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COMPUTATIONAL BIOLOGY

Antigen Potency and Maximal Efficacy Reveal a Mechanism of Efficient T Cell Activation

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T cell activation, a critical event in adaptive immune responses, depends on productive interactions between T cell receptors (TCRs) and antigens presented as peptide-bound major histocompatibility complexes (pMHCs). Activated T cells lyse infected cells, secrete cytokines, and perform other effector functions with various efficiencies, which depend on the binding parameters of the TCR-pMHC complex. The mechanism through which binding parameters are translated to the efficiency of T cell activation, however, remains controversial. The "affinity model" suggests that the dissociation constant (K_D) of the TCR-pMHC complex determines the response, whereas the "productive hit rate model" suggests that the off-rate (k_{off}) is critical. Here, we used mathematical modeling to show that antigen potency, as determined by the EC_{50} (half-maximal effective concentration), which is used to support K_D-based models, could not discriminate between the affinity and the productive hit rate models. Both models predicted a correlation between EC_{50} and K_D, but only the productive hit rate model predicted a correlation between maximal efficacy (E_{max}), the maximal T cell response induced by pMHC, and k_{off}. We confirmed the predictions made by the productive hit rate model in experiments with cytotoxic T cell clones and a panel of pMHC variants. Thus, we propose that the activity of an antigen is determined by both its potency (EC_{50}) and maximal efficacy (E_{max}).

INTRODUCTION

T cell activation is a tightly regulated event that is critical for adaptive immune responses. It is driven by interactions between T cell receptors (TCRs) on the surface of T cells and peptide-bound major histocompatibility complexes (pMHCs) that are presented on the surfaces of antigen-presenting cells (APCs). These interactions are confined to the T cell–APC contact interface, termed the immunological synapse, and may persist for hours (*1*). On this time scale, the efficiency of T cell activation, as assessed by measurement of downstream functional responses, such as cytokine release, degree of cell lysis, and proliferation, depends on the binding parameters of the TCR-pMHC interaction (*2*–*15*). Over the past decade, two main models have emerged to explain how these chemical binding parameters affect the functional response of T cells. The "affinity model" states that the number of TCR-pMHC complexes at equilibrium is the primary determinant of the T cell response. Because that number is governed by the TCR-pMHC dissociation constant, K_D, it is argued that K_D is the primary determinant of the T cell response (*3*, *4*, *9*, *15*). In its simplest form, the second model, the "productive hit rate model," consists of two components. The first is that a single pMHC can initiate multiple signaling cascades by serially binding, or "hitting," multiple TCRs (*16*). The second component is that only TCR engagements of sufficient duration can lead to productive downstream signaling (*17*, *18*). When combined, these components predict that maximum stimulation of T cells may be achieved by pMHCs with an intermediate k_{off} (*6*, *7*, *12*). Put another way, an intermediate k_{off}, or equivalently, an intermediate half-life ($t_{1/2} = \ln 2/k_{off}$) enables TCR-pMHC binding events of sufficient duration to generate productive intracellular signaling without compromising the turnover of receptor-ligand encounters. In this model, the off-rate balances the probability of signaling during each binding event with the total number of binding events in a fixed time interval. In contrast to the affinity model, the productive hit rate model emphasizes the productive turnover of TCR-pMHC bonds rather than their absolute number. Over the years, these basic models have been extended to include additional observations; however, which model best determines activation efficiency remains controversial.

A mechanistic model that can predict activation efficiency on the basis of the bond parameters of the TCR-pMHC complex could be used for the rational design of TCRs and peptides to enhance the immune response in various therapies. For example, several immunotherapies aim to modify TCRs and consequently perturb the reaction rates of TCR-pMHC complexes, to modulate immune responses with potential applications in treating cancers, viral infections, autoimmune diseases, and allergies (*19*–*21*). This is the working business model of several pharmaceutical companies; however, many seemingly paradoxical results have been observed; for example, low-affinity TCRs can outperform high-affinity TCRs depending on the dose of antigen, and no single bond parameter can predict therapeutic outcomes (*14*, *22*–*24*).

Much of our knowledge of receptor-mediated cellular activation is derived from dose-response experiments, in which a downstream readout of activation is measured at various doses of a ligand specific for a cell surface receptor. These experiments can often be summarized by two numbers: the maximal response that the ligand produces (E_{max}, the maximal efficacy of the ligand) and the ligand potency (EC_{50}, the concentration of ligand that gives rise to a half-maximal response). In pharmacological studies, advances have been made in understanding

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heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptor (GPCR)–mediated cellular activation with mathematical modeling of dose-response assays. In these models, the steady-state concentration of the multiple GPCR states is calculated and used to predict how mutations in the ligand or GPCR might alter ligand potency, maximal efficacy, or both. These studies have systematically refined our understanding of GPCR-mediated cellular activation (*25*, *26*). In lymphocyte biology, dose-response experiments are routinely performed to assess the efficiency of T cell activation by measuring the amount of secreted cytokines after several hours of incubation with various doses of antigen (*2*–*5*, *9*, *13*–*15*). Studies have largely focused on antigen potency (EC_{50}) as the predictive parameter of cellular activation; indeed, EC_{50} is ubiquitously used not only to evaluate therapeutics but also to support specific models of T cell activation. Here, we used mathematical modeling to show that antigen potency (EC_{50}), the functional correlate used to support the affinity model, could not be used to discriminate between the affinity and the productive hit rate models of T cell activation. We showed theoretically that both models predicted a correlation between EC_{50} and K_D, but only the productive hit rate model predicted a correlation between maximal efficacy (E_{max}) and k_{off}. Dose-response experiments with $CD8^+$ T cells and a panel of pMHC variants confirmed the predictions of the productive hit rate model. We discuss how the productive hit rate model can explain previous findings, as well as its implications for TCR signaling models, and we relate the model to the pharmacological theory of dose response.

