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Insights into the initiation of TCR signaling

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Abstract

The initiation of T cell antigen receptor signaling is a key step that can result in T cell activation and the orchestration of an adaptive immune response. Early events in T cell receptor signaling can distinguish between agonist and endogenous ligands with exquisite selectivity, and show extraordinary sensitivity to minute numbers of agonists in a sea of endogenous ligands. We review our current knowledge of models and crucial molecules that aim to provide a mechanistic explanation for these observations. Building on current understanding and a discussion of unresolved issues, we propose a molecular model for initiation of T cell receptor signaling that may serve as a useful guide for future studies.

T cell antigen receptors (TCR) recognize peptides bound to proteins of the major histocompatibility complex (MHC). These peptide-MHC complexes are displayed on diverse cell types. The peptide-MHCs with peptides derived from a pathogen's proteome are the molecular signatures of pathogens displayed on infected cells. It is important to note that these same cells also display abundant numbers of peptide-MHC molecules in which the peptide is derived from endogenous host proteins. T cells discriminate between such self and foreign peptide– MHC molecules with high sensitivity and selectivity. For example, at least *in vitro*, as few as 1–10 agonist peptide–MHC molecules in a sea of thousands of self

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peptide–MHC molecules can trigger T cell activation[1–4]. Very recent imaging studies suggest that even a single agonist peptide–MHC molecule can trigger cytokine production from some naïve T cells[1]. Productive interactions of TCRs with cognate ligands are among the weakest protein-protein interactions that can initiate a biological response. A T cell needs to discriminate selectively between foreign peptide–MHCs and self peptide–MHC molecules, even though the differences in affinity and kinetics of binding to self and foreign peptide–MHCs are not large[5–7]. Therefore, the earliest events in TCR signaling must reflect these remarkable dual features of high sensitivity and selectivity for agonist peptide–MHC.

Here we will consider some TCR signaling models that could account for such selectivity and sensitivity and review some recent insights into the molecular machinery that initiates TCR signaling and probably serves to initiate downstream signaling that leads to cellular responses. We will then build on existing models of TCR signaling to propose additional features of regulation that are likely to contribute to TCR signal initiation.

The concept of kinetic proofreading

Because the difference in binding properties of the TCR to endogenous peptide–MHC and foreign agonist peptide–MHC is not large, it seems that the selectivity with which T cells discriminate between them should be modest. For example, suppose that the consequence of binding is to induce a single biochemical transformation that initiates major downstream signaling. In this hypothetical situation (Fig. 1a), the TCR binds to peptide-MHC (either agonist or endogenous), and this complex then undergoes a biochemical transformation that results in downstream signaling. For simplicity, consider the situation in which the endogenous and agonist ligand are differentiated only by the off-rates of the TCR–peptide-MHC complex dissociation; that is, their k_{on} values are the same, but their off-rates are different. One can calculate an error coefficient, f, as the ratio of the rates at which downstream signaling molecules are produced upon binding to endogenous peptide–MHC versus agonist peptide–MHC. If the rate of producing the downstream signaling molecules (k_p) is much larger than the off-rates of either peptide-MHC to the TCR (Fig. 1a), there will be no discrimination between these ligands ($f \sim 1$) because both TCR–endogenous peptide–MHC and TCR–agonist peptide–MHC complexes will be converted to downstream signals as soon as they are formed. Thus, it does not matter which ligand is bound to the TCR. When a single biochemical transformation can lead to productive downstream signaling, the best discrimination possible is when this rate of production of downstream signals (k_p) is much smaller than the off-rates for TCR–peptide-MHC binding. This is because the TCR–peptide-MHC complex with the agonist ligand has a longer lifetime than that with the endogenous ligand, and thus is more likely to be slowly converted to downstream signals. But, even in this extreme limit favoring the agonist, if the off-rate for the agonist is one-tenth the off-rate for the self peptide–MHC, TCR triggering would occur erroneously 10% of the time. This error rate climbs to ~20% if the rate of production of downstream signaling molecules is comparable to the off-rate for the agonist. This degree of selectivity is inferior to the ability of T cells to differentiate between self and foreign peptide–MHC that bind to a TCR with a similar difference in off-rates.

Two insightful studies[8,9] pointed out that kinetic proofreading is the mechanism that allows biological systems to discriminate with high selectivity between two substrates in spite of relatively small differences in binding affinities or off-rates for the substrates[8]. One of these applied the concept of kinetic proofreading to TCR signaling[9]. Kinetic proofreading is predicated on two key characteristics. First, after the initial binding event, a number of biochemical modifications must occur before formation of the major product that initiates major downstream signaling events (Fig. 1b). Second, these biochemical transformations must be driven out of equilibrium by being coupled to energy-consuming reactions. Each biochemical modification in the sequence of steps can be reversed (Fig. 1b), thus hindering their completion and productive downstream signaling. It would seem that the agonist ligand that binds more strongly to the TCR is more likely to stay bound until these biochemical modifications are completed and that this advantage grows greater with higher numbers of required modifications. However, one can show mathematically that there is no amplification of selection over a mechanism that does not involve a sequence of steps if the set of biochemical reactions is at thermodynamic equilibrium. Significant amplification of selectivity can occur only when the intermediate biochemical modifications are driven far from equilibrium. Why is this true?

The effect of the sequence of biochemical reactions may be viewed as a means to introduce a waiting time before major signaling can occur, and the ligand that stays bound longer is more likely to be able to ‘wait’ for the duration that it takes for the sequence of steps to be completed. The longer the waiting time (the more biochemical modifications in the sequence), the greater the difference between the ligands' probabilities of inducing productive downstream signaling. The concept of waiting time is meaningless for a system at equilibrium, because such a system does not progress with time. As the existence of a waiting time is the key to ligand discrimination in the kinetic proofreading mechanism, the set of biochemical modifications must be driven out of equilibrium (Fig. 1b). In TCR signaling, many biochemical modifications that occur after TCR ligation are phosphorylation reactions that consume ATP (hence energy) and thus are driven out of equilibrium.

