 in its kinase domain activation loop (18). For this explanation to be correct, the effect of high ITAM multiplicity is predicated on the requirement for ZAP70 activation to occur through trans-autophosphorylation (Fig. 4A). However, this question remains unresolved, and there is limited experimental evidence for an autophosphorylation mechanism for ZAP70 activation (19, 20). Indeed, a recent study predicted that the ZAP70 activation loop sequence should be a poor substrate for its own kinase domain (21) and suggested that ZAP70 is primarily activated by LCK (Fig. 4A).

We wanted to provide direct evidence as to whether the ZAP70 autophosphorylation was indeed important for its own activation, which would speak to the advantage of high ITAM multiplicity on the TCR. To do this, we used a previously characterized reconstituted cellular system, which relies on expressing the minimal set of proteins (TCR, LCK, ZAP70, CD45, CSK/CBP and LAT) required to recapitulate proximal TCR signaling events in HEK-293T, a non-immune cell-type (13). This approach allows us to quantitatively investigate the proximal events of T-cell activation within a cellular system that expresses essentially none of the T-cell proteins that could potentially confound the interpretation of our results (13). We therefore measured the kinetics of proximal signaling through the reconstituted protein network, using phosphatase inhibition to synchronously initiate signaling (Fig. 4B and fig. S6A) and quantified the intensity of ZAP70^{Y493} phosphorylation over time by fluorescent phospho-western analysis.

By co-expressing the wildtype forms of both LCK and ZAP70, we could readily observe phosphorylation of ZAP70^{Y493} increasing with time (Fig. 4C). We first confirmed that this measured phosphorylation was solely due to the expressed components, and not any endogenous kinases also present in the reconstituted cells by using a catalytically-inactive mutant of LCK (LCK^{K273R}). This caused ZAP70 phosphorylation to become undetectable (Fig. 4C), confirming the stringent dependence of LCK in the initiation of TCR signaling in the reconstituted system (13). However, repeating this assay with a kinase-dead version of ZAP70 (ZAP70^{K369R}) also caused a very substantial reduction in ZAP70^{Y493} phosphorylation (Fig. 4C). We believe this provides direct evidence that ZAP70 activation has a strong dependence on its own kinase activity, with the activation mechanism predominantly relies on ZAP70 trans-autophosphorylation (22). This requirement was not absolute though, as there was still some Y493 phosphorylation mediated by LCK when

ZAP70 was catalytically inactive (Fig. 4C). This is perhaps an expected requirement to 'prime' the bound ZAP70 so that it can initiate the autophosphorylation process. We have recently demonstrated the requirement for auto-phosphorylation in ZAP70 activation using a complementary assay (23).

Both the NFAT-driven reporter (Fig. 1) and Ca^{2+} flux experiments (Fig. 2) depend on the upstream enzymatic activity of the phospholipase $\text{PLC}\gamma 1$ (24). This enzyme is recruited to the plasma membrane by binding to LAT^{Y132} when it becomes phosphorylated during proximal TCR signaling (25). To confirm that this phosphorylation was dependent on ZAP70 kinase activity, and so would be influenced by ITAM multiplicity, we measured the kinetics of LAT^{Y132} phosphorylation. We found that ZAP70 was indeed the kinase responsible for this modification (Fig. 4D), with LCK showing undetectable phosphorylation of this site over the time period studied (Fig. 4D), as has also been shown recently (21).

To see whether the requirement for ZAP70 autophosphorylation could also explain the kinetics of our Ca^{2+} flux (Fig. 2) and phospho-ERK data (Fig. 3), we created network models that invoked the known proximal steps of TCR triggering using BioNetGen (26) that could be scaled with receptor ITAM density, and used LAT phosphorylation as a 'readout' of receptor activation (fig. S6, B and C). Using a model that relied on LCK-mediated ZAP70 activation could not readily replicate our experimental data (fig. S6B) but one that relied on ZAP70 autophosphorylation within a single receptor complex could (fig. S6C), which was most evident in the substantial increase in cell output from one to two ITAMs.