RESULTS

E_{max} discriminates between the affinity model and the productive hit rate model

We began by mathematically formulating the two proposed models. The system that we aimed to model was the immunological synapse, the contact area between a T cell and an APC or between a T cell and a synthetic antigen-presenting surface. Given that the reaction rates are rapid (with a time scale of seconds) compared to the time scale of activation assays (which have a time scale of hours), we assumed that the system was at steady state. With this assumption, we could determine the concentration of TCR-pMHC complexes, C, as:

$$C = (P_T + T_T + K_D - ((P_T + T_T + K_D)^2 - 4P_T T_T)^{1/2})/2$$

where P_T and T_T are the total concentrations of pMHC and TCR, respectively. K_D, the bond dissociation constant, is defined as the ratio of the kinetic parameters, namely, $K_D = k_{off}/k_{on}$, where k_{off} is the bond off-rate and k_{on} is the bimolecular bond on-rate. We plotted the number of TCR-pMHC bonds, CA (where A is the area of the contact interface), as a function of k_{off} for a fixed value of the bimolecular reaction on-rate (k_{on}) (Fig. 1A). In contrast to the affinity model, the productive hit rate model predicted that the rate of productive TCR-binding events determined the efficiency of T cell activation. At equilibrium, the rate of TCR-binding events was equal to the turnover of bonds ($k_{off}CA$), and therefore, the number of productive TCR-binding events can be given by:

$$R = k_{off} CAf(k_{off})t$$

where t is time (in seconds) and $f(k_{off})$ is a decreasing function of k_{off} between 1 and 0 that determines the probability that a binding event produces a productive downstream signal. In this equation, $k_{off}CAf(k_{off})$ is then the rate of productive hits. The exact functional form of f is irrelevant here, but is expected to be determined by the organization of the TCR-proximal signaling events. For example, a kinetic proofreading scheme predicts that

$$f(k_{off}) = (k_p/k_p + k_{off})^N$$

where N is the number of signaling steps, and k_p is the rate of each step (*17*). We related this derivation to the classical serial binding analysis (see the Supplementary Materials for details). We plotted the number of productive hits after 4 hours as a function of k_{off} (Fig. 1B). Both models captured only TCR-proximal events, and therefore, the functional T cell response (often in the form of secreted cytokines) is expected to be proportional rather than equal to the model predictions.

Most studies investigating these models perform dose-response assays (*2*–*5*, *9*, *13*–*15*) in which the efficiency of T cell activation is measured as a function of the dose of antigen. Support for the affinity model has come from studies that find statistically significant correlations between antigen potency (EC_{50}, the concentration of antigen that gives rise to a 50% maximal response in T cells) and K_D but poor correlations between EC_{50} and k_{off}. However, in many studies, a large correlation between K_D and k_{off} is found, which is thought to prevent the determination of which model is correct. In contrast, evidence for the productive hit rate model has not come from dose-response studies, but from various other assays (*6*, *11*, *12*, *27*).

To resolve this debate, we used the mathematical models derived above to predict the result of a dose-response assay for both models (Fig. 1, C and D). To extract the predicted value of the maximal response (E_{max}) from each model, we considered the limit of a large concentration of pMHC ($P_T \gg T_T, K_D$). Taking this limit revealed that

$$E_{max} = T_T A$$

for the affinity model and that

$$E_{max} = k_{off} T_T A f(k_{off}) t$$

for the productive hit rate model (see Supplementary Materials). This result showed that despite having large concentrations of antigen that saturated all of the TCRs, the productive hit rate model, but not the affinity model, predicted antigen discrimination on the basis of k_{off}. Unexpectedly, solving these equations for antigen potency, as measured by EC_{50} (the antigen concentration that gives rise to $E_{max}/2$), gave an identical prediction for both models (Fig. 1, C and D), namely, that

$$EC_{50} = K_D + T_T/2$$

Therefore, both proposed models were consistent with the published data, and the models could only be distinguished from each other by E_{max}, but not EC_{50}.

The predicted values of EC_{50} and E_{max} in the productive hit rate model, but not the affinity model, suggest that dose-response curves will be qualitatively different for antigens with different k_{off}. In the case of the affinity model, where only EC_{50} is predicted to vary between antigens, a greater concentration of antigen is predicted to always be able to compensate for a larger k_{off} (Fig. 1C). In contrast, the productive hit rate model predicts that both EC_{50} and E_{max} will depend on k_{off}, and therefore, the reduced T cell response to antigens with larger k_{off} cannot be compensated for by just increasing the concentration of antigen (Fig. 1D).