If only one more biochemical transformation is added to the scheme (Fig. 1a), and the reactions are strongly driven out of equilibrium, selectivity can be almost as high as f^2. So, instead of a best-case error rate of 10%, the error rate could be only 1% when the off-rates of the agonist and self peptide–MHC differ by a factor of ten. It is worth pointing out that a system such as that described here (Fig. 1b) can be at steady state owing to phosphorylation and dephosphorylation events, but it is not at thermodynamic equilibrium because there is energy input (consumption of ATP). It is also worth noting that for the specific realization of kinetic proofreading described here (Fig. 1b), greatly enhanced selectivity is accompanied by very low amounts of downstream signaling. However, this effect is mitigated if the dissociation rates for the later biochemical modifications are lower than those of the earlier steps[9]. This can be achieved by cooperative binding of a number of downstream signaling molecules to a species created by a previous biochemical modification. An example is the cooperative formation of the Lat signalosome in TCR signaling. We will return to the concept of kinetic proofreading a number of times below, and note that an ‘adaptive sorting’

mechanism has also been proposed as a variant scheme that may enable discrimination between ligands[10].

ITAM sequestration

The earliest biochemical modifications that occur upon ligation of the TCR in model T cell lines and cultured primary T cells are phosphorylation of the tyrosine residues on the TCR-associated cytoplasmic CD3 and ζ-chains by the nonreceptor tyrosine kinase Lck[11–14]. The tyrosines are part of the immunoreceptor tyrosine-based activation motifs (ITAMs). ITAMs are contained as single copies within the CD3ε, CD3δ and CD3γ chains and as three copies within the ζ-chain. Why does ITAM phosphorylation not occur unabated leading to TCR triggering in the absence of TCR ligation? One possible reason[15] is that the inner leaflet of the plasma membrane is negatively charged, and the proximal cytoplasmic domains of the CD3 and ζ-chains have a net positive charge. A fluorescence resonance energy transfer (FRET)-based assay and nuclear magnetic resonance (NMR) experiments with *in vitro* systems that aim to mimic the cellular situation suggest that this electrostatic attraction leads to the burial of the aromatic tyrosines in the ITAMs of the cytoplasmic domains of the CD3ε and the ζ-chain, such that the peptide backbone is located at the interface of the hydrophobic tails and hydrophilic head groups of the cell membrane or the bicelle mimics used in these experiments. It was further suggested that ligand binding leads to pulling the cytoplasmic domains of the CD3 and ζ-chains away from the membrane, thus making the tyrosines of the ITAMs accessible to multiple phosphorylation events mediated by Lck.

The original reports that ITAMs are sequestered in the plasma membrane has been contradicted by another study that shows that mutant chimeras in which the tyrosines are not shielded by the membrane lipids are not phosphorylated in the basal state[16]. A follow-up study then reported that although sequestration of ITAMs is not involved in the earliest events in TCR signaling, it is implicated in downstream events that result from the TCR-induced downstream calcium increase[17]. This would suggest that TCR signaling is initiated by a small number of receptors that induce a large calcium increase which, in turn, somehow mediates exposure of the ITAM tyrosines in other TCRs that engage additional signaling molecules, thereby serving as an amplification mechanism. Problems with this model include the nonphysiologic concentrations of calcium used for the biophysical studies and that other divalent cations are able to induce this release of ITAMs. Magnesium concentrations in the cell far exceed concentrations of free calcium and would seem to pose a problem for this mechanism. Moreover, recent studies suggest that a magnesium transporter is responsible for a magnesium influx that regulates downstream TCR signaling events such as phospholipase C-γ1 (PLC-γ1) tyro-sine phosphorylation, which subsequently leads to calcium increases[18]. Finally, the release of ITAMs from the membrane does not account for the fact that, in most *ex vivo* isolated thymocytes and $CD4^+$ T cells, the ζ-chain ITAMs are largely constitutively phosphorylated (discussed below)[19–21]. Thus, release of ITAMs from the plasma membrane is unlikely to be a requisite step in the initiation of TCR signaling.

The TCR as a mechanosensor

It has been suggested that TCR binding to peptide-MHC causes conformational changes that make the CD3 and ζ-chains accessible to phosphorylation reactions. NMR cross-correlation analyses show that agonist antibodies bind CD3 in a different orientation from that of antibodies to CD3 that do not trigger downstream signaling[22,23]. The binding sites of the agonist antibodies seem to be exposed in the TCR complex. These results are consistent with early studies[24,25] that showed that different antibodies to CD3 and TCR stimulate varying amounts of signaling, perhaps owing to different architectures of the TCR-CD3 complex. Those findings are also in harmony with the finding that when a bound TCR's position over a peptide-MHC complex is very different from its usual orientation, the TCR does not induce signaling, perhaps owing to an unproductive architecture of the TCR-CD3 complex (although other possibilities cannot be excluded)[26].

One possible way for the TCR to mediate conformational change is by acting as a mechanosensor. A force associated with the binding event could cause a deformation or conformational change in the TCR component chains. When a T cell crawls on an antigen-presenting cell (APC), forces act on bound TCR–peptide-MHC complexes. It has been suggested that such forces result in a torque that causes conformational changes that lead to interactions of the TCR with CD3 at the binding sites of agonist CD3 antibodies[27], thus triggering signaling (Fig. 2). In support of this idea, when beads coated with the nonagonist CD3 antibody interacted with T cells, application of a 50-pN force with optical tweezers resulted in a calcium increase[22]. Similarly, T cells transgenic for a TCR interacting with beads coated with an agonist peptide–MHC increased calcium only after a 50-pN force was applied. Intriguingly, nonagonist peptide–MHC did not elicit a calcium increase regardless of whether a force was applied.

This last observation highlights a conundrum presented by the mechanosensor model of TCR triggering. The transmission of force due to T cells' crawling on APCs, cytoskeletal motion or external application acts on the TCR–peptide-MHC bond and results in mechanical work. Thus, the off-rates of both the agonist and the endogenous ligands should be higher in the intercellular environment[28,29]. Therefore, it is difficult to see why, upon the application of force, the effective off-rates of both the endogenous and agonist ligands do not become too high for productive signaling to ensue.

