Fast on-rates allow short dwell time ligands to activate T cells

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Two contrasting theories have emerged that attempt to describe T-cell ligand potency, one based on the $t_{1/2}$ of the interaction and the other based on the equilibrium affinity (K_D). Here, we have identified and studied an extensive set of T-cell receptor (TCR)-peptide-MHC (pMHC) interactions for CD4$^+$ cells that have differential K_Ds and kinetics of binding. Our data indicate that ligands with a short $t_{1/2}$ can be highly stimulatory if they have fast on-rates. Simple models suggest these fast kinetic ligands are stimulatory because the pMHCs bind and rebind the same TCR several times. Rebinding occurs when the TCR-pMHC on-rate outcompetes TCR-pMHC diffusion within the cell membrane, creating an aggregate $t_{1/2}$ (t_a) that can be significantly longer than a single TCR-pMHC encounter. Accounting for t_a, ligand potency is K_D-based when ligands have fast on-rates (k_{on}) and $t_{1/2}$-dependent when they have slow k_{on}. Thus, TCR-pMHC k_{on} allow high-affinity short $t_{1/2}$ ligands to follow a kinetic proofreading model.

affinity | kinetic proofreading | MHC | rebinding | T cell receptor

T cell receptors (TCRs) expressed on T cells bind host MHC proteins presenting both self- and foreign pathogen-derived peptides (pMHCs). Depending on the signal emanating from these interactions, diverse biological outcomes ensue. In the thymus, these TCR-pMHC-mediated signals shape the specificity of the mature T-cell repertoire and prevent overtly self-reactive T cells from escaping (1). In the periphery, naive T cells require continual TCR engagement with self-pMHC complexes to receive a homeostatic survival signal, whereas engagements with foreign peptides induce rapid T-cell division and the acquisition of effector functions (2). How T cells interpret the interaction between their TCR and pMHC ligands leading to these different biological outcomes is greatly debated.

Two competing models of T-cell activation have been proposed, with ligand potency being a function of TCR-pMHC equilibrium affinity (K_D) (3–7) or $t_{1/2}$ (8–11). Evidence supporting K_D-based receptor occupancy models of TCR signaling comes from sets of ligands that show a correlation between the K_D and ligand potency (3, 5) and from the fact that ligands induce qualitatively distinct biological outcomes depending on their concentration (12).

In sharp contrast to receptor occupancy models, $t_{1/2}$-based kinetic proofreading models hypothesize that the TCR must be engaged long enough to complete a series of signaling events, including coreceptor recruitment and TCR phosphorylation (13). Increases in the $t_{1/2}$ of the TCR-pMHC engagement raise the probability that any single TCR-pMHC engagement will surpass the threshold amount of time required to initiate T-cell activation (14). Recently, this threshold amount of time has been predicted to be at least 2 s (9, 15). Whether there is, in addition, an optimal $t_{1/2}$ that balances these kinetic proofreading requirements and the serial triggering of TCRs has been debated (16, 17).

Further evidence supporting $t_{1/2}$-based kinetic proofreading models arises from the discovery of antagonist pMHC ligands (18). TCR antagonists induce partial but not complete phosphorylation of the TCR complex and fail to activate T cells fully

at any ligand concentration (18). The subsequent discovery that antagonist ligands bind TCRs with a shorter $t_{1/2}$ than stimulatory agonist-pMHC complexes further suggests that activating ligands must engage a specific TCR for a long enough period to allow a series of signaling events to occur (19, 20).

As compelling as the arguments are for $t_{1/2}$ models of T-cell activation, discoveries of highly potent T-cell ligands with a short $t_{1/2}$ suggest that T-cell activation may not be solely dependent on the dwell time (4–6, 21, 22). In an attempt to reconcile why neither K_D nor $t_{1/2}$ fully predicts ligand potency, we have identified low-, medium-, and high-potency T-cell ligands that have medium and fast binding kinetics. The potency of these ligands fails to be described by either a K_D or $t_{1/2}$-based model. By mathematically modeling the biophysical mechanisms leading to T-cell activation using standard assumptions, our results indicate that fast k_{on} allow an individual TCR to bind and rebind rapidly to the same pMHC several times before diffusing away. The rebindings lead to an aggregate $t_{1/2}$ (t_a) that can be significantly longer than individual TCR-pMHC interactions. Importantly, ligand potency correlates closely with this t_a regardless of whether the ligands have fast or slow k_{on} or $t_{1/2}$s. These findings demonstrate that K_D and $t_{1/2}$ models of T-cell activation are not mutually exclusive, because both emerge from a t_a model. In particular, the t_a depends on the $t_{1/2}$ or K_D alone when k_{on} are low or high, respectively. The t_a allows strong K_D/fast-binding kinetic ligands to follow a kinetic proofreading model of activation.