Dose-response experiments confirm the predictions of the productive hit rate model

To test the above predictions, we used the G10 CD8$^+$ cytotoxic T cell (CTL) clone, which is specific for the HIV gag epitope (*28*) and binds to a panel of 11 different pMHCs with various binding parameters (table S1). We generated these pMHC variants by introducing point mutations into the human leukocyte antigen A2 (HLA-A2) MHC at sites that are not expected to alter peptide loading (see structure in fig. S3). We determined the efficiency of T cell activation by incubating T cells with 32 titrations of 11 different pMHCs for 4 hours, after which we measured the concentration of interferon-γ (IFN-γ) in the culture medium. We used this large number of titrations rather than performing repeated measurements at fewer titrations because it provided more accurate estimates of EC_{50} (see the Supplementary Materials for details).

The resulting dose-response curves for five representative pMHCs revealed differences in both EC_{50} and E_{max} (Fig. 2A). The use of a conformationally sensitive antibody against A2 ensured that the observed E_{max} was not an artifact of pMHC saturation on the plate and that MHC mutations did not alter peptide loading (Fig. 2A, solid black line, and fig. S4). In agreement with previous reports, we found a correlation between EC_{50} and K_D (Fig. 2B); however, we also observed a correlation between E_{max} and k_{off} (Fig. 2E), which confirmed the mathematical modeling predictions of the productive hit rate model. We also observed correlations between EC_{50} and k_{off} (Fig. 2C) and between E_{max} and K_D (Fig. 2D), because the correlation between K_D and k_{off} in this system was large ($R^2 = 0.84$, $P < 0.001$). These data showed that a larger concentration of antigen could not compensate for larger off-rates. Together, these results provide support for the productive hit rate model.

Differences in E_{max} are the result of differences in the rate of IFN-γ secretion

The mathematical model predicted that differences in E_{max} resulted from differences in the rate of IFN-γ secretion, which itself was determined by the rate of productive hits. Alternative possibilities for differential E_{max} values included a differential lag in initial secretion of IFN-γ or the existence of different proportions of responsive cells. To examine the former possibility, we performed a time-course experiment at a single concentration of pMHC that gave rise to the maximal rate of IFN-γ secretion (that is, a concentration beyond which no further increases in IFN-γ secretion were observed for the particular pMHC). We found a linear increase in the secretion of IFN-γ after a time lag of 90 min, which was common to all of the pMHCs (Fig. 3A). This common time lag was likely associated with changes in transcription that ultimately increased the production of IFN-γ. The rate of IFN-γ secretion (as determined by the slope) directly correlated with k_{off} (Fig. 3B) despite the use of saturating amounts of pMHC.