Improving CAR activation and discrimination

Chimeric antigen receptors (CARs) are a new class of cancer therapy that use genetically engineered T cells to attack malignant cells. These constructs splice the high affinity binding of antibodies onto the ITAMs of the TCR, including costimulatory motifs to maintain their expression in the host (fig. S7A). While much work has gone into improving the latter sequences, there has been very limited work on optimizing the signaling potency mediated by the ITAMs (27). Current CARs employ the 3 ITAMs from the $\text{CD3}\zeta$ chain of the TCR, but we hypothesized that the efficiency of CAR activation could be improved by simply increasing the ITAM density of the construct. To do this, we duplicated the $\text{CD3}\zeta$ sequence to create a 6 ITAM CAR variant (fig. S7A). We also made equivalent versions that had 0-2 ITAMs as well, to quantify the relationship between the number of ITAMs and the CAR-mediated response.

As a proof-of-principle, we used a CAR that is reactive to CD19, a B-cell specific surface protein that has been previously used to target CAR-expressing T cells against lymphoblastic leukemias (28, 29). We transduced human primary CD4^+ T cells (fig. S7B) to express $\alpha\text{-CD19}$ CAR variants with 0-6 ITAMs. As surrogate target cells, we used K562 myeloma cells that were transduced to express different levels of the CD19 antigen, spanning over the normal range of CD19 expression (fig. S7C). CAR-T cells were conjugated with target cells for 24 hours to drive CD19-induced T-cell activation that was quantified by increased expression of CD137 (fig. S7D), a robust marker of activation that correlates well with cell proliferation (30).

As expected, the ‘standard’ CAR construct harboring 3 ITAMs caused activation of transduced T cells when mixed with target cells presenting all three antigen densities (Fig. 5A). However, increasing the number of ITAMs from 3 to 6 increased the efficiency of T-cell activation by ~15% for all target cells (Fig. 5, A and D). By using the same analysis as the for the synthetic receptors, we again found that the effect of increased ITAM number primarily caused an increase in the fraction of activated cells (Fig. 5B) and not the absolute level of the cell output (Fig. 5C). This effect held true for all three levels of CD19 expression on the target cells (Fig. 5, B and C), although activation was clearly enhanced with higher target density suggesting CAR-mediated signaling was not saturated. Similar results were found using CD69 as the readout for T-cell activation, although it appeared the activation period allowed for subsequent down-regulation of CD69, clearly evident in the ‘high’ target-cell data, which slightly confounded the analysis (fig. S7E-G).

An unanticipated result from this dataset was that by decreasing the number of ITAMs in the receptor construct from 3 to 2 ITAMs, the CAR-T cells were now substantially more selective for target cells over-expressing the CD19 target antigen (Fig. 5D), something that CARs are not currently optimized for. This agreed with the synthetic receptors experiments described above, which showed that equivalent T-cell activation with fewer ITAMs can only be achieved with more engaged receptors, which would be the case for the target cells over-expressing CD19. To expand on this finding, we reasoned that manipulating the available target density on the CD19-expressing target cells could be an alternative means to make the CAR-T cells respond more selectively to K562 over-expressing the target protein, so sparing target cells expressing more-physiological CD19 levels. To achieve this, we repeated the experiment using the original CAR with 3 ITAMs but now included the full-length antibody that the α -CD19 CAR is constructed from during T-cell activation, to sterically ‘block’ most of the available target sites. We hypothesized that this would artificially shift the effective target densities to a regime where the CAR could be more selective towards target cells over-expressing CD19. This was indeed what we found (Fig. 5E); antibody addition ‘protected’ the target cells expressing physiological levels of CD19 while allowing the over-expressing cells to still be targeted (Fig. 5F).

Discussion

We have developed a synthetic receptor system to quantitatively investigate how the number of ITAMs encoded by a cell surface receptor modulates its ability to drive intracellular signaling in T cells. We found that greater receptor ITAM multiplicity enhanced the potency of the receptor, by increasing the efficacy, or transduction efficiency of ligand binding being converted into an intracellular signal. This effect manifested as an increase in the fraction of T cells that became activated with a greater number of ITAMs with a more synchronous initiation of downstream signaling, and was consistent over five distinct downstream signaling outputs, providing strong support for our conclusions. Through reconstitution experiments, we showed that there is a substantial requirement for autophosphorylation to drive ZAP70 kinase activity, which could provide a mechanistic explanation for the increased efficacy observed with high ITAM multiplicity. Unfortunately, it was not technically possible to investigate ZAP70 activation at the single-cell level to directly confirm this hypothesis.