Results

Identification of High, Medium, and Low K_D TCR–pMHC Interactions with Fast Rates of Association and Disassociation. During our previous study of TCRs specific for IA^b/3K, we noticed that several of these TCRs bound IA^b/3K with a strong K_D using very fast binding kinetics (22, 23). However, because some of the k_{off} were exceptionally fast, with loss of all specific binding for some occurring in less than 1 s, the original measurement had a significant error range. Using surface plasmon resonance (SPR) focusing on obtaining TCR-pMHC disassociation rates, we measured the binding kinetics of the B3K506 and B3K508 TCRs interacting with the previously reported and additional IA^b/3K altered peptide ligands (APLs) (Fig. 1).

Although the B3K506 and B3K508 TCRs interact with the IA^b/3K complex with a conventional K_D for agonist ligands (7 μM for the B3K506 and 29 μM for the B3K508), the binding kinetics of the interaction of the B3K506 TCR with IA^b/3K are
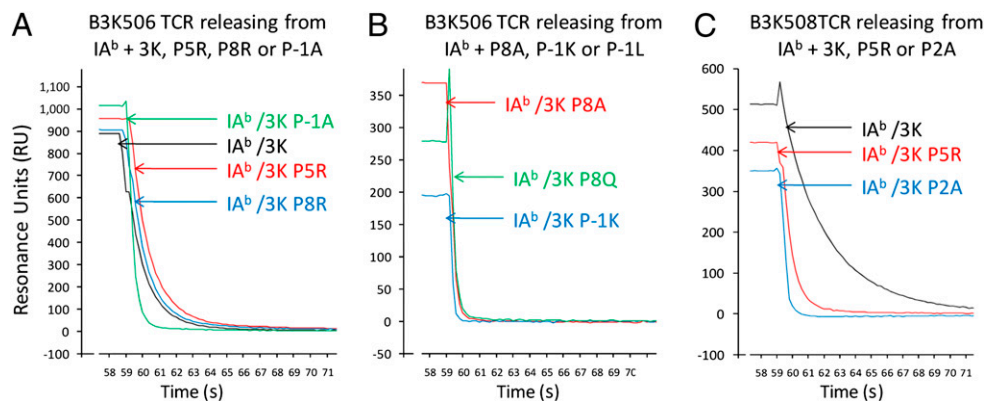
Fig. 1. Release of soluble IA^b/3K and APLs from immobilized B3K506 or B3K508 TCR, monitored SPR. Soluble IA^b/3K, P5R, P8R, or P-1A (A); P8A, P5Q, or P-1K loaded onto B3K506 TCRs (B); or IA^b/3K, P5R, or P2A loaded onto B3K508 TCRs (C) was allowed to disassociate for 60 s at a flow rate of 20 μL/min at 25 °C. Data were collected at 0.2-s intervals and fit to a 1:1 Langmuir binding model to determine the dissociation rate (k_{off}) and $t_{1/2}$ of the MHC/TCR complex. Curves are examples of three independent experiments.