Published work suggests a possible solution to this problem[30]. This group used an apparatus called a biomembrane force probe to measure the kinetics of binding of the Ovalbumin peptide–specific OT1 TCR to a set of its peptide-MHC ligands in a two-dimensional membrane environment. In a setup that the authors considered to be applicable when no external force was applied on the bond, they had previously reported the surprising result that antigen potency is inversely correlated with the lifetime of the TCR–peptide-MHC bond in the membrane environment[29]. This finding was incongruent with measurements of TCR–peptide-MHC bond lifetimes in the membrane environment by other groups using different experimental apparatus[28,31]. Subsequent studies using the biomembrane force probe have reported that, lifetimes of the different TCR–peptide-MHC pairs correlate with antigen potency, as expected, but only upon application of a moderate amount of tensile force to the

bond. The authors further report that, for agonists, the lifetime increases with the application of moderate amounts of force and decreases upon application of too much force, whereas for nonstimulatory ligands, the application of force reduces the lifetime of the TCR–peptide-MHC bond. Thus, the authors posit that agonists form ‘catch bonds’ that lead to increased bond lifetime upon application of moderate force, while nonstimulatory ligands form ‘slip bonds’, which always result in a decrease of bond lifetime with force[30]. They propose a mechanism for what makes the lifetime of the TCR–peptide-MHC bond longer for agonists upon application of force; a finding that potentially resolves the conundrum noted above.

Several points are worth noting, however. Experiments were done without the CD8 coreceptor, which has an important role in mediating CD8 T cell functional responses. There are a number of stimulatory ligands with potency between that of A2 and G4 (two ligands studied)[5], and it is unknown whether these agonists also form catch bonds. The molecular reasons that lead to formation of catch bonds and slip bonds also remain unknown. Finally, it is important to note that this work provides a possible mechanism for why agonist peptide–MHC–TCR bonds have a longer lifetime than bonds formed by nonstimulatory ligands in the intercellular environment—it does not address why this longer lifetime is necessary for productive signaling to ensue.

We note also that agonists that have a high on-rate can trigger T cells when their off-rates are comparable to that of some nonstimulatory ligands[32,33]. This observation has been attributed to the importance of rebinding, a phenomenon that has been noted in published reports[29,30]. Clearly, more research is required to clarify the role of force in TCR signaling.

The TCR as an allosteric receptor

Other reports have implicated TCR conformational changes in initiation of TCR signaling. Notably, an alteration in the position of the A-B loop in the TCR α-chain constant region in the bound and unbound states has been reported[34]. Interestingly, the A-B loop, whose position changes, is presumed to interact with the extracellular domains of CD3 chains, thereby making this an attractive means to transmit information from the ligand-binding chains of the TCR to its invariant signaling components. However, the change in the position of the bound and unbound TCR α-chain A-B loop has only been observed for a single pair of TCR bound and unbound structures. Therefore, the significance of this change in TCR structure is uncertain.

An alternate conformational change that involves an intracellular proline-rich sequence (PRS) N-terminal in the CD3ε ITAM has been described in a series of reports[35–37]. The PRS becomes accessible after TCR stimulation and can bind to the N-terminal Src homology 3 (SH3) domain of the adaptor Nck, which then is thought to facilitate downstream signaling. Nck interacts with a number of signaling molecules, many of which interact with the actin cytoskeleton[38]. However, the importance of a conformational change involving the PRS has also proven controversial, as mutation of this site has led to discordant results[35,39,40]. In short, whether and how TCR binding to cognate ligands results in conformational changes that enable ITAM phosphorylation or signal initiation remains unknown.

The kinetic segregation model

Another model for TCR signal initiation is that of kinetic segregation[41]. In this model, it is proposed that TCR binding to agonist peptide–MHC along with other similarly sized molecules is sufficiently strong to result in apposition of the T cell and APC membranes at a distance of 15 nm. The ectodomain of the abundant and important phosphatase CD45 is substantially longer than this. Because the bending rigidity of cellular membranes is not small, CD45 is excluded from the region where TCR and peptide-MHC are bound[42,43]. CD45 is a receptor-like tyrosine phosphatase with high catalytic activity. Hence, according to this model, its exclusion would promote TCR triggering.

This concept derives from the fact that stimulating the TCR induces well-characterized tyrosine phosphorylation events that are crucial for the activation of downstream signaling. In this model, CD45 is viewed as having a major negative regulatory function to sustain the inactive basal state. Thus, by excluding CD45 from regions adjacent to the stimulated TCR, TCR tyrosine phosphorylation and its induced downstream tyrosine phosphorylation is protected from CD45 action. Supporting the kinetic segregation model are studies in which the extracellular domain of CD45 has been shortened through engineered chimeric molecules containing the intracellular CD45 phosphatase domains[44,45]. This model has also been recently supported in a reconstituted heterologous cell system, although that study suggests that size may not be the only determinant of segregation of molecules from the TCR[43]. Thus, the kinetic segregation model focuses on the putative negative regulatory role for CD45 as a major controller of the tyrosine phosphorylation landscape around the stimulated TCR.

The kinetic segregation model is challenged by the fact that CD45 unequivocally has a positive regulatory role on the activity of Lck in the earliest signaling events following TCR stimulation[46–48]. Lck, which noncovalently associates with the coreceptors CD4 and CD8, has a crucial initiating role in TCR signaling by its ability to phosphorylate the two tyrosines in each CD3 or ζ-chain ITAM[13,14]. In support of this positive regulatory role of CD45, shortening the ectodomain of another phosphatase, CD148, is in apparent concordance with the kinetic segregation model[44]. However, CD148 has been shown to inhibit TCR signaling by acting downstream of the TCR complex (targeting Lat, and possibly PLC-γ)[49,50], rather than by affecting the initiation of TCR signaling. Therefore, the positive regulatory role of CD45 on Lck activity remains at odds with the kinetic segregation model. Lck phosphorylates ITAMs and, importantly, it also phosphorylates and contributes to the activation of Zap70 (as discussed below). Thus, understanding the regulation of Lck by CD45 is crucial for understanding its role in TCR signaling.