We then tested the idea that the number of ITAMs increased receptor potency by demonstrating that we could improve the therapeutic potential of CARs. We found that increased ITAM count on an α -CD19 CAR led to improved activation of primary human T cells, a result that should be applicable to all current CARs being trialed and the effect is likely to be significantly higher when the target antigen is expressed at low levels. Conversely, decreasing the number of ITAMs, or using different ITAM sequences with weaker binding affinities (31) could be an interesting new avenue to provide some selectivity to target expression levels.

These findings contrast with the prevailing view that ITAM multiplicity is principally for intracellular signal amplification, which should lead to greater absolute levels of signaling output. We believe our experiments at single-cell resolution demonstrate that this is not the primary effect of high number of ITAMs. Instead, we find that greater ITAM multiplicity leads to a decrease in the number of triggered receptors required to cause a half-maximal output response. Equivalent levels of signaling were possible with fewer ITAMs but this required more receptors to be engaged. By only measuring the mean response of a population of T cells, as essentially all other experiments on ITAM number have been done, it would not be possible to separate these two explanations. Our results suggest that ITAM multiplicity of the receptor influences the conversion of ligand binding into an intracellular signal. Although it could be argued that simply having more bound ZAP70 is amplification *per se*, we believe this is not an appropriate use of the term because of the change in input modality, with amplification normally only defined as an increase in a signal amplitude.

A key advantage of our experiments using the synthetic receptor is that a gain-of-function approach to investigate ITAM multiplicity obviates the need to directly disrupt the underlying signaling network, which can often lead to unintended consequences such as systemic adaptation. There are of course caveats to our approach too. By expressing the synthetic receptors in an endogenous T-cell line we are inherently increasing the total density of ITAMs at the cell surface, which could alter basal signaling. We were therefore careful not to over-express the receptor, which increased the total ITAM density by no more than ~10% at the highest receptor expression used. The synthetic receptor also relied on the FKBP/FRB interaction that has a higher affinity than the normal TCR/pMHC equivalent, although the kinetics of the FKBP unbinding under tension have never been measured and so may not be so different to that for pMHC dissociation. The presence of the endogenous TCR in our synthetic receptor-expressing T cells could potentially lead to signal augmentation by co-opting these receptors. However, we saw no evidence for wildtype TCR downregulation in our assays even with potent stimulation, suggesting that only the engaged receptors were providing signal input to the cells.

While the synthetic receptors used in this study may not replicate all the features of the endogenous TCR, we believe the work presented here can explain the requirement for 10 ITAMs being present within the TCR complex. Why does the TCR have this requirement? The unique aspect of TCR-mediated signaling is that it must function at very high sensitivity, with potentially only a few ligands being sufficient to drive cell activation (32, 33). To achieve this, the TCR must be able to convert cognate pMHC binding events as effectively as possible into a triggered receptor; this efficacy in signal transmission across

the plasma membrane is then derived from the high ITAM multiplicity, something not required for most other immune receptors. Furthermore, having the ITAMs distributed over multiple chains, rather than encoded by a single large sequence might increase the efficiency of receptor triggering by increasing the effective local concentration of ZAP70 binding sites. It is also possible ligand-induced receptor clustering (34) or pre-formed TCR ‘nanoclusters’ in the plasma membrane (35) could further enhance the efficacy of receptor triggering to low levels of pMHC ligands by increasing the effective number of ITAMs that could drive autophosphorylation and hence proximal signaling.

Materials and Methods

Construct design and cell transduction

All DNA vector constructs, cell culture, and transient and lentiviral transduction procedures are detailed in the Supplementary Methods.

Chemically-inducible synthetic receptor system

Construction of the vectors for the FKBP-CD86-CD3 ζ receptor (previously described as FKBP^{Ex} ζ ^{Int}), its ligand, FRB^{Ex}, and the Raji B-cell line expressing this ligand have been previously described (13), although the synthetic receptor now included an HA-epitope at the mature N-terminus for quantification of expression. The first Tyr in each ITAM was mutated to Phe to sequentially decrease the number of ITAMs. To engineer a receptor complex with 10 ITAMs, we fused FKBP to the mature N-terminus of the TCR α sequence from Jurkat T cells (along with an HA-epitope), which was incorporated into the endogenous Jurkat TCR complex through competition with the wildtype equivalent TCR α chain. A clonal Jurkat derivative (J.NFAT) was created that expressed GFP on TCR stimulation using NFAT-response elements, as well as constitutively expressing the fluorophore iRFP713 for identification. The synthetic receptors were transduced in J.NFAT cells and expression was measured using the HA-epitope, which could be quantified using fluorescent calibration beads as described in the Supplementary Methods.