extremely fast: k_{on} = 101,918/M·s and k_{off} = 0.7/s, leading to a $t_{1/2}$ of 0.9 s (Fig. S1 and Table S1). The K_Ds of other B3K506 and B3K508 TCR ligands range from 7–175 μM, all with fast or medium binding kinetics.
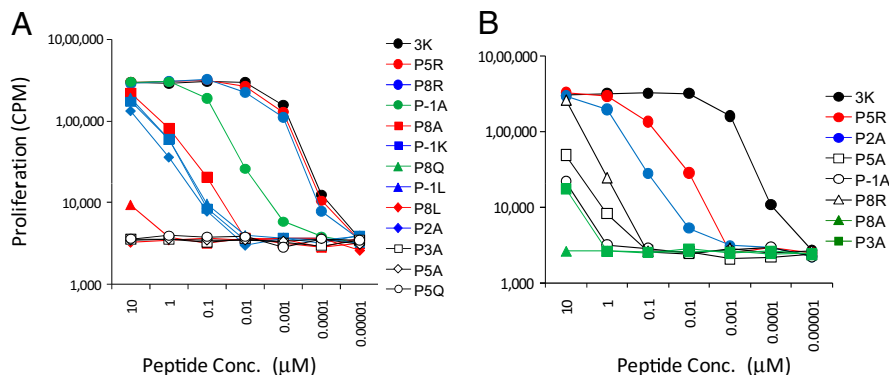
B3K506 and B3K508 CD4 T Cells Proliferate in Response to High, Medium, and Low K_D Ligands with a Very Short $t_{1/2}$. To determine the potency of high, medium, and low K_D ligands with differing binding kinetics, mature CD4 T cells from B3K506 and B3K508 Rag1$^{-/-}$ TCR transgenic (Tg) mice were incubated with titrating concentrations of peptides and assessed for proliferation (Fig. 2). Because the peptides with a K_D or $t_{1/2}$ beyond the SPR detection limit failed to induce significant activation, we do not consider them in our subsequent analysis. Of critical importance, except for a 2-fold increase in binding by the 3K P2A peptide to IA^b, the peptides all bind similarly to IA^b proteins (24). Furthermore, mature B3K506 and B3K508 CD4 T cells are equally sensitive to anti-CD3-mediated T-cell signaling, suggesting that the responses of these different T cells to stimulatory ligands can be directly compared (Fig. S2). Our data confirm that fast kinetic ligands can signal, suggesting that the 2-s limit on $t_{1/2}$ is not absolute. Notably, the B3K506 undergoes proliferation at submicromolar peptide concentrations by the 3K, P5R, P8R, and P-1A ligands ($t_{1/2}$ = 0.9, 0.9, 0.8, and 0.3 s, respectively) (Table S1).

Some T-cell ligands with a shorter $t_{1/2}$ than the immunizing ligand can induce superagonist or partial T-cell effector functions if the TCR complex is not efficiently ubiquitinated (18, 25).

To determine whether B3K506 and B3K508 T cells undergo complete activation in response to fast kinetic ligands, we chose two additional cellular functions to explore: (*i*) ligand-induced TCR down-regulation as a measure of receptor phosphorylation, ubiquitination, and degradation by Cbl-b (26) and (*ii*) cytokine production by T cells. Consistent with inducing complete phosphorylation of the TCR complex and T-cell activation, fast kinetic ligands induce TCR down-regulation and TNF-α production (Fig. S3 and Table S1).

Ligand Potency of 3K or APLs Fails to Obey Straightforward K_D or $t_{1/2}$ Model. Individually, ligand potency for the B3K506 or B3K508 T cells loosely follows the overall trend of both K_D- and $t_{1/2}$-based models. However, when B3K506 and B3K508 T-cell activation data are compared, neither model suffices (Fig. 3 and Table S1). In regard to K_D, the B3K508 T cells are hyperresponsive. For example, the 3K ligand induces proliferation of B3K506 and B3K508 T cells at a similar nanomolar range concentration, despite having significantly different K_Ds (7 vs. 29 μM). In another example, the B3K506 TCR binds IA^b/P-1A (26 μM) with a similar K_D as the B3K508 TCR binding IA^b/3K (29 μM), yet the B3K506 T cells proliferate at an EC_{50} that is 23-fold less than that of the B3K508 T cells. A failure of K_D to define the ligand potency is further apparent when additional 3K APLs are tested (Fig. 3*A* and Table S1).

In reverse correlation from K_D, ligand potency does not correlate with $t_{1/2}$ because the B3K506 T cells are hyperresponsive.

Fig. 2. Activation of 3K-reactive T cells to differing K_D ligands. B3K506 (A) and B3K508 (B) T cells proliferate when challenged with 3K and APLs. 3K APLs are listed next to each panel by decreasing K_D. Data are representative of at least three independent experiments.
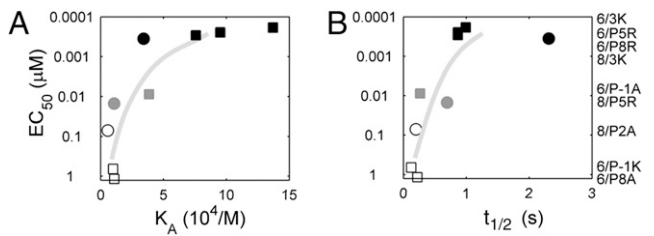
Fig. 3. Failure of K_D or $t_{1/2}$-based models to predict ligand potency. EC_{50} values, based on proliferation, are shown with respect to K_A (A) and $t_{1/2}$ (B). Data points are labeled by T cell, B3K506 (squares) or B3K508 (circles) and grouped by ligand potency: highest (black), intermediate (gray), and lowest (white). Specific TCR-pMHC pairs are listed to the right, ordered according to EC_{50}. The EC_{50} values are averaged over three measurements.