Regulating Lck function

Lck, like all Src family kinases, has two major tyrosine phosphorylation regulatory sites. Tyr394 in the catalytic domain activation loop is phosphorylated by trans-autophosphorylation or by Fyn, the other Src kinase expressed in T cells. Phosphorylation of Tyr394 stabilizes the active conformation of the catalytic domain and, therefore, represents a positive regulatory site of tyrosine phosphorylation. Tyr505 represents a highly conserved

negative regulatory site present in all Src family kinases. Phosphorylation of Tyr505 promotes the autoinhibited conformation of Lck in which intramolecular binding of its SH2 domain with phosphorylated Tyr505 and the interaction of the SH3 domain with a proline-containing motif N-terminal to the kinase domain stabilizes the inactive conformation of the catalytic domain (Fig. 3). In cells, Tyr505 is thought to be phosphorylated mainly by the kinase Csk[51,52]. Strong genetic and biochemical data suggest that CD45 dephosphorylates this residue[53–58]. T cell lines, T cell clones or primary thymocytes deficient in CD45 have Lck molecules that are hyperphosphorylated on Tyr505 and have blocks in the earliest events associated with TCR triggering, including ITAM phosphorylation as well as downstream signaling events leading to activation or even thymocyte positive selection[46,47,53,57,58]. Two independent studies of different allelic series of mice that express varying amounts of CD45 have found that the abundance of CD45 and the phosphorylation of Lck Tyr505 are inversely correlated[53,58]. Similar effects are observed for Fyn as well as for other SFKs expressed in B cells, although in those cells another receptor-like protein tyrosine phosphatase, CD148, can partially compensate for the loss of CD45 (refs. 59,60).

There is evidence that CD45 can have a negative regulatory function[49,53,58,61,62]. High amounts of CD45 have a somewhat negative impact on the phosphorylation status of Tyr394 (refs. 53,58); however, whether this reflects a direct or indirect effect is less clear[63]. This inhibitory impact of CD45 on the negative regulatory site in Src family kinases is seen in T cells but not in B cells[53,58,64]. Moreover, in contrast to *in vitro* assays, use of the transgenically expressed reporter Nur77-GFP as an *in vivo* indicator of TCR signaling strength failed to reveal the negative regulatory impact of physiologic amounts of CD45 in thymocytes[65]. Finally, other cytoplasmic tyrosine phosphatases, such as PTPN22 and PTP-PEST, have been reported to act on this Tyr394 and comparable activation loop residues in other Src family kinases[63,66].

In the unperturbed steady state in T cells and in thymocytes, the counteracting forces of CD45 and Csk maintain a steady state basal activity of Lck. The importance of the positive regulatory role of CD45 is further highlighted by experiments designed to study the consequences of inhibition of the activity of Csk[67,68]. A mutant of Csk that is sensitive to a bulky analog of the general kinase inhibitor PP1 was generated. Inhibition of Csk using this construct led to a rapid increase of Tyr394 phosphorylation and Lck activity as well as evidence of spontaneous TCR signaling without TCR ligation[68]. Interestingly, similar experiments in Jurkat T cells have found that CD45 expression as well as Lck is required for such spontaneous TCR signaling[67]. These results suggest that perturbing the balance between the actions of Csk and CD45 can result in enhanced activation of Lck and TCR triggering. Importantly, segregation of CD45 and its phosphatase activity away from the TCR and the downstream tyrosine phosphorylated molecules is not necessary for the induction of TCR signaling by Csk inhibition. This finding highlights the positive regulatory role of CD45 in TCR signaling and supports the argument that kinetic segregation (of CD45) is not a requisite event for the initiation of TCR signaling, although the influence of CD45 segregation in physiologic TCR signaling for its later maintenance needs to be addressed. The studies with Csk also suggest that ITAM sequestration is not the limiting factor in the initiation of TCR signaling. Nonetheless, the question of how TCR ligation by agonist peptide–MHC perturbs the basal steady state remains unknown.

The TCR and serial triggering

Almost two decades ago, pioneering experiments suggested that T cells are sensitive to just one agonist peptide–MHC ligand presented on an APC[3]. Since then, single-molecule–counting experiments have made clear that T cells can be triggered by as few as 1–10 ligands in a sea of thousands of endogenous peptide–MHC molecules[1,2,4,69–72]. This is an extraordinary 'signal-to-noise' ratio that is unprecedented even in synthetic electronic circuits. Several proposals for the mechanism that results in such extraordinary sensitivity of T cells for antigenic peptide–MHC have been described. The first of these is the serial triggering model, which posits that a single agonist peptide–MHC can serially trigger many TCRs[73].

The serial triggering model, when juxtaposed with the requirements of kinetic proofreading, makes a clear prediction. Serial triggering is enhanced by ligands that bind the TCR weakly, and kinetic proofreading mechanisms lead to higher selectivity for ligands that bind the TCR more strongly. Thus, there should be an optimal TCR–peptide-MHC binding affinity (or off-rate) that balances these conflicting requirements. Very little evidence exists in clear support of this prediction. In one report, mathematical models were combined with experiments that assayed TCR expression 5 h after incubation of T cells with APCs bearing variants of a vesicular stomatitis virus (VSV) peptide that bound the TCR with different half-lives[74]. TCR downregulation was taken to be the readout of signaling, and the range of VSV peptide half-lives spanned a factor of ~20. A peak in TCR downregulation was observed as a function of half-life. Definition of the peak relied on the extent of downregulation measured for two peptides with similarly long half-lives.

Experimental data suggest that there is no optimal off-rate for TCR–peptide-MHC binding and that stronger binding always leads to more stimulation. Using yeast surface display methods, investigators have obtained variants of the 2C TCR that bind to the same cognate peptide–MHC ligand with half-lives up to two orders of magnitude longer than that for 2C (~30–1,500 s)[75]. The more strongly binding TCR variants confer greater responsiveness on a T cell hybridoma, as measured by interleukin 2 (IL-2) production, and there is no optimal window of half-life.

A study combining single-molecule imaging and single-cell cytokine assays has found that a single cognate peptide–MHC molecule presented on APCs can trigger production of IL-2 and tumor necrosis factor (TNF) in a small fraction of what would be considered a homogeneous population of T cells[1]. This study reports that greater numbers of cognate peptide–MHC molecules result in cytokine secretion by a greater proportion of T cells, but the rate and amount of cytokine production by triggered T cells remains the same. This finding is consistent with T cell signaling being digital[76,77]. The authors also observed that a single peptide-MHC recruits many TCRs to form a cluster[1]. TCR engagement of peptide-MHC has been reported to result in coupling of the TCR to the cytoskeleton and migration to the center of the immune synapse[78]. Thus, the clustering of TCRs stimulated by a single peptide-MHC has been suggested to be due to serial triggering of TCRs by a single peptide-MHC. However, as we describe below, this interpretation may be confounded by other phenomena.