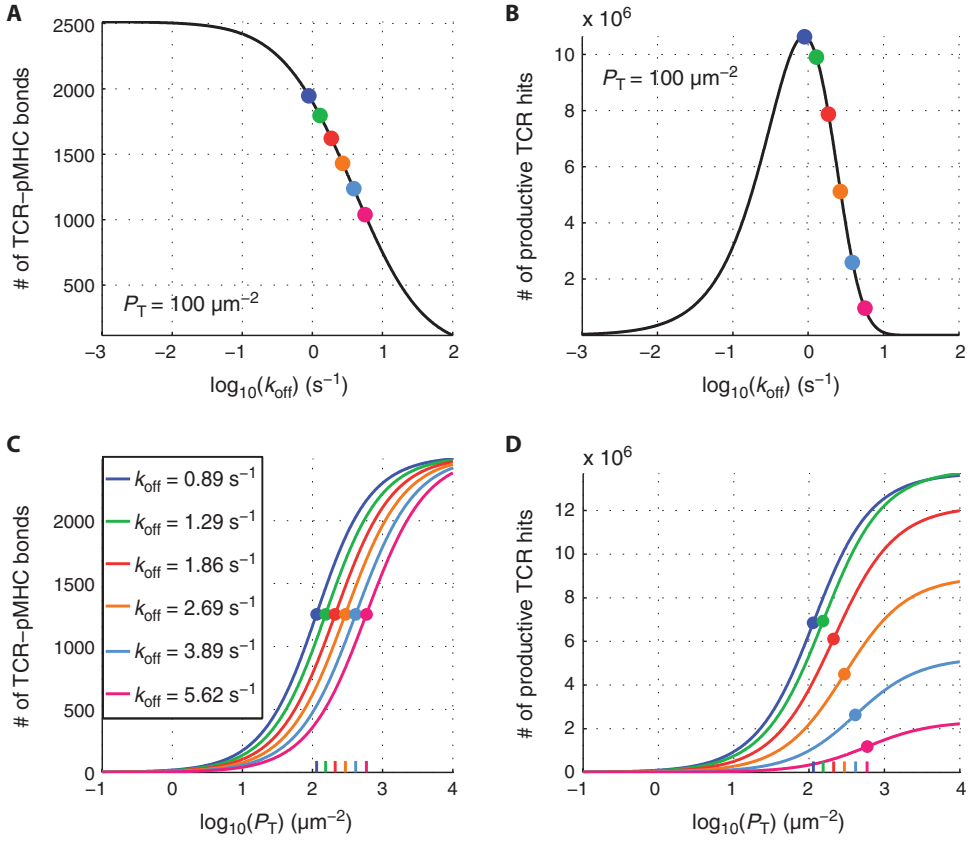

Fig. 1. Mathematical modeling predicts differences in maximal efficacy (E_{max}), but not potency (EC_{50}), between the affinity and the productive hit rate models. A steady-state model of TCR-pMHC binding at the contact interface was used to calculate the number of TCR-pMHC bonds and the number of productive TCR hits. (**A**) The affinity model proposes that the number of TCR-pMHC bonds (*y* axis) will determine the efficiency of T cell activation; therefore, decreases in k_{off} (or K_D) will always increase the activation efficiency. (**B**) The productive hit rate model proposes that the number of productive TCR binding events (*y* axis) will determine the efficiency of activation. In this model, increases in k_{off} will increase the number of TCR hits but will simultaneously decrease the probability that any given hit will last sufficiently long to transduce a productive intracellular signal. Therefore, an optimal k_{off} emerges. (**C** and **D**) The mathematical models were used to predict the dose-response assay for the (C) affinity and (D) productive hit rate models. Both models predicted the same relationship for antigen potency ($EC_{50} = K_D + T_T/2$); however, whereas the affinity model predicted that the maximum response was $E_{max} = T_T A$, the productive hit rate model predicted that $E_{max} = k_{off} T_T A f(k_{off}) t$. The dose responses in (C) and (D) are shown for six simulated antigens, with off-rates shown in (C) and represented as colored circles in (A) and (B). Filled circles and corresponding vertical ticks on the *x* axis [(C) and (D)] show the doses of antigen that gave rise to 50% of the maximal response (EC_{50}). Parameters: $k_{on} = 0.05$ μm^2/s; $T_T = 50$ μm^{-2}; $A = 50$ μm^2; $t = 4$ hours; $f(k_{off}) = (k_p/(k_p + k_{off}))^N$, where $N = 10$ is the number of signaling steps, and the rate of each step was defined by $k_p = 10$ s^{-1}. The exact functional form of $f(k_{off})$ is not important provided that it decreases from 1 to 0 as k_{off} increases.

To determine whether different proportions of cells were responding, we stimulated T cells as described earlier but blocked the secretion of IFN-γ for the last 2 hours of the incubation period, during which we expected IFN-γ production to linearly accumulate intracellularly over time.

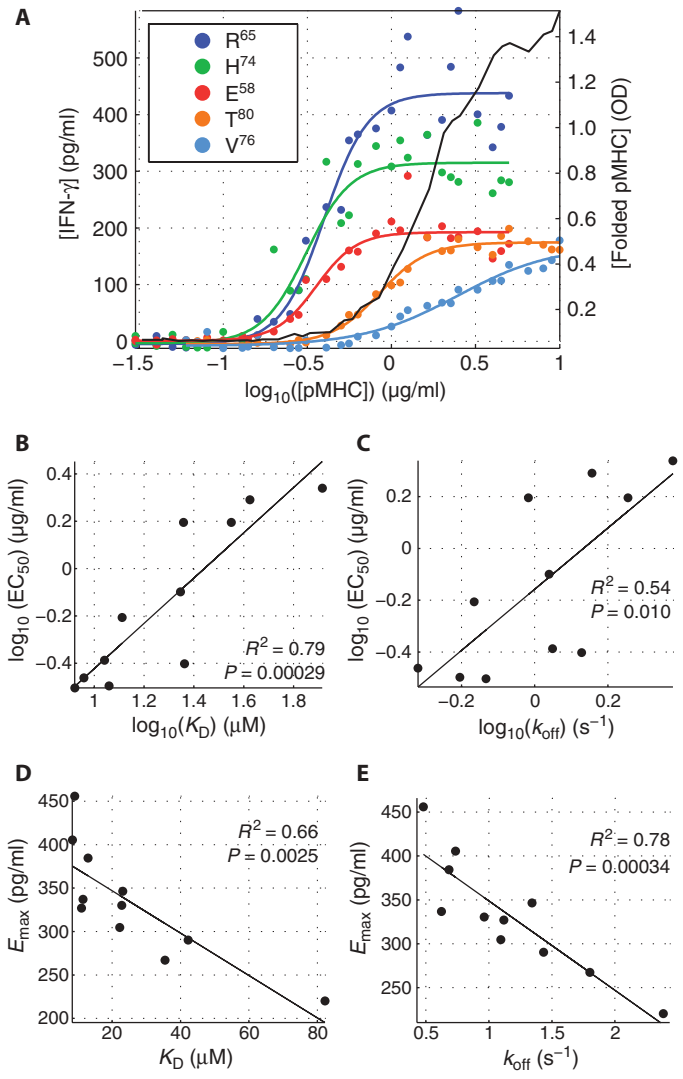

Fig. 2. Dose-response experiments support the productive hit rate model. (A) The concentration of secreted IFN-γ (colored circles) by G10 CTLs in response to 4 hours of incubation with the indicated plate-bound pMHCs at various doses was determined. A Hill function was fitted to the data (solid lines) to extract estimates of EC_{50} and E_{max} for each pMHC. The mean amount of pMHC on the plate (solid black line) indicates that IFN-γ saturation occurred before pMHC was saturated on the plate. Additional pMHCs and individual traces of pMHC amounts are shown in fig. S4. (B to E) Correlations between TCR-pMHC reaction parameters and EC_{50} [(B) and (C)] and E_{max} [(D) and E)]. As predicted by both models, a correlation between EC_{50} and K_D was observed (B). As predicted by the productive hit rate model, but not by the affinity model, a correlation was observed between E_{max} and k_{off} (E). Correlations with k_{on} can be found in fig. S5, and individual pMHC parameters are summarized in table S1. Optimization of the dose-response assay is discussed in the Supplementary Materials.