The 3K ligand induces similar proliferation of the B3K506 T cells ($t_{1/2}$ = 0.9 s) as the B3K508 T cells ($t_{1/2}$ = 2.2 s) (Table S1). In addition, the P5R ligand is significantly less potent in activating the B3K508 T cells than the 3K ligand is in activating the B3K506 T cells, despite having a similar $t_{1/2}$ (0.7 and 0.9 s, respectively). Multiple discrepancies can be observed when comparing other 3K APLs (Fig. 3B and Table S1). The finding that each T cell in isolation loosely follows both K_D- and $t_{1/2}$-based models appears to be an artifact of limited variation in the kinetics among the ligands for each T cell. A failure of K_D or $t_{1/2}$ to predict ligand potency is true for cytokine production as well, suggesting that the proliferation response is not anomalous (Fig. S3 and Table S1).

Consistently, activating ligands for B3K506 T cells use a fast k_{on} or strong K_D to compensate for a short $t_{1/2}$. (Because there is a simple relation among them, only two of the three parameters describing the interaction are independent.) Vice versa, B3K508 T cells compensate for a weak K_D by engaging IA^b/3K ligands for a longer $t_{1/2}$. These results suggest that ligand potency is determined by an interplay between the TCR-pMHC k_{on} and $t_{1/2}$ (or K_D and $t_{1/2}$) in a way that allows for enhanced signaling by fast kinetic ligands.

Does a Combined K_D/$t_{1/2}$ Model or Serial Triggering Predict T-Cell Ligand Potency? In an attempt to reconcile how the interplay of K_D and binding kinetics influences T-cell activation, we evaluated whether straightforward merging of the two predicts ligand potency. A combined K_D and $t_{1/2}$ model suggests that increasing the frequency or total number of TCRs engaged by pMHCs would stochastically result in an increase in the number of uncharacteristically long TCR-pMHC interactions. To test this, we identified the change in receptor occupancy required for a strong K_D fast kinetic ligand to be bound to an equal number of TCRs, on average, for at least 2 s as compared with a medium kinetic medium K_D ligand.

To approximate how frequently each pMHC ligand is bound to a TCR, we assume that a quasiequilibrium between TCRs and pMHCs occurs on the time scale of cell-cell contact and that TCRs are far in excess of the relevant pMHCs. The probability that a pMHC is bound to a TCR then depends on the equilibrium association affinity (K_A) through a simple saturation curve (3):

$$\frac{c_{pMHC-TCR}}{c_{pMHC}^0} = \frac{K_A c_{TCR}^0}{1 + K_A c_{TCR}^0} \quad \textbf{[1]}$$

The parameter c_{pMHC}^0 denotes the concentration of pMHCs on the antigen presenting cell (APC), c_{TCR}^0 denotes the concentration of TCRs in the interface, and $c_{pMHC-TCR}$ denotes the concentration of bound pMHC. c_{TCR}^0 was estimated to be 20 TCRs per square micrometer (10,000 TCRs per T cell per 500-μm^2 surface area of a T cell; SI Text). Within TCR islands, c_{TCR}^0 can be locally much

higher (80–430 per square micrometer) (27); however, increasing this value had little effect on our results. To convert the measured K_A of the TCR-pMHC pair in solution to K_A when the TCRs and pMHCs are membrane-bound, we have used a confinement length measured for the 2B4 TCR interacting with the MCC88-103 ligand (1.2 nm, corresponding to a conversion factor of 0.262 nm) (8).

The TCR-pMHC saturation curve from Eq. **1** contains a threshold K_D, K*, above which pMHC ligands are bound at least 50% of the time. Using the above approximations, K* is 130 μM and pMHC ligands with a 43-μM K_D are bound 75% of the time (Fig. 4). These values mirror measurements made by Grakoui et al. (8), in which the majority of a 60-μM K_D pMHC ligand was bound to a TCR when located within the interface of T cells and APCs. As a result of ligand saturation, strengthening K_D above 100 μM has only a modest effect on the overall frequency of TCRs bound to pMHCs. This saturation curve can be used to show that changes in TCR-pMHC occupancy do not describe ligand potency (SI Text).