Endogenous peptide–MHC can amplify TCR triggering

Another mechanism suggested for signal amplification by a few agonist peptide–MHC ligands does not, unlike the serial triggering mechanism, sacrifice selectivity for sensitivity. This mechanism posits that endogenous peptide–MHC molecules are not just bystanders during the earliest events in T cell activation when a few agonists are present. TCRs interact weakly with endogenous peptide–MHC molecules, and these interactions are thought to deliver signals as T cells scan APCs[79–82]. Evidence for such signaling could be reflected by the high basal level of the phosphorylated ζ-chains in thymocytes and T cells[19,20]. Recent work shows basal signaling differences, reflected by CD5 expression and phosphorylated ζ-chain, studied in the context of transgenic expression of TCR[81,82]. These studies not only support the concept of ongoing basal signaling, but they also provide evidence that such basal signaling has consequences for modulating the sensitivity of TCR signaling to subsequent agonist encounter.

For both $CD4^+$ and $CD8^+$ T cells, experimental data show that endogenous peptide–MHC molecules are important for signal amplification[4,69–72,83]. One important difference between these cell types is the observation that amplification is sensitive to the identity of the endogenous ligand for $CD4^+$ T cells, but not for $CD8^+$ T cells (for example, ref. 70). Recently, by combining experiments and computer simulations, a conceptual framework has been proposed that unifies these observations as manifestations of the same mechanism for signal amplification by endogenous ligands. This mechanistic picture is summarized below in the context of coreceptor function.

Coreceptor function and its role in kinetic proofreading

It has long been thought that coreceptors stabilize the TCR–peptide-MHC complex and recruit Lck to the receptor complex[84]. Biophysical measurements and computer simulations have led to a more nuanced view. FRET measurements in the two-dimensional cellular environment combined with inhibition of CD4–MHC class II interactions using antibodies to CD4 show that CD4 does not have a stabilizing effect on TCR–peptide-MHC class II interaction[28]. In contrast, CD8 has a modest stabilizing effect for TCR–peptide–MHC class I interactions[85,86]. Biophysical measurements show that both CD4 and CD8 binding to MHC is characterized by rapid off-rates, with CD8 binding longer to MHC class I molecules[87]. Stochastic computer simulations in the two-dimensional membrane environment using mostly measured biophysical parameters showed that these fast off-rates precluded any significant increase in TCR–peptide-MHC lifetimes as a consequence of coreceptor binding, with only a modest effect for CD8 and essentially none for CD4 (ref. 88). The modest enhancement for $CD8^+$ T cells is mediated by cooperative bivalent interactions between CD8 and MHC and CD8-associated–Lck with the TCR complex. These computational studies also show how the coreceptor has a critical role in Lck recruitment to the receptor complex. Upon TCR–peptide-MHC binding, if free Lck approaches the TCR complex, it can either associate with the TCR complex or diffuse away. Measurements of protein diffusion coefficients and estimates of the rate at which Lck may associate with the TCR complex show that the probability that it will diffuse away is much higher. The measured on-

rate for coreceptor binding to MHC is very large and competes effectively with diffusion, thus localizing coreceptor-associated Lck to the TCR complex.

In an extension of this study using computer simulations, experiments with two different T cell lines (OT1 and F5) that bind to their cognate ligands with different affinities, MHC mutants that bind to CD8 with varying affinity and different endogenous ligands, investigators have proposed a mechanism for signal amplification when T cells are stimulated by small numbers of agonist peptide–MHCs[70]. Following TCR binding to agonist peptide–MHC, Lck is recruited by the coreceptor to the TCR complex and potentially activated. This is a key kinetic proofreading step in a series to follow. Endogenous ligands bind too weakly to allow this step to occur with high probability. After phosphorylation of the TCR ITAMs (or the tyrosine kinase Zap70, discussed below) by active Lck, Lck can bind weakly to a single phosphorylated ITAM or diffuse away. The latter process is facilitated by the high off-rate characterizing coreceptor-MHC interactions. The coreceptors bearing activated Lck that were recruited by the agonist can now bind to vicinal MHC molecules that bind to endogenous peptide–MHC. Thus, the first kinetic proofreading step is obviated, allowing ITAM phosphorylation in spite of the fleeting interactions of TCR with endogenous ligands. This mechanism of signal amplification is facilitated by TCR microclusters that have been observed on T cells[89,90] because it allows rapid signal spreading before Lck is deactivated.

This mechanism also provides a possible explanation for the observation that a single agonist peptide–MHC can recruit many TCRs[1]. If TCR bound to endogenous ligands can be triggered by roving coreceptor-associated activated Lck molecules, they would bind to the cytoskeleton and be recruited to the center of the synapse. If there were more agonist peptide–MHCs, more coreceptors bearing activated Lck would be generated or recruited, thereby enhancing the number of TCRs that could undergo ITAM phosphorylation when bound to endogenous ligands. Thus, a greater proportion of T cells are likely to be activated because an early kinetic proofreading step is more likely to be successful.

Reducing the binding affinity of CD8 to MHC bearing the agonist peptide makes the identity of the endogenous peptide important for signal amplification; endogenous peptide–MHC that binds more weakly to TCR can no longer support signal amplification[70]. According to the proposed mechanism, this is because weaker coreceptor binding would make coreceptor-mediated Lck recruitment (and potential activation) less likely. Thus, there would be fewer available coreceptor-associated active Lck molecules. Signal amplification would require a higher probability of these coreceptor-bound Lck molecules encountering endogenous peptide–MHC-TCR complexes—that is, more stable endogenous peptide–MHC-TCR interactions. Similarly, weakening the interactions of CD8 with the MHC molecule bearing the endogenous peptide also reduces signal amplification because the coreceptor bearing activated Lck is less likely to bind to endogenous peptide–MHC-TCR complexes to enable ITAM phosphorylation[70]. This proposed mechanism also suggests that signal amplification by endogenous ligands is more sensitive to the identity of the endogenous ligand for $CD4^+$ T cells because CD4 binds to MHC class I molecules more weakly than CD8 does to MHC class I molecules.

Signal amplification by endogenous ligands is a type of serial triggering mechanism. Rather than individual agonist peptide–MHC molecules serially binding to different TCRs to trigger them, coreceptor-bound activated Lck molecules can serially trigger vicinal TCRs bound to endogenous ligands until the Lck is deactivated. This mechanism of serial triggering does not sacrifice kinetic proofreading requirements enabled by stronger TCR binding to agonists to gain sensitivity. However, the serial triggering mechanism and the mechanism that proposes signal amplification by endogenous ligands for sensitivity have a potential problem. Both mechanisms implicitly assume that after ITAM phosphorylation, the next steps necessary for productive TCR signaling, such as the binding and activation of Zap70, either occur very rapidly or do not require that TCR is bound to ligand.

