

Activated TCRs remain marked for internalization after dissociation from pMHC

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To assess the roles of serial engagement and kinetic proofreading in T cell receptor (TCR) internalization, we have developed a mathematical model of this process. Our determination of TCR down-regulation for an array of TCR mutants, interpreted in the context of the model, has provided new information about peptide-induced TCR internalization. The amount of TCR down-regulation increases to a maximum value and then declines as a function of the half-life of the bond between the TCR and peptide-major histocompatibility complex (pMHC). The model shows that this behavior, which reflects competition between serial engagement and kinetic proofreading, arises only if it is postulated that activated TCRs remain marked for internalization after dissociation from pMHC. The model also predicts that because of kinetic proofreading, the range of TCR-pMHC-binding half-lives required for T cell activation depends on the concentrations and localization of intracellular signaling molecules. We show here that kinetic proofreading provides an explanation for the different requirements for activation observed in naïve and memory T cells.

The activation of T lymphocytes is mediated by the association of specific T cell receptors (TCRs) with antigenic peptides bound to major histocompatibility complex (pMHC) on antigen-presenting cells (APCs). Variation in the sequence of a peptide or TCR can change a fully activating peptide (agonist) into one with a reduced ability to activate (partial agonist) or to a peptide that inhibits T cell activation (antagonist)^{1–5}. There is a positive correlation between the lifetime of the TCR-pMHC bond and T cell activation: agonists have the longest lifetimes and antagonists have the shortest^{2,6–8}; however, exceptions to this trend are well documented^{1,3,9,10}. Efficient T cell activation requires that the half-life for the bond between an agonist peptide and a TCR reside within an optimal limited range³. Consistent with this is the observation that weak agonists can have either shorter or longer half-lives than the wild-type agonists from which they were derived¹.

The observation that there is an optimal range of half-lives for T cell activation was anticipated¹¹ because of the simultaneous requirement for kinetic proofreading^{12,13} and serial engagement^{14,15}. Kinetic proofreading works at the level of the individual receptor: to become activated, a bound TCR must complete a series of biochemical modifications—for example, phosphorylations of immunoreceptor tyrosine-based activation motifs (ITAMs), association of ZAP-70, activation of ZAP-70, recruitment and phosphorylation of additional signaling molecules—before dissociating from the pMHC. Thus, the half-life of the

TCR-pMHC bond must be long enough to allow completion of the signaling events required for TCR activation. On the other hand, serial engagement works at the cell level. Because under physiological conditions the density of cognate pMHC on the APC is low, the half-life of the TCR-pMHC bond must be short enough to permit a single peptide to serially engage the multiple TCRs required for T cell activation.

Evidence that large numbers of nonagonist peptides may participate in T cell signaling, provided that agonist peptides are also present¹⁶, has called into question the biological relevance of serial engagement. However, evidence that certain alterations of the TCR, peptides or MHC result in an increase in the lifetime of the TCR-pMHC bond but a decrease in signaling^{1,5} suggest that serial engagement is a determining factor in whether a peptide behaves as a strong or weak agonist.

To test the hypothesis that a trade-off between serial engagement and kinetic proofreading leads to an optimal TCR-pMHC half-life for T cell activation, we developed a mathematical model for analyzing pMHC-induced TCR down-regulation. The model is based on fundamental processes, including binding, diffusion, biochemical modifications of TCRs and internalization, with no *ad hoc* assumption of serial engagement. Analysis of the model shows that competition between serial engagement and kinetic proofreading can account for the existence of an optimal TCR-pMHC dwell-time for TCR internalization (that is, a bond half-life that maximizes internalization)¹¹. However, we found

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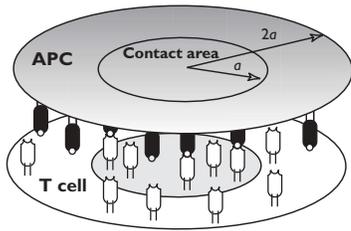