Calcium flux and phospho-ERK analysis

Synthetic receptor-expressing T cells were loaded with Indo-1, a fluorescent Ca²⁺ indicator prior to conjugation with Raji B cells expressing the cognate binding domain (FRB^{Ex}). Cell conjugates were gated on by flow cytometry and the Ca²⁺ flux of these cell conjugates was measured, after baseline acquisition, after the rapalog drug was added. We used a similar approach for measuring phospho-ERK levels, where cell conjugates were first formed in the absence of the rapalog drug. The conjugates were then incubated with the rapalog for a defined period at 37°C before fixing using 4% formaldehyde. Fixed cells were then methanol-extracted and subsequently stained with a directly-conjugated fluorescent antibody to phospho-Erk1/2 (T202/Y204). Further details, including data analysis are detailed in the Supplementary Methods.

Downstream cellular activation

J.NFAT cells expressing the appropriate synthetic receptor were conjugated to Raji-FRB^{Ex} cells for 30 min as described for the Ca²⁺ flux assay, except that the cells were conjugated in

medium that included the rapalog drug at the prescribed concentration. Conjugates were then cultured for 16 hours to allow activation-induced expression of GFP from the NFAT-responsive promoter. The supernatant of the activated cells was also collected at this point to assay for IL-2 cytokine secretion. Cells were stained with a fluorescently-conjugated antibody against the HA-epitope prior to running the sample on a flow cytometer to measure both receptor and GFP expression. Further details, including data analysis are given in the Supplementary Methods.

Cellular reconstitution of TCR phosphorylation kinetics

HEK-293T cells were transduced to express all the proteins required to reconstitute the proximal events of TCR triggering, as described previously (13). HEK-TCR cells, which stably expressed the entire TCR complex (1G4 clone), were transiently transfected and sorted by flow cytometry to purify a homogeneous population of cells expressing all components (LCK, ZAP70, LAT, CD45 and CSK/CBP), which were fluorophore tagged for detection. These sorted cells were then activated at 21°C using pervanadate. Aliquots of the cells were taken at defined time-points and rapidly quenched by snap-freezing. Frozen samples were lysed on ice, pelleted to remove perinuclear material and heated at 70°C in reduced LDS sample buffer. Samples were subjected to gel electrophoresis, before blotting onto nitrocellulose and probing for the required (phospho-) protein by Western analysis in conjunction with fluorescent secondary antibodies. Blots were imaged using an Odyssey Imaging system. Further details, including band quantification and data analysis are detailed in the Supplementary Methods.

Modeling effect of ITAM number on receptor triggering

BioNetGen (26) was used to model TCR triggering. The two Tyr residues of an ITAM were modeled as a single phosphorylation event to minimise complexity. Up to 6 ZAP70-binding sites could be computed (over ~20 hours) but more than this became unfeasible. The code used to model the network, along with further details are detailed in the Supplementary Methods.

Chimeric antigen receptor activation

The anti-CD19 chimeric antigen receptor (CAR), including mutations of the ITAMs of the CD3 ζ sequence as described for the synthetic receptor above, was lentivirally transduced into human primary CD4⁺ T cells and left to proliferate over ~10 days until there was a clear decrease in cell volume, judged by scatter on a flow cytometer. To construct a CAR variant with 6 ITAMs, the CD3 ζ intracellular sequence was duplicated, and all synonymous mutations in one half were made. This sequence was synthesized and inserted into the CAR vector. K562 target cells were transduced to express the CD19 protein using different promoters to drive differential surface densities. Cell sorting was used to select three different target cell lines. T cells were conjugated with these target cells over 24 hours and stained with fluorescently-labeled antibodies against CD137 and CD3 and were then measured by flow cytometry. When required, the target cells were pre-incubated with an anti-CD19 antibody to block the CAR epitope, and the antibody was present throughout the subsequent incubations. Further details, including data analysis are detailed in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a Sir Henry Dale Fellowship (JRJ) jointly funded by the Wellcome Trust and the Royal Society (Grant Number: 099966/Z/12/Z).