Insights into Lck and Zap70 regulation

Recent studies of the regulation of Lck and of Zap70 pose some problems for the models of the initiation of TCR signaling described above. Most investigators have found it difficult to detect TCR-induced Lck activation on the basis of an increase in Tyr394 phosphorylation, although ligation of CD4 alone can upregulate Tyr394 phosphorylation[91]. A model that incorporates TCR-mediated Lck activation is not necessary, as work from many labs has shown that a substantial percentage of Lck molecules is already phosphorylated on Tyr394 at steady state[53,58,92]. Some Lck molecules are even doubly phosphorylated in that they are phosphorylated on both Tyr394 and Tyr505, the activating and inhibitory sites, respectively[92]. Detecting Lck activation in response to TCR stimulation may be difficult owing to methods used to detect an overall increase in Tyr394 phosphorylation after TCR stimulation. Most investigators have used steady-state measures based on immunoblotting, phospho-flow or mass spectroscopic analysis. Such methods do not provide insight into ongoing dynamic turnover of phosphates at each site, which presumably is based on the concerted actions of CD45, Csk and PTPN22. Such dynamic turnover may be required to maintain the basal state and also to allow for a pool of active Lck that could be recruited to contribute to TCR signaling. The idea that the turnover at these phosphorylation sites in the basal state is highly active is supported by the very rapid and large increase in Tyr394 that occurs when Csk activity is inhibited in the absence of receptor perturbation. Likewise, titration of CD45 abundance inversely influences Tyr505 phosphorylation status[53,58]. Varying CD45 expression has more complex effects on Tyr394 phosphorylation, with intermediate amounts of CD45 optimally promoting Tyr394 phosphorylation, again in the absence of ligand.

This discussion of Lck phosphorylation status is not meant to rule out the possibility of an influence of TCR ligation on Lck structure or function. Indeed, it is difficult to fully exclude the possibility that a small pool of Lck molecules, proximal to the stimulated TCR and, perhaps, bound to a coreceptor, is activated during TCR stimulation. Indeed, a Lck biosensor has detected presumed Lck activation following TCR stimulation on the basis of an allosteric change in a small localized pool of Lck biosensor molecules[93]. Super-resolution microscopic techniques have suggested that Lck is in small clusters in the basal state but becomes more highly clustered and less mobile with stimulation by anti-TCR immobilized to a surface[94]. Curiously, this clustering is independent of Lck phosphorylation status, its N-terminal membrane myristoylation and palmitoylation sites and the participation of its SH2

domain. The basis for clustering was best attributable to an open Lck conformation. CD45 was excluded from clusters that contained Lck and the TCR. However, how such clustering is controlled and whether it contributes to TCR signal initiation or occurs in response to signaling needs further study under more physiologic stimulation conditions.

The models of TCR triggering discussed above have focused largely on ITAM phosphorylation by Lck as the initiating event in TCR signaling. This is based on work with T cell lines, such as the Jurkat or Hut78 lines, or with T cell lymphoblasts or clones that have been maintained in culture or have been cultured *ex vivo* for many hours. However, such studies have ignored findings[20,21,81,95] that the ζ-chain in unstimulated *ex vivo* thymocytes or T cells is constitutively tyrosine phosphorylated. This basal phosphorylation only modestly increases with robust TCR stimulation. Even Csk inhibition, which robustly increases downstream phosphorylation events, has minimal effect on ζ-chain phosphorylation on *ex vivo* thymocytes or T cells[68]. Interaction with coreceptors and with endogenous peptide–MHC seems important for such constitutive ζ-chain phosphorylation[19,95]. If ζ-chain ITAMs are phosphorylated, why do subsequent downstream signaling events not occur?

At least one subsequent step in TCR signaling does occur: the binding of Zap70 via its tandem SH2 domains to the doubly phosphorylated ITAM in *ex vivo* thymocytes and unstimulated T cells[19]. However, although bound to a phosphorylated ζ-chain, Zap70 is itself neither phosphorylated nor activated. Insights into how Zap70 might bind ITAMs but not become activated come from the crystal structure of autoinhibited wild-type Zap70 (ref. 96). Binding of the Zap70 tandem SH2 domains to a doubly phosphorylated ITAM frees its SH2 domains and interdomain A from stabilizing interactions with the back of the kinase domain. However, another level of autoinhibition is revealed in a more recent Zap70 crystal structure[96]. The N-terminal lobe of the catalytic domain is held in an inactive conformation through a previously unappreciated interaction of Tyr319 in interdomain B with the N-terminal lobe of the catalytic domain, constraining the catalytic domain from adopting an active conformation. Phosphorylation of Tyr319 leads to its inability to interact with the N-terminal lobe of the catalytic domain and thereby allows the kinase to adopt an active conformation. Thus two steps are envisaged to be required for activation of Zap70 from its autoinhibited conformation. The first involves docking via its tandem SH2 domains to a doubly phosphorylated ITAM. This is a stable and highly selective, high-affinity interaction with a K_D in the low nanomolar range. This interaction helps protect the doubly phosphorylated ITAM from the action of phosphatases and prevents phosphorylated ITAM interaction with the Lck SH2 domain. The second step involves the Lck-mediated phosphorylation of Tyr319 (and possibly Tyr315) in ITAM-bound Zap70. One question remains: if Lck has phosphorylated the ITAM *in vivo*, what prevents it from also phosphorylating Tyr319 of Zap70? We speculate below on plausible mechanisms that may resolve this enigma.