By comparing ligands with similar EC_{50}s of proliferation yet different $t_{1/2}$s, we tested whether a merged K_D/$t_{1/2}$ model describes ligand potency. Specifically, the tests evaluate whether a stronger K_D for the B3K506 TCR engaging the pMHC generates enough additional bindings to overcome the lower probability of the bindings being long-lived. One comparison is the B3K506 TCR interacting with 3K/P-1A peptide (K_D = 26 μM, $t_{1/2}$ = 0.3 s, EC_{50} = 9 nM) and the B3K508 TCR interacting with the 3K/P5R peptide (K_D = 93 μM, $t_{1/2}$ = 0.7 s, EC_{50} = 15 nM). Assuming that TCRs bind pMHCs with exponentially distributed dwell times, the B3K506 TCR would have to bind 26-fold more IA^b/P-1A ligand than the B3K508 TCR binding IA^b/P5R to generate an equal number of 2-s engagements. However, the 3.6-fold difference in K_D between the two TCR-pMHC pairs leads to only a 1.5-fold difference in receptor occupancy. The effect is qualitatively similar for other comparisons (Fig. S4A) and is largely robust to assumptions about the parameters (SI Text). Thus, a merged K_D/$t_{1/2}$ model does not properly account for ligand potency. Based on similar reasoning, the effects of serial triggering cannot contribute significantly to ligand potency (Fig. S4 B and C and SI Text). It appears that the roles of the k_{on} and K_D in our data are not to increase the number of bindings, either at any given time (receptor occupancy) or over time (serial triggering).

Could Rebinding of TCRs to pMHCs Expand the Dwell Time for Fast Kinetic Ligands? The failure of K_D, $t_{1/2}$, or serial triggering models indicates that other mechanisms must underlie ligand potency.
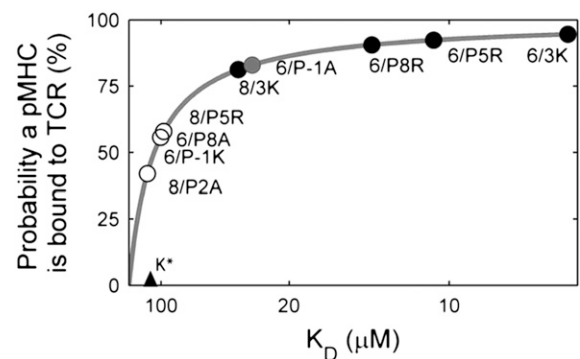
Fig. 4. Receptor occupancy depends only weakly on K_D for pMHC ligands with a K_D stronger than 130 μM. The receptor occupancy predicted by Eq. **1** is plotted, according to the parameter estimates in the text, on a scale that is linear in K_A (1/K_D). The predictions for the actual pMHC-TCR pairs in our experiments are superimposed on the plot (circles), colored (black, gray, or white) according to their actual activity as described in the legend for Fig. 3.

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The hypothesis of serial triggering, that individual pMHCs can sequentially bind multiple TCRs, led us to wonder whether a pMHC can bind multiple times to the same TCR. The ability of a receptor/ligand pair to associate, disassociate, and reassociate in a finite amount of time before complete disengagement is termed "rebinding." Although TCR-pMHC interactions are usually thought of as single binding events, it is theoretically possible that ligands with fast k_{on} may be able to rebind TCRs (28), especially because they are bound on membranes on which diffusivities are typically slower than in solution. If it occurred, TCR-pMHC rebinding would generate an aggregate dwell time (t_a) of interaction, assuming that the rebindings occur faster than the TCR signaling complex disassembles.

To investigate whether TCR-pMHC rebindings are plausible, we have followed an extensive set of work analyzing diffusion-influenced reactions (29, 30). Our approach has been to apply the particular estimate of the t_a, including rebindings, as provided by Bell (31), because of its simplicity and to suggest that the qualitative results are robust to the choice of model (see below and *SI Text*). In applying Bell's model (31), we assume that pMHCs and TCRs move purely diffusively on flat stiff membranes. Neglecting membrane forces is potentially in conflict with emerging work indicating the role of the actin cytoskeleton in breaking TCR-pMHC bonds, decreasing their $t_{1/2}$ (32). However, when k_{on} are fast enough for rebinding to occur, they happen very quickly; thus, it is unclear how much membrane forces could intervene.

The model also assumes that all rebindings occur at the same rate, which neglects any stabilization of binding that may be provided by coreceptors. Stabilization would have the effect of increasing the propensity of rebinding. Furthermore, the model counts only those rebindings that occur almost immediately, before the TCR and pMHC separate by more than a molecular length scale (e.g., 100 Å), on the order of 1 ms using the parameters below. Although the molecular details of TCR activation are not entirely understood (33, 34), TCR activation is not expected to be appreciably reversed on such short time scales.