Figure 1. Model geometry. Binding occurs between a pMHC on the APC and a TCR at the T cell in the contact area ($0 \leq r \leq a$). Beyond the contact area ($a < r \leq 2a$) are transition regions on the APC and T cell. There is a TCR and pMHC concentration gradient in the contact area and transition regions. Outside the transition regions ($r > 2a$), TCR and pMHC concentrations are taken to be spatially uniform, but vary with time as TCRs are internalized and TCRs and pMHC diffuse into and out of the transition region.

that only certain models that include serial engagement and kinetic proofreading are consistent with the existence of a dwell-time that is optimal for TCR down-regulation. We determined the conditions under which this model predicts an optimal dwell-time and found that an optimal dwell-time is inconsistent with the simplest kinetic proofreading model, in which an activated TCR reverts rapidly to the basal state and is no longer subject to internalization after dissociation from pMHC. Rather, the data showing an optimal bond half-life for TCR internalization were consistent only with a model in which an activated TCR remains marked for internalization after dissociation from pMHC. The model also shows how the set of pMHCs capable of eliciting a vigorous T cell response depends on the concentration and localization of signaling molecules. Increased TCR access to intracellular signaling molecules broadens the range of bond half-lives of pMHCs that can activate a T cell.

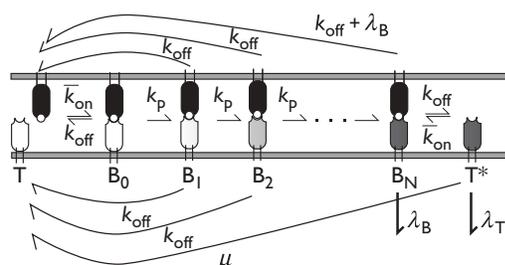
Results

The model

The mathematical model we used to interpret the data is based on assumptions described in the following sections. The model consists of a system of partial differential equations that describe the variation, with respect to time and position, of TCR and pMHC concentrations in distinct states. Details of the modeling and parameter estimation are available in the **Supplementary information** online.

Contact area

The activation of a T cell starts with the formation of a region of contact—a supramolecular activation cluster (SMAC)¹⁷ or immunological synapse¹⁸—between a T cell and an APC. We modeled the contact region as a disk of radius a (Fig. 1). In the model, the contact area remains fixed. A slow systematic variation in the size of the contact area with time has been observed¹⁸, but this is omitted from the model.



We did not model formation of the synapse or the spatial distribution of molecules other than TCR and pMHC. However, variation exists among cell types with respect to the stability, molecular organization and time-course of development of the contact region¹⁹. Our model applies best to experimental conditions under which a mature immunological synapse forms rapidly^{17,18}, with TCR and pMHC located predominantly in a central subdomain, surrounded by a ring of bound adhesion molecules. However, calculations of initial rates of TCR activation and internalization (**Supplementary information** online), which do not depend on the shape of the contact area, indicated that our qualitative results are robust and apply to differently organized contact regions, such as the immature synapse¹⁹. Also, although TCR-pMHC interactions and signaling events begin before stable cell-cell contact is established, these early events have a small impact on model-based predictions and experimental data when TCR internalization is monitored over a period of hours.

TCR-pMHC binding kinetics

We define \bar{k}_{on} (in $\text{cm}^2 \text{s}^{-1}$) as the two-dimensional forward rate constant for the binding of a TCR on the surface of a T cell to a pMHC on the surface of an APC. The bar notation distinguishes the two-dimensional forward rate constant (appropriate for interactions at the cell-cell interface) from the three-dimensional forward rate constant k_{on} (in $\text{cm}^3 \text{s}^{-1}$ or $\text{M}^{-1} \text{s}^{-1}$) that would be measured in a Biacore experiment. The dissociation rate constant is denoted by k_{off} (s^{-1}). Estimates of \bar{k}_{on} and k_{off} are obtained from a combination of experimental measurements, as described²⁰. Simulations based on a model for synapse formation²¹ suggest that not all rate constants lead to stable immunological synapses with disk-like contact areas. When we investigated how TCR internalization varied as a function of the dissociation rate constant, we allowed k_{off} to vary over many orders of magnitude. It is possible that some of the extreme values for k_{off} included in the analysis would not support stable synapse formation.