We then performed flow cytometric analysis to detect intracellular IFN-γ at the cell population level. We found broad distributions of IFN-γ (Fig. 3C), with roughly the same proportion of responding cells (Fig. 3D) but with different efficacies, as observed by the correlation between the mean extent of IFN-γ production and k_{off} (Fig. 3E). Together, these experiments showed that differential E_{max} values were determined by a difference in the rate of IFN-γ production, which was controlled by the rate of productive TCR hits.

Independent TCR systems exhibit differences in E_{max}

The results shown thus far were obtained from experiments with the G10 CTL clone. An important question is whether the results are reproducible in other systems. We therefore confirmed our key findings with the 1G4 TCR system (*2*). As predicted by the productive hit rate model, we found a relationship between K_D and EC_{50} and between k_{off} and E_{max} (fig. S8 and table S2). E_{max} was generally larger for the G10 CTL clone than for the 1G4 CTL clone, a plausible explanation for which is discussed below. Previous reports have focused on antigen potency, but by examining the raw dose-response curves in these studies, it is possible to find differential E_{max} values in other TCR systems (*5*, *7*, *9*, *15*).

Application of the confinement time model improves functional correlations

Our reported correlations are large given that the measured bond parameters were three-dimensional (3D), whereas the interactions took place within a 2D contact interface. We have taken great care to optimize our dose-response protocol (see the Supplementary Materials for details). To improve these correlations further, we and others have constructed models to relate 3D rates to 2D rates, which are more physiological because TCR-pMHC interactions occur in the 2D space of the plasma membrane (*2*, *8*, *13*, *29*). We therefore examined whether the previously proposed confinement time model (*2*, *8*, *30*), in which rapid rebinding between the TCR and the pMHC increases the effective lifetime of the complex, could improve our functional correlations. We found that antigen potency was unchanged in this model but that maximal efficacy (E_{max}) was predicted to be dependent on k_{on} (see Supplementary Materials for details). We found improved fits in the 1G4 system but did not find any improvement in the G10 system (fig. S12 and Supplementary Materials), which was likely a result of small variations in the k_{on} in the G10 system. The conversion between 3D and 2D rates may involve other independent parameters that were not measured here, such as the mechanical strength of the receptor-ligand interaction (*31*, *32*) or various thermodynamic parameters (*13*). Differences in these parameters may lead to larger differences in the 2D rates and may offer an explanation for the observed differences in E_{max} between the G10 and the 1G4 CTL clones, despite their having similar 3D reaction rates for the tested pMHC.

DISCUSSION

Over the past decade, studies have illustrated the effects of antigen dose, affinity, and lifetime in the modulation of lymphocyte cellular activation (*2*–*15*) and, recently, in the peripheral induction of $Foxp3^+$ regulatory T cells (*33*). Here, we showed that a productive hit rate model could accurately predict the relationship between these components in two independent experimental systems. The model predicted not only the observed correlations between K_D and antigen potency but also the correlation between k_{off} and maximal efficacy. In contrast, we showed that, whereas the previously proposed affinity model correctly predicted the correlation between K_D and antigen potency, it could not explain the observed differences in maximal efficacy.

The productive hit rate model that we used assumes that each TCR transmits only a single productive binding event for each pMHC-binding event and that the system is at steady state. We also considered two modifications of this simple model that enabled an activated TCR to continue signaling and another modification that included the non–steady-state dynamics of TCR binding and internalization (see Supplementary Materials). With the former modification, we never observed an optimal k_{off}, and with the latter modification, we observed an optimum as a function of k_{off} that became less pronounced at higher concentrations of antigen, as previously reported (*7*). Both model variants predicted that E_{max} may decrease with increasing k_{off}, as in our simple formulation. Future work using high-affinity engineered TCRs (*9*, *23*, *24*, *34*) in detailed dose-response assays combined with mathematical modeling is required to refine the productive hit rate model.

The TCR-pMHC kinetics reported here are 3D rates that were determined by surface plasmon resonance; however, TCR-pMHC interactions are confined to the 2D area of the plasma membrane, and direct measurements of these 2D kinetics have been made (*10*, *35*). Huang *et al.* (*10*) reported an intriguing negative correlation between 3D and 2D off-rates and in turn predicted that larger 2D off-rates increased antigen potency; these 2D rates were signaling-dependent. It is possible that signaling-induced changes in receptor mobility, clustering, or orientation might affect the measurement of 2D rates (*36*), and future work is required to determine signaling-independent 2D kinetics. Functional correlations between 2D kinetic parameters and antigen potency were performed by Huang *et al.*, but here we showed that antigen potency was a time-independent equilibrium measure, whereas maximal efficacy was not (fig. S7). It will be important to examine how the 2D kinetic parameters relate to E_{max}.