Within this framework, Bell's result (31) for the total dwell time, summing the duration of any rebindings that occur, is:

$$t_a = t_{1/2} + \left[\frac{\ln(2)}{2\pi (D_{TCR} + D_{pMHC})} \right] \cdot K_A \qquad [2]$$

The parameters D_{TCR} and D_{pMHC} represent the diffusivities of TCR and pMHC, respectively. From Bell's result (31), it can be seen that the t_a is dependent on the individual $t_{1/2}$ and K_D. The first term in Eq. **2** accounts for the duration of the first binding, whereas the second affinity-dependent term accounts for any subsequent rebindings. Noting that every individual binding event lasts, on average, as long as any other, the expected number of rebindings between a particular pMHC-TCR pair is:

$$\bar{N} = \frac{k_{on}}{2\pi (D_{pMHC} + D_{TCR})} \qquad [3]$$

The parameter k_{on} denotes the on-rate of the pair on the membrane. The system has qualitatively different dependence on $t_{1/2}$ and K_D when k_{on} are small and large. When k_{on} are fast relative to the diffusion rates, pMHC binds and rebinds the same TCR many times, reaching a quasiequilibrium before diffusing away. As a result, the K_D dominates the duration of the interaction when k_{on} are high. However, when k_{on} are slow, rebinding does not occur and $t_{1/2}$ dominates. Because Eq. **2** can be independently motivated by simple arguments such as these, it is qualitatively robust to the choice of model (*SI Text*).

More generally, Eq. **3** suggests that there is a threshold k_{on} above which rebindings are relevant:

$$k_{on}^* = 2\pi (D_{TCR} + D_{pMHC}) \qquad [4]$$

Whenever the k_{on} exceeds this threshold (Eq. **4**; also known as the diffusion-limited rate), at least one rebinding is expected to occur. Importantly, the specific parameter values are important only insofar as they influence this threshold and not the underlying biophysical event (Figs. S5 and S6).

Rebinding of TCRs to pMHCs Uniquely Explains How Fast Kinetic Ligands Induce T-Cell Activation. To evaluate whether rebinding could have an impact on the dwell time of B3K506 or B3K508 TCRs engaging pMHC ligands, we applied Eq. **2** to our dataset. The diffusivities for a TCR and pMHC were estimated at 0.04 and 0.02 $\mu m^2/s$, respectively, corresponding to midrange measured values (*SI Text*). On-rates measured using SPR were converted to k_{on} on the membrane by assuming (*i*) that k_{off} of membrane-bound TCRs binding pMHCs are identical to SPR measurements and (*ii*) that the K_Ds of membrane-bound TCRs engaging pMHCs are proportional to SPR-measured affinities, as done in our analysis of receptor occupancy. Because of limited data, it is generally difficult to convert SPR-measured k_{on} directly to k_{on} on the membrane (35, 36). We discuss sensitivity to the assumptions in *SI Text*.

Using these parameter values, rebinding likely occurs for TCR-pMHC pairs with fast binding kinetics (Fig. 5). Specifically, this initial model predicts that the threshold on-rate for rebinding is 60,000/M·s. As a result, the expected number of rebindings increases from almost none to 1.7 as the on-rate increases in our sample from 11,000/M·s to 102,000/M·s. Because T-cell activity is generally thought to be very sensitive to $t_{1/2}$, a factor of 2 or 3 can be important. When rebindings are accounted for, the highly potent B3K506 T-cell ligands 3K, P5R, and P8R change from a $t_{1/2}$ of 0.9 or 0.8 s to t_as of 2.7, 1.9, and 1.8 s and the medium potent P-1A ligand converts from a $t_{1/2}$ of 0.27 s to a t_a of 0.72 s. Importantly, the t_a is significantly better at predicting ligand potency than the K_D or $t_{1/2}$ (Fig. 6C and Figs. S4, S7, and S8).

Within the dataset, two groups of high- or medium-potency ligands arise from different TCR-pMHC binding parameters (Table S1). Using these groups, the competing models can be quantitatively evaluated. The four high-potency ligands (3K, P5R, and P8R binding the B3K506 TCR and 3K binding the B3K508 TCR) have K_Ds and $t_{1/2}$s that vary widely by factors of 4.0 and 2.7, but t_as that vary only by a factor of 1.5 (Fig. 6C). The two ligands in the second most potent group (B3K506 TCR binding P2A and B3K508 TCR binding P5R) have K_Ds and $t_{1/2}$s that vary by factors of 3.6 and 2.6, respectively, but t_as that are almost identical, varying only by a factor of 1.1.