Mobility of TCR, pMHC and TCR-pMHC

The pMHC diffuses with diffusion coefficient D_p on the APC and the TCR diffuses with diffusion coefficient D_T on the T cell. The model encompasses alternative assumptions regarding the mobility of the bound complex. If a TCR-pMHC bond forms without inducing cytoskeletal interactions, the diffusion coefficient of the bound complex D_B is $D_B = D_p D_T / (D_p + D_T)$ ²⁰. At the other extreme, cytoskeletal interactions may immobilize the bound complex, resulting in $D_B = 0$. Thus, the diffusion coefficient of the bound complex has values in the range $0 \leq D_B \leq D_p D_T / (D_p + D_T)$.

The dynamics of the interaction between TCR and pMHC in the immunological synapse have been examined with fluorescence photo-bleaching recovery (FPR)¹⁸. After a mature synapse formed between a 2B4 T cell and a substrate expressing the cognate peptide, a spot in the

Figure 2. The monomer model for TCR internalization. A TCR (T) binds to a pMHC with forward and reverse rate constants \bar{k}_{on} ($\text{cm}^2 \text{s}^{-1}$) and k_{off} (s^{-1}). The bound receptor (B_0) undergoes a sequence of biochemical modifications, each with rate constant k_p (s^{-1}). Modifications are reversed if the TCR dissociates from the pMHC before the final, N^{th} , modification. After N modifications, the bound TCR is “activated” and becomes subject to internalization, with rate constant λ_B (s^{-1}). If a fully modified bound TCR dissociates from the pMHC, the free TCR may be internalized, with rate constant λ_T (s^{-1}), and may revert, with rate constant μ (s^{-1}), to the basal state where it is no longer subject to internalization. If $\lambda_B = 0$, only unbound activated TCRs are subject to internalization.

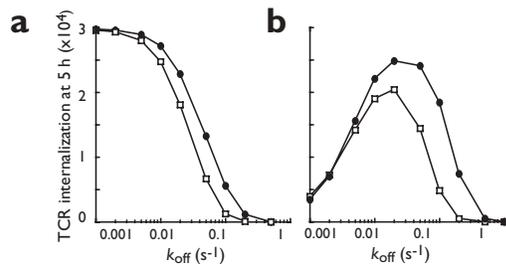


Figure 3. TCR internalization as a function of the dissociation rate constant. Results are shown for the monomer model (closed circles) and the dimer model (open squares). (a) Only activated bound TCRs are internalized, $\lambda_b = \lambda_d = 0.003 \text{ s}^{-1}$, $\lambda_r = 0 \text{ s}^{-1}$. (b) Only unbound activated TCRs are internalized, $\lambda_b = 0 \text{ s}^{-1}$, $\lambda_r = 0.003 \text{ s}^{-1}$.

contact area was bleached. On average only 2% recovery of fluorescence was observed in 400 s. In contrast, when Chinese hamster ovary (CHO) cells expressing glycosyl-phosphatidylinositol (GPI)-linked 2B4 TCRs were used, there was >60% recovery in 400 s. These FPR studies were interpreted as indicating that pMHC in the central cluster ($r \leq a$ in the model) of the immunological synapse were sequestered from the pool of free pMHC complexes and did not exchange and were not released from the cluster. Our model does not assume sequestration, but is consistent with an alternative interpretation of the FPR data. Because many of the TCRs on 2B4 cells are immobile¹⁸, many of the bound TCR-pMHC complexes will also be immobile. If a pMHC bound to a TCR is immobile, diffusing only when it is unbound, then its effective rate of diffusion in the mature synapse will be substantially reduced, and recovery in a FPR experiment will be slowed. Under these conditions, a pMHC will spend more time in the mature synapse, and serial engagement will be increased compared to when the bound pMHC-TCR complex is mobile.

TCR oligomerization

The question of whether or not TCR oligomerization is required for TCR activation remains open^{22,23}. To allow for both possibilities, we considered alternative versions of the model. In the “monomer model”, monomeric TCR-pMHC undergoes a series of biochemical modifications that lead to activation and internalization (Fig. 2). We studied the potential effect of a requirement for oligomerization in the simplest setting, where only dimers form. In this way, we avoided the ambiguities that arise in a kinetic proofreading model where oligomers of size 3 or greater are allowed to form and dissociate. In the “dimer model” (Web Fig. 1 online), ligation of a TCR allows it to aggregate with a second ligated TCR to form a dimer, and only after dimerization can the processes of modification, activation and internalization proceed.