Antigen recognition by T cells is thought to involve a digital or switch-like response, whereby individual T cells either respond or not a few minutes after encountering antigen. This has been well documented, for example, by the all-or-none phosphorylation state of downstream signaling molecules, such as the extracellular signal–regulated kinases (ERKs) (*37*, *38*). However, the present study and a previous one (*39*) have provided evidence that the functional response of individual T cells is analog, whereby individual cells release IFN-γ in proportion to the antigen concentration and k_{off}. Our work is consistent with an early time switch

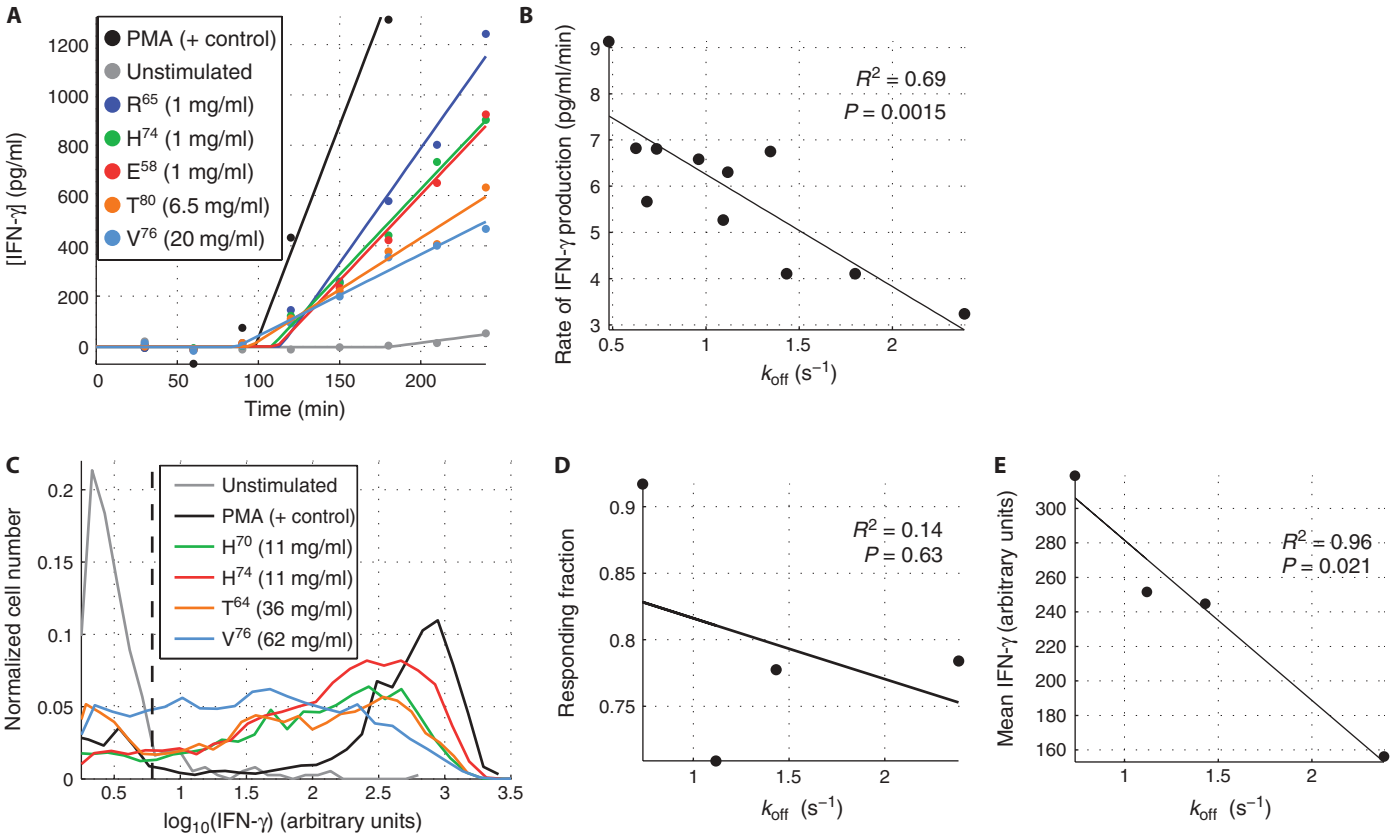

Fig. 3. Differential maximal efficacy (E_{max}) is a result of differences in the rate of IFN-γ production. (**A**) Time course of IFN-γ production for five representative pMHCs (see fig. S6 for additional pMHCs). After an ~90-min time lag common to all pMHCs, a linear increase in IFN-γ production was observed, which was consistent with the predicted linear relationship between productive hits and the downstream response, in this case, IFN-γ synthesis. (**B**) The rate of IFN-γ production, as determined by the slope in (A), correlated with k_{off}. (**C**) Intracellular accumulation of IFN-γ was obtained by blocking secretion with brefeldin-A after 2 hours of incubation with the indicated pMHC. The cells were fixed and analyzed by flow cytometry at 4 hours. Broad IFN-γ distributions were observed. (**D**) The fraction of responding cells (defined as cells that released IFN-γ above background levels, dotted black line) did not vary widely and did not correlate with k_{off}. The fraction of responding cells stimulated by PMA was 0.82. (**E**) As predicted by the productive hit rate model, mean IFN-γ production correlated with k_{off}. The concentration of pMHC used in both experiments was sufficiently large to ensure that IFN-γ production was completely saturated for the particular pMHC. Data are representative of two independent experiments.