Although our t_a model was generated without empirically fitting the data, our estimate for the rebinding threshold, 60,000/M·s, is near the best fit for minimizing the variation in the t_as of the most potent group of ligands (Fig. S5). Quite similarly, for the medium-potent ligands, the best-fit threshold is 45,000/M·s (Fig. 6D). Convergence of the t_a model with empirical data suggests that the assumptions and underlying biophysical process are correct.

Discussion

Binding of two proteins is governed by the K_D, k_{on}, and $t_{1/2}$, any two of which suffice to describe the interaction because the three are simply related. Although ligand potency could be dependent on each of these binding characteristics, research over the past two decades has suggested that only the K_D or $t_{1/2}$ matters. Mechanistically, these two mutually exclusive models have been interpreted to mean that T cells are either (*i*) sensitive to the number of TCRs simultaneously bound to pMHC (3–6) or (*ii*) sensitive to ligands that produce a long enough interaction to phosphorylate the TCR complex fully (8–11, 13). In seeming

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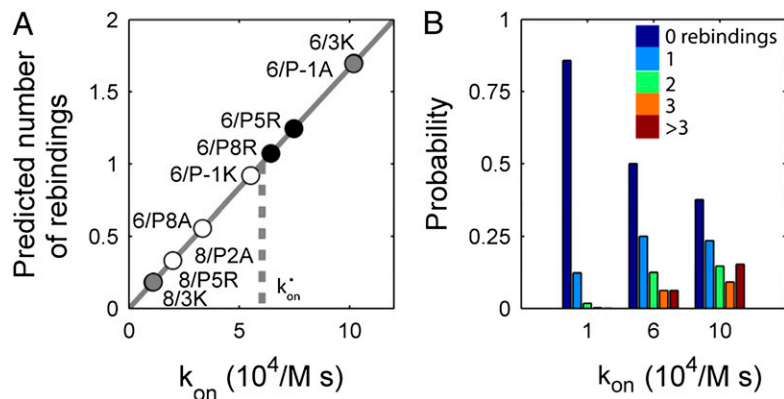
Fig. 5. Fast k_{on} lead to rebinding. (*A*) Average number of rebindings predicted by Eq. 3 is plotted vs. the k_{on}. The threshold for rebinding, k_{on}*, separates pairs expected to rebind at least once from those that rarely rebind. (*B*) Probability of zero, one, two, three, or more than three rebindings between TCRs and pMHCs, according to their on-rate, as predicted from Eq. 2 (*SI Text*).

contradiction to both theories, data presented here suggest that neither the K_D nor $t_{1/2}$ determines the potency of T-cell ligands.

A plethora of data suggests that T cells are increasingly sensitive to long-lived TCR-pMHC engagements, with a $t_{1/2}$ of 2 s being near the shortest allowable time (9, 15). Additionally, T-cell responses are dependent on ligand concentration, suggesting that T cells are also responsive to the frequency of these long-lived bonds. With this as a starting point, we asked how changes in the k_{on} or K_D might allow T cells to be equally re-active to ligands with a different $t_{1/2}$. The IA^b/3K model system is particularly well suited for this analysis because each of the 3K APLs binds IA^b similarly and a relatively large number of TCR-IA^b/3K APL pairs contain several that have similar potency, although using different K_Ds and binding kinetics. These controlled combinations of T cells and pMHC ligands allowed a direct comparison of the different theories of T-cell activation.

Because high-potency T-cell ligands with short $t_{1/2}$s all have fast k_{on}, we hypothesized that TCR-pMHC interactions may be influenced by diffusion rates. Although rebinding is potentially relevant for any binding event, it will be less important for cytosolic reactions because diffusivities in the cytoplasm are relatively high (31). However, when both the receptor and ligand are anchored on membranes, the rates of diffusion are drastically reduced. A recent study of the interaction between membrane-bound CD2 and CD58 using fluorescence recovery after photobleaching (FRAP) suggests that the fast-binding pair may rebind 100 times before separating, significantly increasing the duration of the bonds (37) and potentially explaining the pair's physiological activity (38).

Modeling TCR-pMHC interactions when both are membrane-bound shows that fast k_{on} allow rebinding to occur.

Depending on the k_{on}, this effect can greatly extend bond durations, allowing medium-potency ligands with measured $t_{1/2}$s of 0.3 and 0.7 s to generate a t_a near 1 s. As an independent example, the lymphocytic choriomeningitis virus-specific P14 TCR has been shown to bind its cognate H-2D^b-gp33 ligand with a low $t_{1/2}$ of 0.7 s (21). Because of a fast k_{on} of 400,000/M·s, our rebinding model predicts that the P14 TCR would have a t_a of 5.5 s, which is fully consistent with kinetic proofreading models of activation.