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One-sentence summary

The T-cell antigen receptor uses high multiplicity of ITAM signaling motifs to very efficiently transduce ligand binding into intracellular signaling, a mechanism that can be exploited to increase the efficacy of therapeutically relevant CARs targeted against cancers.

Fig. 1. T-cell signaling potency is enhanced by greater ITAM multiplicity.

(A) Schematic of the synthetic receptors. For receptors encoding 0-3 ITAMs, the FKBP domain was expressed at the extracellular terminus of CD86 and fused to the intracellular sequence of CD3 ζ , with point mutations used to disable the ITAMs when required. For the receptor with 10 ITAMs, the FKBP domain was expressed at the extracellular terminus of the TCR α chain, which was then incorporated into the complete endogenous TCR complex. (B) Schematic of synthetic receptor triggering, with proteins approximately to scale. The receptor is expressed in T cells in the presence of endogenous TCR. Conjugation with cells presenting FRB, the cognate FKBP ligand, drives receptor engagement but only in the presence of the rapalog drug (AP21967). This leads to receptor triggering through LCK-mediated phosphorylation of the receptor and the subsequent recruitment of the cytoplasmic

kinase ZAP70 and its activation, shown as an ‘opening’ of the structure, which causes phosphorylation of proteins such as LAT that initiate downstream signaling.

(C and D) Jurkat T cells expressing the synthetic receptor encoding the number of ITAMs shown in legend were activated with FRB-expressing Raji B cells in the presence of 0.5 μ M (C) or 2.5 μ M (D) rapalog. Representative flow cytometry data from one experiment of T-cell activation is shown, measured by NFAT-mediated GFP expression, with cells gated for equivalent receptor surface expression.

(E and F) Data from C and D were used to calculate the fraction of T cells that had been activated (E) and the mean GFP intensity of this activated fraction (F) at the rapalog concentration shown in the legend. The mean of three independent experiments is shown for each data point, with error bars representing SEM.

(G) Density plots of activation-induced GFP expression against synthetic receptor expression with differing ITAM multiplicity (white box). Vertical lines denote the range of receptor expression analyzed and the horizontal dashed line shows the threshold for designating a cell as activated.

(H and I) The fraction of activated Jurkat T cells plotted against receptor expression is shown for all variants of the synthetic receptor in the presence of 0.5 μ M (H) or 2.5 μ M (I) rapalog. Beads with known amounts of fluorophores were used to convert relative fluorescence data from the flow cytometer to absolute surface density. The mean of three independent experiments is shown at each binned expression level, along with a bounding area representing SEM. Datasets are fit using a logistic function.

(J and K) The receptor density required to cause half-maximal cell activation (EC_{50}) was calculated from datasets (H and I) and is shown as a function of the number of ITAMs encoded by the receptor in the presence of 0.5 μ M (J) or 2.5 μ M (K) rapalog. A 95% confidence interval is shown as a bounding area.

Fig. 2. Increasing ITAM multiplicity drives more synchronous T-cell activation.

(A) Jurkat T cells expressing the synthetic receptor were loaded with the Ca^{2+} indicator Indo-1 before conjugation with FRB-expressing Raji B cells. T cells were activated by 2.5 μM rapalog addition (white arrow) and the Ca^{2+} flux, presented as Indo-1 fluorescence ratio, was recorded with time by flow cytometry. Plots show the flux profile for the synthetic receptors bearing the designated number of ITAMs (white box). The binned density at each point is denoted by color scale. The solid line marks the boundary between responding and non-responding cells used in the subsequent analyses.

(B and C) The fraction of T cells activated over time was calculated when cells expressing the synthetic receptor with number of ITAMs shown in legend were synchronously initiated at 30 s using 0.5 μM (B) or 2.5 μM (C) rapalog. The mean of three independent experiments is shown for each ITAM variant, along with a bounding area representing SEM. Data points have been decimated and the moving-average fit and bounding area smoothed for visual clarity. The panels on right show the derived rate of activation (after smoothing) for these datasets.

