Architecture of a minimal signaling pathway explains the T-cell response to a 1 million-fold variation in antigen affinity and dose

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T cells must respond differently to antigens of varying affinity presented at different doses. Previous attempts to map peptide MHC (pMHC) affinity onto T-cell responses have produced inconsistent patterns of responses, preventing formulations of canonical models of T-cell signaling. Here, a systematic analysis of T-cell responses to 1 million-fold variations in both pMHC affinity and dose produced bell-shaped dose–response curves and different optimal pMHC affinities at different pMHC doses. Using sequential model rejection/identification algorithms, we identified a unique, minimal model of cellular signaling incorporating kinetic proofreading with limited signaling coupled to an incoherent feed-forward loop (KPL-IFF) that reproduces these observations. We show that the KPL-IFF model correctly predicts the T-cell response to antigen presentation. Our work offers a general approach for studying cellular signaling that does not require full details of biochemical pathways.

Significance

T cells initiate and regulate adaptive immune responses when their T-cell antigen receptors recognize antigens. The T-cell response is known to depend on the antigen affinity/dose, but the precise mechanism, and the mechanisms underlying it, are debated. To resolve the debate, we stimulated T cells with antigens spanning a 1 million-fold range in affinity/dose. We found that a different antigen (and hence different affinity) produced the largest T-cell response at different pMHC doses. Using a model identification algorithm, the model predicted the T-cell response from the physiological low-affinity regime into the high-affinity regime applicable to therapeutic receptors.


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Changes in affinity were largely a result of changes in the off-rate of protein 1 (AP-1) and nuclear factor of activated T cells (NFAT) (SI Appendix). We measured the supernatant concentration of IL-8 after 16 h (Fig. 1 A). Using single, double, and triple peptide mutations we produced a T-cell receptor with 14 amino acid substitutions (primarily at the contact interface) but maintained the same binding mode as the parental 1G4 TCR (40). This TCR contains a peptide derived from the cancer antigen NY-ESO-1 in complex with HLA-A*02:01 (Kd ~ 50 pM) (27). We next transduced the c58c61 TCR into primary human CD8 T cells and the Jurkat T-cell line (SI Appendix, Fig. S2 A–C). The observed changes in affinity were largely a result of changes in the off-rate Kd.

We next transduced the c58c61 TCR into primary human CD8+ T cells and the Jurkat T-cell line (SI Appendix, Fig. S2A) before stimulating them with a 1 million-fold range of pMHC concentration. In the case of the primary T cells we measured the supernatant concentration of IFN-γ or macrophage inflammatory protein 1-ß (MIP-1ß) after 4 h of stimulation (Fig. 1 D and E and SI Appendix, Fig. S2B). In the case of the Jurkat T cells we measured the supernatant concentration of IL-8 after 16 h (Fig. 1 F and SI Appendix, Fig. S2C) or the transcriptional activity of activator protein 1 (AP-1) and nuclear factor of activated T cells (NFAT) (SI Appendix, Fig. S2D).

Four striking features were observed. First, the dose–response seemed to exhibit a bell shape with reduced cytokine production at high pMHC concentrations. This bell shape was less pronounced or absent for low-affinity ligands, which is consistent with published studies reporting a sigmoidal dose–response for low-affinity ligands (14, 15, 17, 18, 21, 23, 41). Second, the peak amplitude of the bell-shaped dose–response was similar for pMHCs despite large differences in their affinities. The next two features describe the observation that the pMHC that produced the most cytokine was dose-dependent. At higher concentrations (to the right of the peaks) there is an obvious intersection of the dose–response curves so that different pMHC ligands produce the most cytokine at different doses (e.g., 4A8K or 4A5A in Fig. 1 D–F). In contrast, at lower concentrations (to the left of the peaks) curve intersection is not apparent, so that a single intermediate affinity pMHC produces the most cytokine.