Kinetic proofreading, TCR activation and down-regulation

We assume that after a TCR goes through a sequence of N biochemical modifications, it is activated and becomes subject to internalization and degradation²⁴. Different T cell responses may require different numbers of modifications. In modeling TCR down-regulation, we use the term “activated” to refer to TCRs that have accumulated the N modifications that target the receptor for internalization. For a more general consideration of activation, see the **Supplementary information** online.

As in the original model of kinetic proofreading of TCR activation¹², we assume that the modifications have a common forward rate constant, k_p , and are effectively irreversible as long as the TCR-pMHC bond remains intact. For the response(s) of interest, the true

biochemical cascade is replaced by a series of unidirectional reactions. If the TCR dissociates from the pMHC before the final, N^{th} , modification, the modifications are lost and the TCR returns to the basal (unmodified) state.

The previous model¹² did not include internalization, and different assumptions regarding the steps between activation and internalization lead to different qualitative predictions regarding the dependence of down-regulation on the TCR-pMHC dissociation rate constant k_{off} . To accommodate the alternatives we decided to test, we introduced distinct internalization rate constants that apply to activated TCRs in distinct states (Fig. 2). A monomeric pMHC-bound activated TCR is internalized with rate constant λ_b , resulting in the loss of the TCR and the freeing of the pMHC to undergo further rounds of binding. If an activated TCR dissociates from pMHC before being internalized, we allow for the possibility that for a period of time the TCR remains subject to internalization, with rate constant λ_r . The free activated TCR can also revert to the basal (unmodified) state, where it is no longer subject to internalization. The rate constant for reversion of the activated TCR to the basal state is μ . We allow for the possibility that μ has different values for receptors in the immunological synapse and for those that have diffused out of the contact region. In the dimer model, an activated dimer is internalized with rate constant λ_d , resulting in the loss of two TCRs and the freeing of two pMHCs. If an activated dimer breaks up before internalization, the resulting monomers are considered to be active and are subject to internalization with rate constants λ_b for a bound monomer and λ_r for an unbound monomer.

Recycling and synthesis of TCR

TCRs undergo both constitutive and pMHC-induced internalization²⁵. Constitutively internalized TCRs recycle back to the cell surface, and thus there is an internal pool of TCRs in resting T cells. We omitted TCR recycling and TCR synthesis from the model in the initial applications described here. The model therefore corresponds to a cell line in which TCR synthesis is negligible over a 5-h period, the longest time period we consider, in experiments where recycling was blocked.

The dependence of TCR internalization on k_{off}

As discussed above, the simultaneous demands of serial engagement and kinetic proofreading could result in a peak in TCR internalization as a function of the TCR-pMHC dissociation rate constant (k_{off}). We predicted the amounts of TCR internalized over a 5-h period for a series of peptides that have varying values of k_{off} , but the same value for k_{on} ($1 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$) (Fig. 3). The simulations were for a T cell with 30,000 TCRs and an APC displaying 1000 pMHCs and were generated under alternative assumptions about how internalization works on activated TCRs. Case 1 (Fig. 3a) is where activated TCRs are no

Figure 4. TCR down-regulation as a function of TCR-pMHC half-life.

Data are derived from 5-h TCR down-regulation experiments done with a panel of TCR mutants derived from a K^b -VSV-specific TCR (N30.7). Each point represents the indicated TCR-pMHC pair: 1, V98D- K^b -VSV; 2, V98L- K^b -VSV; 3, G97A- K^b -VSV; 4, G99A- K^b A158T-VSV; 5, G97/99- K^b A158T-VSV; 6, G97A- K^b A158T-VSV; 7, N30.7- K^b A158T-VSV; 8, N30.7- K^b A158T-VSV; 9, G97/99A- K^b -VSV; 10, G99A- K^b -VSV. Half-lives are shown as relative units (RU) normalized to the half-life for the N30.7- K^b -VSV (number 7) interaction⁴. TCR down-regulation data are shown as RU normalized to TCR down-regulation measured for N30.7 hybridomas that were interacting with 10^{-4} – 10^{-5} M VSV peptide.