Most importantly, a rebinding-mediated t_a uniquely predicts ligand potency for B3K506 and B3K508 T cells (Fig. 6). Although our data initially appear to be in conflict with both K_D- and $t_{1/2}$-based activation models, the t_a model is consistent with reports that either $t_{1/2}$ or K_D can be the better predictor of ligand potency. T-cell ligands with slow k_{on} are predicted to follow a strict $t_{1/2}$-based reactivity pattern because rebinding does not occur and the t_a is equal to the $t_{1/2}$ of a single binding event. The canonical $t_{1/2}$-dependent systems, such as the 2B4-IE^k/MCC and 3L.2-IE^k/Hb TCR-pMHC pairs, have slow k_{on} compared with the rebinding threshold we have estimated (45,000–60,000/M·s) (10, 11). Because most T-cell activation studies have been done using these systems, $t_{1/2}$-based models have appeared sufficient and rebindings have not been required to understand ligand potency. For example, the k_{on} for the $t_{1/2}$-dependent 2B4/MCC system studied by Krogsgaard et al. (10) are all less than 6,670/M·s, such that almost no rebindings (<0.15) are predicted to occur.

In contrast to the canonical $t_{1/2}$ models, most T-cell activation studies suggesting that K_D is a better predictor of ligand potency have k_{on} larger than or close to the rebinding threshold (5, 6). Our data suggest that these correlations with K_D occur because
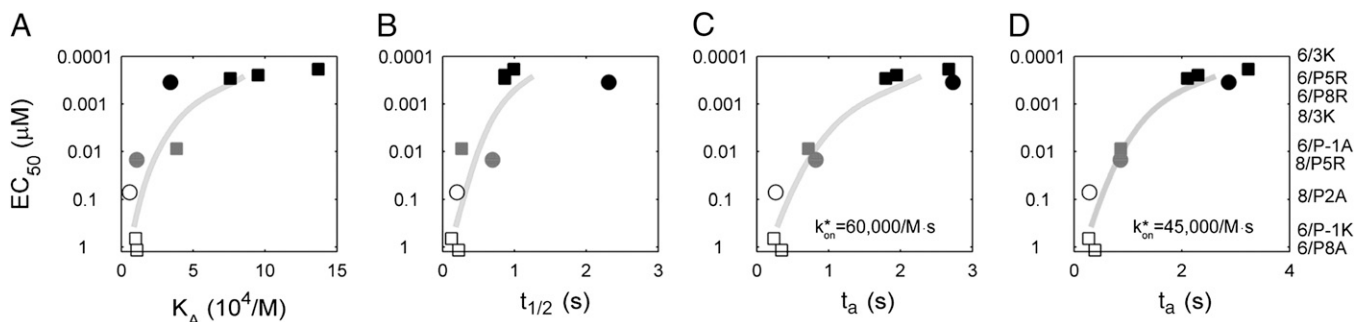
Fig. 6. The t_a is the best predictor of ligand potency for 3K-reactive T cells. EC$_{50}$ values, based on proliferation, are shown with respect to K_D (*A*), $t_{1/2}$ (*B*), and t_a (*C*), with the rebinding threshold set at 60,000/M·s, and (*D*) t_a, with rebinding threshold set at 45,000/M·s.

of rebinding. For example, the K_D dependence of the two peptides studied by Ely et al. (6) is consistent with a dependence on the t_a, with the more potent peptide having a 14-fold faster k_{on} and a predicted 1.3- to 1.4-fold longer t_a according to our model. Thus, observations that ligand potency is dependent on K_D or $t_{1/2}$ are not in conflict with each other; rather, they are different manifestations of the interaction between the T cell and APC when the k_{on} is very fast or very slow. With the continuing emergence of T-cell ligands with very fast k_{on} (4), our findings are likely to have an impact on a large repertoire of T cells.

On completion of this work, we have become aware of results for CD8$^+$ T cells that are in harmony with our conclusions (39).

Materials and Methods

C57BL/6 mice were purchased from the Jackson Laboratory. Rag1$^{-/-}$ B3K506 and Rag1$^{-/-}$ B3K508 TCR Tg mice have been previously described (22). All mice were maintained in a pathogen-free environment in accordance with institutional guidelines in the Animal Care Facility at the University of Massachusetts Medical School. Peptides were purchased from the Medical Research Council at the National Jewish Medical Center. Additional details are provided in *SI Materials and Methods*.

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