A model of TCR triggering for future exploration

We close by proposing a model of TCR signal initiation (Fig. 4) that takes into account some of the observations noted above and builds on existing models. The control of Lck activity in

the basal state depends on the steady-state regulation of its phosphorylation status, which is governed by Csk, CD45 and PTPN22. Thus, in the basal state there is an amount of already active Lck that may or may not be associated with coreceptor. This is a dynamic steady state with ongoing phosphate turnover and is regulated by negative feedback circuitry that may operate to control spontaneous Lck trans-autophosphorylation. Negative feedback may control Csk activity or its membrane localization. This steady state will adapt to minor perturbations from noise or variation in endogenous peptide encounter and will act somewhat as a buffer to resist change in the system. Thus, this steady state sets a threshold to prevent Lck from increasing its activity, but this basal activity is sufficient to allow for significant ITAM phosphorylation under the conditions in which transient interactions between coreceptors, TCR, and endogeneous peptide–MHC occur. Stronger interactions with endogenous peptide–MHC[81,95] allow for more ζ-chain phosphorylation and more basal signaling by the TCR, as further evidenced by CD5 expression.

Unphosphorylated Zap70 is bound to phosphorylated ITAMs but is still autoinhibited as a consequence of the interaction between Tyr319 in interdomain B and the N-terminal catalytic domain. Several mechanisms might explain why the available active Lck does not phosphorylate the Tyr319 site to activate Zap70. Perhaps the timing of Zap70 binding does not allow it to interact with the TCR complex soon enough for Lck to phosphorylate Zap70—that is, Lck may diffuse away before Zap70 binds to the doubly phosphorylated ITAM. But why does Lck not return (as it does to phosphorylate ITAMs) later? Perhaps, when the TCR is unligated, Zap70 binding to phosphorylated ITAMs is very dynamic with fast turnover. Thus, there is not enough time for coreceptor-associated Lck to return and find bound Zap70 with high probability. This model may also explain why stronger binding of TCR to endogenous ligands leads to more signaling—this enables a higher probability of binding coreceptors, whose active Lck can phosphorylate Zap70 even if it turns over quickly.

Productive downstream TCR signaling is initiated when an agonist peptide–MHC complex serves to colocalize coreceptor-associated active Lck to TCR-associated ITAM-bound Zap70. Lck phosphorylation of Zap70 at Tyr319 relieves the autoinhibited constraint imposed by Tyr319 on the Zap70 catalytic domain, thereby leading to Zap70 activation via trans-autophosphorylation of Zap70 Tyr493. Tyr493 in the Zap70 activation loop is ‘preferentially’ phosphorylated by transphosphorylation by Zap70, rather than by Lck[97]. The presence of multiple dimeric ITAMs may, thus, serve to position pairs of Zap70 molecules across from each other so that they can mediate trans-auto-phosphorylation and activation following release from autoinhibition by Lck phosphorylation of Tyr319.

Phosphorylated Tyr319 is also a docking site for the SH2 domain of Lck, thereby stabilizing the active conformation of Lck, which confers upon it the ability to resist negative feedback mechanisms that could promote inactivation of Lck. Stabilization of the active conformation of Lck promotes its ability to further phosphorylate other Lck molecules on Tyr394 and Zap70 molecules associated with vicinal TCRs that are bound to other agonist or endogenous ligands, thereby generating a positive feedback mechanism that could then overwhelm other negative feedback loops that maintain the resting state. The binding of the Lck SH2 domain to ZAP70 also prevents the passive diffusion of Lck and its associated coreceptor from the ligated TCR. Indeed, the coreceptor interaction with MHC and the Lck

SH2 interaction with phosphorylated Tyr319 will help maintain the coreceptor in close proximity with the agonist peptide–MHC-bound TCR. Thus, this represents a positive feedback loop whereby the recruitment of a single Lck molecule can have an amplifying effect to phosphorylate other ITAM-bound Zap70 molecules in the vicinity that are bound to either agonist or endogenous peptide–MHC. It also allows for Lck phosphorylation of the Zap70 activation loop and Zap70 transphosphorylation of neighboring ITAM-bound Zap70 molecules. Triggering this positive feedback loop may represent the TCR signaling threshold that must be achieved before the TCR disengages from agonist peptide. The binding of coreceptor to MHC and of Lck to phosphorylated Zap70 may also promote rebinding of the same or neighboring TCRs to the agonist peptide–MHC.

We find this model attractive and consistent with older and more recent experimental findings. Some studies support the importance of the Lck SH2 domain as well as its interaction with Zap70 Tyr319, which has a crucial role in our proposed model of TCR signal initiation. First, mutation of the sequences C terminal to Tyr319 to a more optimal Lck SH2 domain binding site (from Tyr-Glu-Ser-Pro to Tyr-Glu-Glu-Ile) has been shown to increase TCR signaling[98]. In other studies, an effort was made to free Zap70 from its autoinhibited constraint by mutating Tyr319 (and Tyr315) to alanine. This resulted not in the hypermorphic variant of Zap70 anticipated but in a hypomorphic variant, perhaps because the Lck SH2 binding site was eliminated[99]. Finally, an elegant study aiming to understand coreceptor function reconstituted CD4 coreceptor function in a Lck-sufficient antigen-specific hybridoma with CD4-Lck fusion proteins[100]. The Lck SH2 domain, not its catalytic function, is crucial for coreceptor function, although some Src family kinace catalytic function is probably required and provided by endogenous Lck or Fyn present in the hybridoma. The model we have proposed is also consistent with several other models discussed here but adds potentially important features that are congruent with *in vivo* observations of substantial ITAM phosphorylation and Zap70 binding in the basal state. As with all models of TCR signaling, more experimental, orthogonal approaches are needed to test this model.

Conclusions

Here we have summarized and discussed some of the progress made in efforts to understand the selectivity and sensitivity of the TCR and its role as a signal transduction machine. Clearly, considerable progress has been made, but many of the remarkable features of the TCR remain enigmatic. We here suggest a new, nuanced model of TCR triggering in an effort to stimulate further discussion and experimentation in the field.

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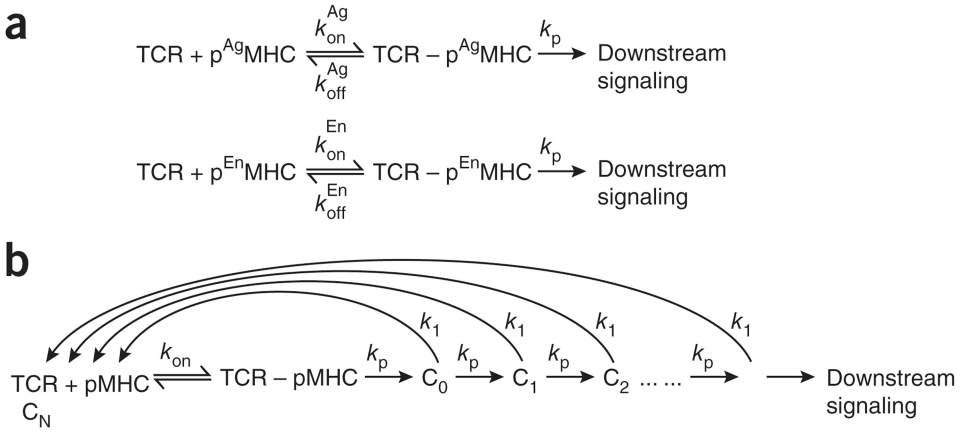

Figure 1.