based on the rate of productive hits. We suggest that once the switch-like determination to respond is made, T cells will still exhibit a functional response that is proportional to the rate of productive hits. In other words, the analog information contained in the rate of productive hits is transmitted into an analog output despite the digital nature of intracellular signaling. More work is necessary to understand how analog-analog conversion is faithfully transmitted through digital intracellular signaling.

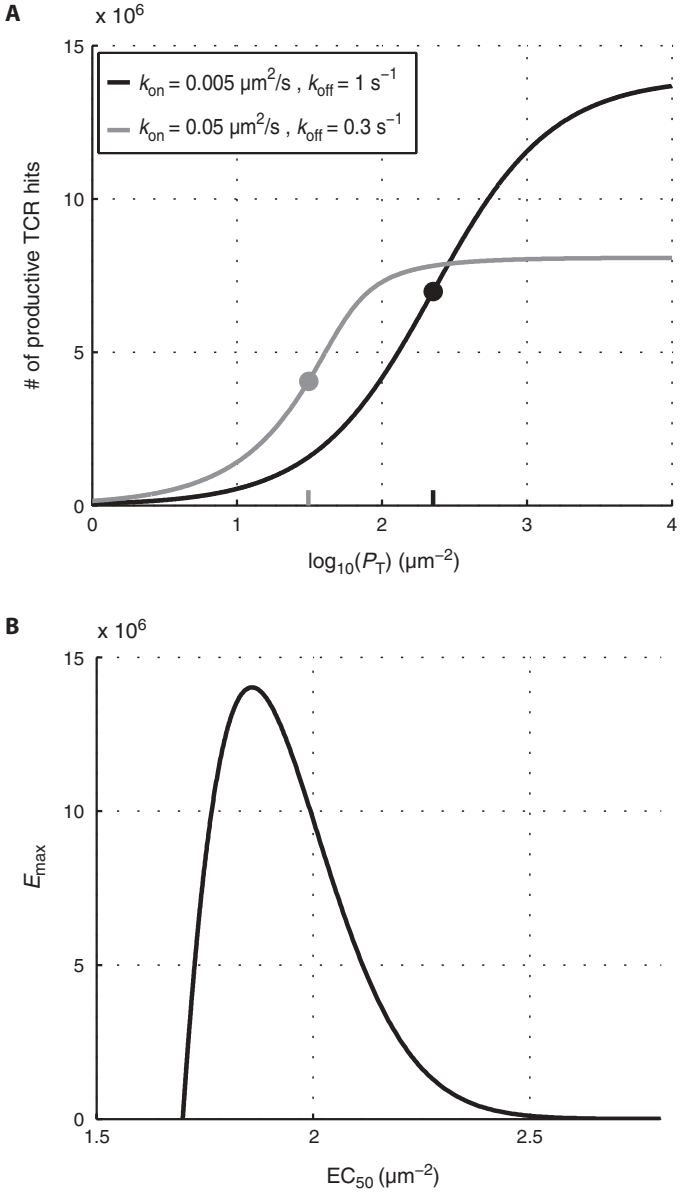

Fig. 4. Implications of the productive hit rate model. (A) Simulated dose-response curves, with the productive hit rate model, for antigens differing only in the indicated binding parameters. Antigen potency is indicated by solid circles and corresponding *x*-axis tick marks. (B) The predicted relationship between EC_{50} and E_{max} was obtained by varying k_{off} (k_{on} = 0.05 μm²/s) with the productive hit rate model. The model predicted that dose-response curves might intersect [shown in (A)], and therefore, the relationship between antigen potency and maximal efficacy is not monotonic [shown in (B)]. It follows that potency alone may not be sufficient to characterize the activity of antigens. All parameters are as indicated in Fig. 1 for the productive hit rate model.

The productive hit rate model has implications for dose-response curves. Given that EC_{50} is a monotonic function of k_{off}, whereas E_{max} can exhibit a maximum, it is possible that dose-response curves intersect (Fig. 4A). Therefore, the productive hit rate model predicts that potency alone may not be sufficient to characterize the activity of antigens (Fig. 4B). This may explain the finding that highly potent peptides in vitro do not always provide the best immune response in vivo, where antigen is encountered at various doses (*14*, *22*, *40*, *41*). Studies aimed at improving adoptive immunotherapy with T cells have provided evidence that increased TCR-pMHC affinity can decrease the T cell response depending on the antigen dose (*23*, *24*, *41*), as predicted by the productive hit rate model (Fig. 4). Therefore, affinity (or potency) alone is not sufficient to predict the T cell response and, as discussed earlier, additional estimates of k_{off} and maximal efficacy (E_{max}) will be important to improve predictions of the activity of potential therapeutics.