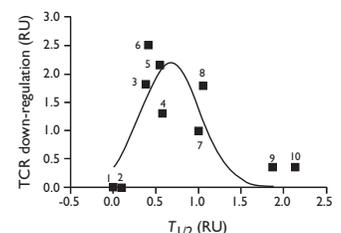
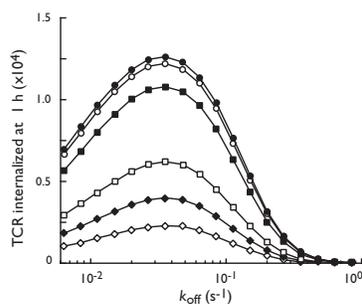


Figure 5. Relation between peak TCR internalization and the lifetime of the unbound activated state. The average time, τ , that the T^* state remains targeted for internalization is related to the rate constant μ for reversion of an activated TCR to the unmodified state by the equation $\tau = 1/\mu$. The graph shows the predicted number of TCRs internalized in 1 h as a function of the dissociation rate constant k_{off} for six values of τ . The symbols correspond to points at which the simulations were done. For the six curves, the values of the mean lifetime τ are 0.5 min (open diamonds), 1 min (closed diamonds), 2 min (open squares), 10 min (closed squares), 30 min (open circles) and 60 min (closed circles).



longer subject to internalization after dissociation from pMHC (Fig. 2, $\lambda_T = 0$). In this case, no peak was observed when internalization was plotted as a function of k_{off} . In contrast, case 2 (Fig. 3b) is where a bound activated TCR must dissociate from the pMHC before it can be internalized ($\lambda_B = 0$). In this case, a peak was evident.

A peak can be observed if both bound and free activated TCRs can be internalized. The outcome depends on the balance between λ_B and λ_T and on the values of the other parameters. However, if the bound activated state is the only one subject to internalization, TCR internalization over any fixed period will be a monotonically decreasing function of k_{off} (Fig. 3a) and a monotonically increasing function of the half-life of the TCR-pMHC bond.

Predicted amounts of TCR internalization were similar for the monomer and dimer models. For parameters typifying physiological conditions, the model predicts extensive TCR internalization, whether or not TCR oligomerization is a prerequisite (Fig. 3).

These predictions were supported by analysis of the initial rate of internalization, before extensive depletion of TCR or spatial redistribution of TCR and pMHC (Supplementary information online). The calculations do not depend on the shape of the contact region. To observe a peak in the initial rate of internalization as a function of k_{off} , it is necessary that activated TCRs remain subject to internalization for a period after dissociation from pMHC.

When TCR down-regulation was evaluated for a panel of TCR mutants that bind pMHC with different bond half-lives, an optimal window of half-lives for TCR internalization was observed (Fig. 4). Values for bond half-lives and TCR down-regulation are relative to those measured for the wild-type TCR (see Methods). The experimental observation of a peak provides evidence against case 1, in which bound activated TCRs are subject to internalization, but receptors are no longer subject to internalization when they dissociate from pMHC. To obtain the peak in TCR internalization (Fig. 4), unbound activated TCRs must remain marked for internalization for a period after dissociation from pMHC.

How long does the unbound activated TCR remain subject to internalization? The simulations summarized in Fig. 5 suggest that fully modified TCRs remain targeted for internalization for at least several minutes after dissociation from pMHC. Otherwise, we would not expect to see the pronounced peak observed experimentally in TCR internalization as a function of the half-life of the TCR-pMHC bond (Fig. 4). The six curves in Fig. 5 correspond to different rate constants, μ , for reversion of the activated TCR state (T^* , Fig. 2) to the basal state. The average lifetime of the activated state is $\tau = 1/\mu$. For lifetimes of the order of a minute (Fig. 5, lower three curves, average lifetimes 30 s,

1 min, 2 min), the predicted peak would be difficult to observe experimentally. For average lifetimes of 10 min to 1 h (Fig. 5, upper three curves) or more, predicted peaks are sufficiently prominent to be detectable.