These four phenotypic features are summarized as follows:

- Bell-shaped dose-response for high- but not low-affinity ligands.
- Peak amplitude of bell-shaped dose-response is independent of affinity.
- A single intermediate affinity ligand produces largest response at low doses (left of the peak).
- Different intermediate affinity ligands produce the largest response at high doses (right of the peak).

Although a bell-shaped dose–response can be a result of activation-induced cell death (42), this is unlikely to be the case here. We found that the dose–response for cytokine production appeared bell-shaped at all times with continued cytokine production to the right of the peak (Fig. 2A). Moreover, direct detection of apoptosis by annexin V binding revealed a maximum of only 10%, which is insufficient to explain the observed reduction in cytokine production (Fig. 2B). Further, lower levels of cytokine observed to the right of the peak were associated with lower levels of annexin V, which is inconsistent with the hypothesis that lower cytokine is a result of apoptosis.

Results

T-Cell Activation in Response to a 1 Million-Fold Variation in Antigen Affinity/Dose. As a first step to identify a T-cell signaling model (Fig. I4) we established a TCR/pMHC system with a large range of affinities by using the therapeutic affinity-matured c58c61 TCR that recognizes a peptide derived from the cancer antigen NY-ESO-1 in complex with HLA-A*02:01 (Kd ~ 50 pM) (27). This TCR contains 14 amino acid substitutions (primarily at the contact interface) but maintains the same binding mode as the parental 1G4 TCR (40). Using single, double, and triple peptide mutations we produced a panel of 11 pMHCs that span a 1 million-fold range in affinity (Fig. 1 B and C and SI Appendix, Table S1 and Fig. S1). The observed changes in affinity were largely a result of changes in the off-rate Kd.

SI Appendix

Fig. 1. T-cell activation in response to a 1 million-fold variation in pMHC affinity and dose. (A) Schematic illustrating that the signaling architecture linking the TCR to cytokine production is unknown. (B) Affinities (displayed as dissociation constants, Kd) and (C) kinetics of the c58c61 TCR interacting with 11 pMHC ligands determined using surface plasmon resonance (see also SI Appendix, Fig. S1 and Table S1). T-cell activation as measured by supernatant (D) IFN-γ and (E) MIP-1ß in primary T cells after 4 h and (F) IL-8 in Jurkat T cells after 16 h transduced with the c58c61 TCR (insets show the three lowest-affinity ligands in each experiment). Ligand color scheme is identical across all panels. Additional data, including additional concentrations, additional ligands, pMHC immobilization controls, and TCR expression levels, are summarized in SI Appendix, Fig. S2.
result of increased apoptosis at high pMHC doses. These observations suggested a reduced rate of cytokine production per cell at high pMHC doses, which we confirmed using single-cell cytokine production in Jurkat T cells (Fig. 2 C) and in primary T cells (SI Appendix, Fig. S2 E).

We observed interdonor variability that could not be explained by differential TCR expression or pMHC activity. For example, the pMHC that produced the largest response to the left of the peak varied between 4A, 5Y, and 8S (compare Fig. 1 D and SI Appendix, Fig. S2 B). However, the feature that a single pMHC of intermediate affinity produced the largest response to the left of the peak was consistent. Therefore, although the quantitative features of the data exhibited variability, we observed a high level of consistency for the key qualitative phenotypic features. Similarly, we found that Jurkat T cells express CD8α, their reduced sensitivity may be related to the absence of CD8β, which has previously been shown to increase antigen sensitivity (12).

Sequential Model Rejection Identifies the KPL-IFF Model as Sufficient to Explain T-Cell Responses. We next identified a T-cell signaling network consistent with all key features by sequentially rejecting models. To do this, we tested models of increasing complexity starting with the simplest possible cellular mechanism, namely the occupancy model, which reduces cellular signaling to a single reaction (Fig. 3 D). In this model, the pMHC ligand binds to the TCR to form a complex that directly activates a node \( P \) that is assumed to be linearly proportional to cytokine production. By examining the predicted dose–response curve for this model it is clear that the model is insufficient to explain the phenotypic features (e.g., it does not produce a bell-shaped dose–response, feature 1) and therefore we reject this model as a plausible model of T-cell signaling.