The debate over the affinity and productive hit rate models in the T cell literature is reminiscent of an earlier discussion in the pharmacology literature. In a pioneering work, Clark showed that the response of a given tissue is proportional to the number of receptors occupied by the drug (*42*). His occupation theory is analogous to the affinity model described in the T cell literature. In 1961, Paton proposed rate theory, which postulated that the action of drugs is not determined by the number of occupied receptors but by the rate of receptor occupation (*43*). Rate theory is analogous to the serial binding hypothesis (*16*) but is different from the productive hit rate model because it does not stipulate a requirement for long-lived interactions. Modern pharmacological theory relies on extensions of the original occupation theory (*25*), such as the cubic ternary complex model (*26*). In these models, the ligand is parameterized not only by its ability to bind to the receptor but also by its ability to, for example, recruit a G protein to the receptor. This additional independent parameter is required because receptor-ligand reaction rates alone cannot explain differential E_{max} values. Our results suggest that this variation could be accounted for by differences in receptor-ligand turnover that result from differences in bond lifetimes. The importance of bond lifetime to the action of drugs has recently been discussed (*44*), and the model presented here may prove useful for future studies.

MATERIALS AND METHODS

pMHC generation

All MHCs were expressed and refolded with synthetic altered peptide ligands (APLs), as previously described (*2*).

Analysis of binding parameters

TCR expression and refolding and the measurement of pMHC-TCR binding parameters by surface plasmon resonance were performed as previously described (*2*).

Stimulation assays

Specific doses of pMHC were incubated in High Binding Capacity Reacti-Bind Streptavidin-Coated Plates (96-well, Thermo Scientific) for 90 min at 25°C. To generate the full dose-response curves, we prepared four initial concentrations from which eight twofold dilutions were made. In this way, three pMHCs could be tested on a single 96-well plate. Specific numbers of CTLs (12,000 for experiments with the 1G4 system and 10,000 for experiments with the G10 system) were incubated at 37°C with plate-

bound pMHC for the indicated times. The concentration of secreted IFN-γ was measured in the culture medium by enzyme-linked immunosorbent assay (ELISA) with the OptEIA Human IFN-γ Set (BD Biosciences). To evaluate the amounts of plate-bound pMHC, we performed an ELISA with a conformation-sensitive antibody (W6/32), which is a mouse antibody against human MHC class I, in combination with horseradish peroxidase–conjugated donkey antibody against mouse immunoglobulin G (Jackson Immunoresearch). Additional details were described by Aleksic *et al.* (*2*). For analysis by flow cytometry, CTLs were stimulated as described earlier, except that brefeldin-A (at a 1:1000 dilution, BioLegend) was added after 2 hours. After 4 hours, the CTLs were harvested and fixed in 4% paraformaldehyde for 15 min at room temperature. After two washes, cells were permeabilized by resuspension in ice-cold 0.1% Triton X-100 and incubation for 10 min on ice. After two washes, the cells were blocked in 5% mouse serum for 15 min at room temperature. Cells were then incubated with phycoerythrin-conjugated antibody against IFN (R&D Systems, #1C285, at a 1:10 dilution) for 15 min and analyzed by flow cytometry. As a positive control, CTLs were stimulated with a combination of phorbol 12-myristate 13-acetate (PMA) (at a final concentration of 50 ng/ml) and ionomycin (at a final concentration of 0.67 μM).

Data analysis

To estimate EC_{50} and E_{max} values from the full dose-response curves, we fitted the following Hill function to the experimental data:

$$y = A_1 + E_{max}/(1 + 10^{n(EC_{50} - x)})$$

where A_1 is the baseline and n is the Hill number. To determine the rate of IFN-γ production from the time-course data (Fig. 3A), we fitted the following function:

$$y = b_0 + b_1(t - t_0)H(t - t_0)$$

where b_0 is the baseline, b_1 is the rate of IFN-γ production, t_0 is the time offset, and H is the Heaviside step function. Correlations of reaction parameters with functional data were analyzed by linear regression.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/4/176/ra39/DC1
Methods
Fig. S1. Comparison of the affinity model with three formulations of the productive hit rate model.
Fig. S2. Productive hit rate model with internalization.
Fig. S3. Structure of the gag peptide in complex with HLA-A2.
Fig. S4. Complete dose-response data for the G10 TCR system.
Fig. S5. Additional correlations between reaction parameters and functional responses for G10 CTLs.
Fig. S6. Time-course data for additional pMHCs.
Fig. S7. Confirmation of the time dependency of E_{max} and the time independency of EC_{50}.
Fig. S8. Confirmation of the relationships between K_D and EC_{50} and between k_{off} and E_{max}.
Fig. S9. Simulated dose-response curves using protocols 1 and 2.
Fig. S10. Histogram of estimated EC_{50} and E_{max} values from protocols 1 and 2.
Fig. S11. Standard deviations in the EC_{50} histograms from protocols 1 and 2.
Fig. S12. Effects of TCR-pMHC rebinding in a confinement time model on maximal antigen efficacy.
Table S1. Reaction and functional parameters for the G10 TCR.
Table S2. Reaction and functional parameters for the 1G4 TCR.
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