The curves were generated under the assumption that the rate of reversion of an unbound activated TCR to the basal unmodified state is the same when the TCR is in the contact region and after it has diffused out of the contact region (Fig. 5). The predicted lifetime of the activated state is lengthened if, instead, TCRs lose the biochemical modifications that target them for internalization only after they diffuse out of the contact region. This condition could arise from the exclusion of specific phosphatases from the contact region²⁶.

T cell signaling molecules and TCR-pMHC half-lives

If activated TCRs are internalized only after dissociation from pMHC, the initial rate of internalization is maximal when k_{off} equals

$$k_{\text{off}}^{\text{max}} \approx \frac{k_p}{N-1} \quad (1)$$

k_p is the rate constant for each of N biochemical modifications in the signaling cascade leading to T cell activation. Because $k_{\text{off}}^{\text{max}}$ is proportional to k_p , equation (1) suggests that the optimal TCR dissociation rate constant for a peptide agonist should be cell-specific, depending in part on the concentration and accessibility of molecules that participate in the signaling pathway.

The approximation given by equation (1) holds if $\bar{K}T \gg 1$ when $k_{\text{off}} = k_{\text{off}}^{\text{max}}$, where $\bar{K} = \bar{k}_{\text{on}}/k_{\text{off}}$ is the two-dimensional equilibrium constant for TCR-pMHC binding and T denotes the concentration of unbound TCR in the contact area between a T cell and antigen-presenting surface. From a previous set of experiments¹⁸, estimated values of $\bar{K}T$ at 37 °C are 6 and 33 for two agonists, in two experimental systems²⁰.

The simulations in Fig. 3b show a value of $k_{\text{off}}^{\text{max}} = 0.02 \text{ s}^{-1}$, which is lower than the value $k_{\text{off}}^{\text{max}} 0.05 \text{ s}^{-1}$ predicted by equation (1) for the parameters used in the simulations. The discrepancy shows that the value of the optimal dissociation rate constant depends on the duration of an experiment. As noted, equation (1) is derived under conditions that hold at the start of an experiment.

Experiments in two systems show an increase in CD8⁺ T cell responsiveness over time, which correlates with increased Lck expression²⁷ or increased association of Lck with CD8²⁸. Either up-regulation or redistribution of Lck to the plasma membrane would correspond, in the kinetic proofreading model, to an increase in k_p , the parameter that governs

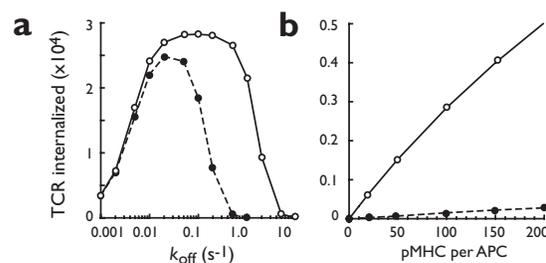


Figure 6. Effect of increasing the rate constant for biochemical modification. Predicted TCR internalization for the monomer model is plotted for two values of k_p , the rate constant for biochemical modification. The broken and solid curves correspond to $k_p = 0.25 \text{ s}^{-1}$ and $k_p = 2.5 \text{ s}^{-1}$, respectively. (a) TCR internalization as a function of k_{off} at 5 h when there are 1000 pMHC per APC. (b) TCR internalization at 1 h as a function of number of peptides per APC, for $k_{\text{off}} = 0.2 \text{ s}^{-1}$.

the rate of biochemical modifications. To address these experiments using our model, we simulated TCR internalization, varying the value of k_p . Increasing k_p from 0.25 to 2.5 s^{-1} broadened the window of k_{off} values for which TCR internalization is high, so that for a range of k_{off} values, there was considerably more TCR internalization with the high than the low k_p value (Fig. 6a). Thus, by increasing TCR access to signaling molecules, the range of pMHCs to which the T cell responds is broadened. An increase in k_p allows weakly binding pMHC (that is, pMHC with a high k_{off}) that would not induce internalization at the lower k_p value to trigger robust internalization. The prediction that increasing a T cell parameter, k_p , can change a weak agonist into a strong agonist also is illustrated in another way (Fig. 6b). For a pMHC with $k_{off} = 0.2 s^{-1}$, there was little peptide-induced TCR down-regulation with $k_p = 0.25 s^{-1}$, but extensive down-regulation at the higher value, $k_p = 2.5$, corresponding to a T cell with more, or more accessible, signaling molecules (Fig. 6b).