(D and E) Equivalent datasets as in B and C, now showing the mean Indo-1 ratio of the activated fraction of T cells over time when synchronously initiated at 30 s using 0.5 μM (D) or 2.5 μM (E) rapalog. The mean of three independent experiments is shown for each ITAM variant, along with a bounding area representing SEM. Data points have been decimated and the moving-average fit and bounding area smoothed for visual clarity. The panels on right show the derived rate of activation (after smoothing) for these datasets.

Fig. 3. Effect of increased ITAM multiplicity on ERK phosphorylation kinetics.

(**A** and **B**) Jurkat T cells expressing equivalent levels of the synthetic receptor encoding the number of ITAMs shown in legend were conjugated with FRB-expressing Raji B cells. Cell conjugates were then incubated in the presence of 0.5 μ M (**A**) or 2.5 μ M (**B**) rapalog for 5 min before fixation and intracellular staining for phospho-ERK1/2. Representative flow cytometry data from one experiment of T-cell activation is shown, where the fluorescence intensity distribution is derived solely from cell conjugates.

(C and D) Data from A and B were used to calculate the fraction of T cells that had been activated (C) and the mean phospho-ERK intensity of this activated fraction (D) at the rapalog concentration shown in the legend. The mean of three independent experiments is shown for each data point, with error bars representing SEM.

(E and F) An equivalent experiment as presented in (B) was performed but now 2.5 μ M rapalog was added for times ranging from 0 – 5 min prior to fixation. The fraction of activated T cells at each time-point is shown (E), along with the mean phospho-ERK intensity of this activated fraction (F). The legend indicates the number of ITAMs encoded by the synthetic receptor expressed in the T cells. The mean of three independent experiments is shown for each data point, with error bars representing SEM.

Fig. 4. ZAP70 activation is primarily driven by autophosphorylation.

(A) Alternative representations for the mechanism of ZAP70 activation through Y493 phosphorylation, which could be driven by LCK-mediated kinase activity (left) or through ZAP70 trans-autophosphorylation (right).

(B) Schematic of the kinetic experiment. HEK-293T expressing the entire TCR complex were transfected with the minimal components of the TCR triggering apparatus (LCK, ZAP70, LAT, CD45, CSK/CBP), sorted on expressing cells and synchronously activated by inhibiting CD45 phosphatase using pervanadate (PerVi). At defined time-points, aliquots of

the activated cells were removed and snap-frozen in tubes pre-frozen in a metal block, before being lysed and analyzed by phospho-western analysis.

(**C** and **D**) Plots of the kinetics of ZAP70 phosphorylation at Y493 (**C**) or LAT phosphorylation at Y132 (**D**) are presented, when either the wildtype ('WT') or kinase-dead ('Dead') versions of LCK and ZAP70 are expressed in the reconstituted TCR triggering system, as shown in legend. All datasets were collected at 21°C, and the mean of three independent experiments is shown at each time-point, along with a bounding area representing SEM. Datasets were fit using a logistic function.

Fig. 5. Chimeric antigen receptor potency can be modulated by ITAM multiplicity.

(A) Human CD4⁺ T cells expressing a CD19-reactive CAR with the number of ITAMs shown in legend were mixed with K562 target cells expressing the designated level of CD19. The expression of CD137 (4-1BB) activation marker was determined 24 hours later by flow cytometry, shown as representative histograms from one experiment. CAR expression in T cells was equivalent between all ITAM variants.

(B and C) Data from A were used to calculate the fraction of T cells that had been activated (B) and the mean CD137 intensity of this activated fraction (C) when T cells were stimulated with target cells expressing the level of CD19 shown in the legend. The mean of three independent experiments is shown for each data point, with error bars representing SEM.

(D) The data in B is replotted to show the effect of target density on the fraction of activated cells, for each CAR expressing the number of ITAMs shown in legend.

(E) T cells expressing the wild-type CAR (3 ITAMs) were activated with target cells, either in the absence or presence of a blocking antibody against CD19. Legend shows the target cells that were used for each dataset. Representative flow cytometry plots of CD137 expression are presented from one experiment.

(F) Data from E were used to calculate the fraction of CAR-expressing T cells that had been activated by target cells expressing different CD19 levels, performed either in the absence or presence of a blocking α -CD19 antibody, as shown in legend. The mean of three independent experiments is shown for each data point, with error bars representing SEM.