Schematic depiction of kinetic proofreading. Models of TCR binding and kinetic proofreading. (**a**) A model where TCR binds to two different ligands. The complexes thus formed can lead to downstream signaling with rate k_p. The agonist and endogenous ligands bind to TCR with different on- and off-rates (k_{on}^{Ag}, k_{off}^{Ag}, k_{on}^{En}, k_{off}^{En}). Ag, agonist; En, endogenous; p^{Ag}MHC, agonist peptide–MHC; p^{En}MHC, endogenous peptide–MHC. (**b**) A model that includes kinetic proofreading. Following TCR binding to peptide–MHC (pMHC), a series of biochemical transformations must occur to form intermediates (C_0, C_1 and so on) before product downstream signaling can occur. Each modification can be reversed completely, with rate k_1. This series of biochemical modifications must be driven out of equilibrium by ATP consumption.

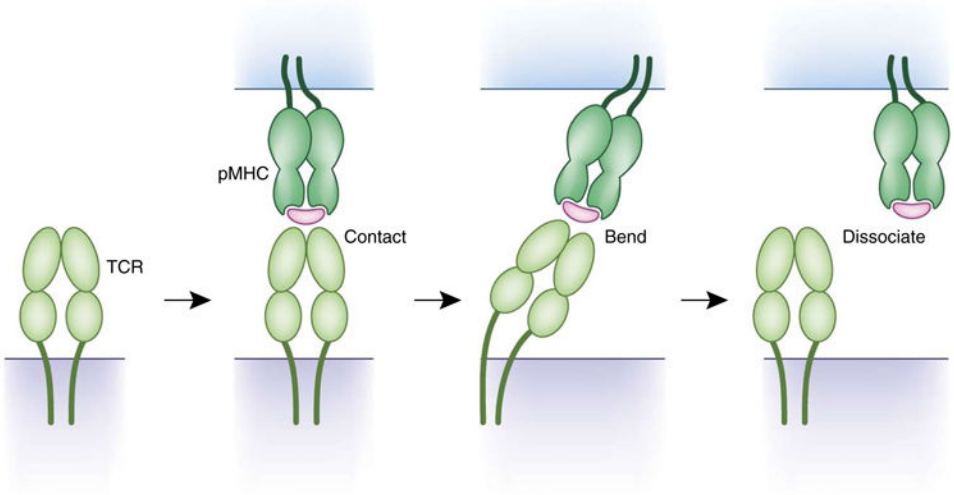

Figure 2.

Schematic representation of the mechanosensor model of TCR signal initiation. After TCR binds to the ligand, the crawling of the T cell on the APC results in a force that imparts a torque on the TCR, thus positioning the TCR-CD3 complex in the architecture necessary for signaling to ensue. This schematic depicts how torque can cause a conformational change or reorientation of the TCR to help facilitate downstream signaling.

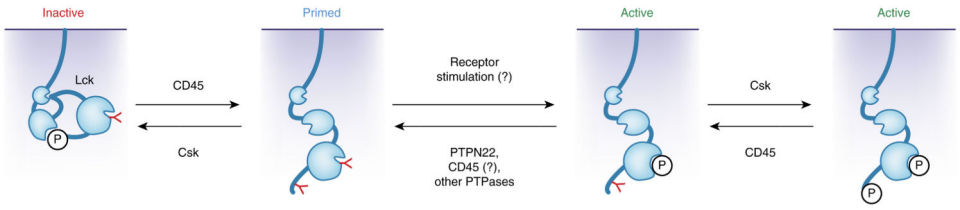

Figure 3.

The regulation of Lck by phosphorylation. Shown are the four states of phosphorylation described in this review, based on phosphorylation of Tyr394 in the activation loop of the catalytic domain and of Tyr505 in the negative regulatory segment. Also shown are the molecules thought to regulate the transition between the different phosphorylation states.

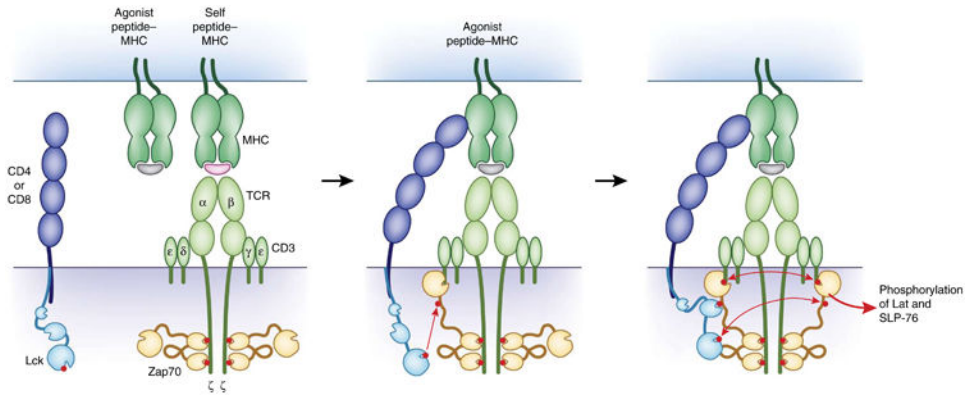

Figure 4.

TCR activation as a consequence of the combined relocalization of CD4 and CD8 coreceptors and bound Lck. In the basal state, the ζ-chain ITAMs are phosphorylated (red dots) and bound to autoinhibited Zap70. Lck initiates activation by phosphorylating Zap70 Tyr319 in interdomain B, relieving Zap70 autoinhibition and creating a binding site for the Lck SH2 domain, further stabilizing the activated state of Lck and, thereby, initiating a positive feedback loop in which Lck can promote further local phosphorylation events. Zap70 trans-autophosphorylation of its activation loop results in its catalytic activation.