Discussion

Two main conclusions follow from the comparisons of model-based predictions and experimental data we have presented here. First, activated TCRs that dissociate from the activating pMHC continue to be targeted for internalization. This conclusion follows from the experimental observation that TCR down-regulation peaks as a function of the half-life of the TCR-pMHC bond. We have shown that the existence of an optimal half-life is inconsistent with a model in which, after dissociating from a pMHC, an activated TCR reverts rapidly to the basal (unmodified) state and is no longer marked for internalization.

For measures of TCR activation other than internalization, the existence of an optimal half-life does not distinguish between models with and without an activated unbound TCR state in which receptors are subject to internalization. Either model could be consistent with an optimal bond half-life for interleukin 2 production, proliferation or other measures of T cell activation. The behavior of down-regulation, as opposed to other measures, is essential in order to rule out a model in which an activated TCR is no longer subject to internalization after dissociation from pMHC.

Kinetic proofreading and serial engagement have competing effects, and there should be an optimal range of TCR-pMHC bond half-lives that are long enough to permit bound TCRs to progress through the signaling pathway and short enough to permit one pMHC to engage many TCRs^{5,11,29}. A result of the mathematical model we present here is that some models incorporating kinetic proofreading and serial engagement are inconsistent with the observation of an optimal half-life for TCR down-regulation. The predictions of alternative models hinge on the fate of the pMHC when a bound TCR is internalized. Serial engagement requires repeated freeing of bound pMHC. If the internalization process acts predominantly on bound and activated TCRs, stretching and breaking the TCR-pMHC bond and leaving the pMHC free on the APC when the T cell internalizes the TCR, then internalization enhances serial engagement. Even peptides that form long-lived bonds with TCRs can, in this case—because of TCR internalization—be liberated to engage new TCRs. pMHCs that dissociate rapidly still induce more serial engagement than pMHCs with long bond half-lives, but differences in the number of TCRs engaged by pMHCs with different dwell-times are reduced if internalization provides an additional mechanism for recycling peptide.

It is not obvious that the boost in serial engagement provided by peptide released when a bound TCR is internalized is sufficient to result in the qualitative behavior we observed (that is, no optimal TCR-pMHC dwell-time for TCR internalization unless activated TCRs remain marked for internalization after dissociation from

pMHC), but this is what analysis of the model showed. If internalization acts primarily on bound activated TCRs, kinetic proofreading always wins over serial engagement, that is, long-lived bonds are always better at activating T cells. It is also not obvious that activation should differ from internalization with regard to the quantitative effects of the competition between serial engagement and kinetic proofreading. Although there is no optimal TCR-pMHC dwell-time for TCR internalization in the case where activated TCRs are no longer subject to internalization after dissociation from pMHC, there may still be an optimal dwell-time for TCR activation. We found that the concentration of fully activated TCRs may or may not have a maximum, depending on the parameter values.

In the model we used to analyze the relationship between TCR internalization and the half-life of the TCR-pMHC bond, we did not assume *a priori* that serial engagement occurs. Rather, we modeled TCR-pMHC binding and dissociation using parameters estimated from published data^{18,20}. The model predicts that serial engagement will be extensive under some conditions but not others. Serial engagement occurs when peptides are in limited supply relative to TCRs and the forward and reverse rate constants for TCR-pMHC binding are large enough to permit multiple cycles of binding and dissociation. The model predicts that there will be little serial engagement when the TCR-pMHC dissociation rate constant is too low, if—as we concluded—internalization does not act primarily on bound TCRs, thereby freeing pMHC to interact with other TCRs. Consequently, the model predicts that TCR internalization will be low if TCR-pMHC complexes dissociate too slowly. The fact that little internalization is observed experimentally under conditions where little serial engagement is expected argues for the biological relevance of serial engagement.

Our second conclusion is that increasing the concentration or accessibility of signaling molecules required for T cell activation increases T cell sensitivity in two respects. T cells with higher numbers of signaling molecules available to interact with TCRs respond to lower concentrations of specific pMHC and respond to additional pMHC that form shorter lived bonds with the specific TCR. The first of these effects was observed over the course of the T cell response in BALB/c and TCR-transgenic mice infected with lymphocytic choriomeningitis virus (LCMV)²⁷. Maturation in the sensitivity of the T cell response was not accompanied by a marked increase in TCR affinity for pMHC or by an increase in the expression of adhesion molecules²⁷. Instead, the observed T cell sensitivity to antigen correlated with an increase in Lck content only in antigen-specific T cells. Lck-dependent differences in T cell sensitivity have also been observed²⁸. Naïve, but not effector and memory, CD8⁺ T cells require costimulatory signals for robust T cell activation²⁸. This difference was attributed to an increased association of Lck with the CD8 coreceptor in the effector and memory T cells compared to naïve T cells.

In our model, a single parameter, k_p (the effective rate of biochemical modification), reflects the availability and activity of the many intracellular signaling molecules that interact with a TCR during signal transduction. Although it does not make sense to identify this complex parameter with a single signaling molecule, increased availability of any of the many components of the signaling pathway (for example, Lck^{27,28}) enters the model as an increase in k_p .

A prediction of the model is that increased availability of signal transduction molecules increases cross-reactivity. We have shown that there is a broader set of dissociation rate constants of pMHC that are predicted to trigger a T cell clone when the modification rate constant k_p is higher, as in the case of effector and memory T cells^{27,28}. The forward rate of a kinase-mediated reaction along the activation pathway depends

on an intrinsic rate constant but also on the local concentration of the kinase. When activation reactions can proceed more rapidly, because of increased availability of kinases, the half-life of the TCR-pMHC bond can be shorter and still provide sufficient time for completion of the steps leading to TCR internalization, satisfying the kinetic proofreading requirement. Although it is not clear that cross-reactivity is a desirable feature of mature T cells, there is experimental evidence that memory T cells are more cross-reactive than naïve T cells^{30,31}.

On the other hand, if the T cell content of a signaling enzyme goes down, our model predicts a narrowing of the range of pMHCs that can trigger the cell. ZAP-70, a key kinase in T cell signal transduction, was down-regulated along with TCR over the course of a 5-h exposure of T cells to APCs³². It is possible that the effect is large enough that some pMHCs that bind to a specific TCR with a long enough half-life to induce TCR internalization in the initial stages of a response are no longer effective late in the response, when the pool of ZAP-70 has been depleted.

In summary, the mathematical model we have presented here provides a framework for testing hypotheses about the dependence of T cell signaling on the lifetime of the TCR-pMHC bond. Two ideas that have been both influential and controversial since they were introduced in 1995^{12,14} to identify requirements for T cell activation are serial engagement and kinetic proofreading. Using the model in conjunction with experimental results, we found evidence for the separate opposing effects of both serial engagement and kinetic proofreading. The model also showed how to extract an additional conclusion from the data: the existence of a TCR state (activated, no longer bound to pMHC and still targeted for internalization) that had not been postulated previously. Also, the kinetic proofreading component of the model suggested an explanation for observed differences between naïve and mature T cells. A testable prediction of the model is that TCR cross-reactivity should increase with increased availability of intracellular signaling molecules.

Methods

T cell receptor internalization model. Model is explained in detail in **Supplementary information** online.

T cell hybridoma activation by peptide-pulsed R8 cells and determination of TCR down-regulation. H-2K^b R8 cells (1×10^5) pulsed with different concentrations of vesicular stomatitis virus (VSV) peptide were cocultured with transfectant T cell hybridomas (2×10^5) expressing either the N30.7 TCR or each of the following TCR mutants: G97A, G99A, G97/99A, V98L and V98D, as described³³. After a 5-h incubation at 37 °C, T cell hybridomas were tested for TCR expression by flow cytometry with the use of the monoclonal antibodies phycoerythrin (PE)-anti-TCR β (H57) or PE-anti-V α 2 (B20.1), which were both from PharMingen (San Diego, CA).

Note: Supplementary information is available on the Nature Immunology website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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