Bell-shaped dose–response curves can be produced by incoherent feed-forward motifs (Fig. 3 B), which are common architectures in transcriptional networks (43). In this model, the TCR–pMHC complex directly inhibits \( P \) and indirectly activates \( P \) (by activating \( Y \), which itself is able to activate \( P \)). The model can produce inhibition at high pMHC concentrations if the activatory pathway (through \( Y \)) saturates, allowing inhibition to dominate at the highest pMHC concentrations. The appearance of the bell shape in this model can explain the observation that a different pMHC affinity produces the largest response to the right of the peak (feature 4). However, this model is also rejected because it produces a bell-shaped dose–response for all pMHC ligands independent of their affinity (feature 1).

Bell-shaped dose–response curves can be produced for high-but not low-affinity ligands by introducing kinetic proofreading (Fig. 3 C). In this model, the pMHC ligand does not trigger signaling immediately upon binding TCR but instead must remain bound until it becomes signaling-competent (denoted as \( C_1 \)).
This delay means that low-affinity pMHCs (with faster \( k_{\text{off}} \)) induce a lower maximal concentration of \( C_1 \) than high-affinity pMHC (see SI Appendix, Fig. S3 for a plot of \( C_1 \) for pMHC with different values of \( k_{\text{off}} \)). If this is below the level at which \( Y \) saturates then inhibition at high pMHC concentrations will not be observed with low-affinity pMHC. As expected, kinetic proofreading has improved antigen discrimination by dramatically decreasing the T-cell response to low-affinity pMHC. This model, however, is also rejected because it predicts that the highest-affinity ligand will produce the largest response left of the peak in contrast to experimental observations (feature 3).

Introducing limited signaling into kinetic proofreading can produce an optimal affinity over a range of pMHC concentrations (9) (Fig. 3D). In this model, activated TCR–pMHC complexes (\( C_1 \)) signal for a limited period before converting to a nonsignaling state (\( C_2 \)), thereby introducing a penalty for pMHCs that remain bound for long periods of time. This model is now able to explain all key features and we therefore accept the KPL-IFF model as a plausible signaling model for T-cell activation.

**Systematic Model Identification Confirms That the KPL-IFF Model Is Unique.** Having identified the KPL-IFF model as sufficient to explain all phenotypic features, we next determined whether other models, with a potentially different underlying mechanism, can also explain all phenotypic features.

We first systematically examined models of equal or lower complexity compared to the KPL-IFF model. To do this, we studied all combinations of three reaction arrows between \( Y \) and \( P \) and the three receptor states (Fig. 4A). Of the 560 possible reaction networks (16 choose 3) only the 304 networks that contain a connection between the ligand and \( P \) were analyzed. For each of these putative signaling networks, we performed an exhaustive search that included a dense parameter scan followed by optimization of the five free parameters (SI Appendix, Fig. S4). The output of the analysis is a list of networks ordered by their ability to reproduce the phenotypic features (Movie S1). As expected, the first network to appear is the KPL-IFF model but, unexpectedly, the 303 subsequent networks were all unable to explain all phenotypic features.

We highlight three models from the network search that are inconsistent with the phenotypic features (Fig. 4 B–D). A mirrored model in which activation is direct but inhibition is indirect cannot produce a bell-shaped dose–response (feature 1) because activation cannot saturate (Fig. 4B). A redirected model where inhibition comes from an earlier complex not subjected to kinetic proofreading (Fig. 4C) cannot explain a different optimal pMHC affinity to the right of the peak (feature 4) or the observation that the peak response is similar for different affinity ligands (feature 2). Finally, models without an incoherent feed-forward loop but with negative feedback, although able to produce oscillations of \( P \) in time, cannot produce a bell-shaped dose–response (Fig. 4D and SI Appendix, Fig. S5; see also SI Appendix for a mathematical proof).

To determine whether more complex models can explain all key features using different mechanisms, we performed the same systematic network analysis on models with four reaction arrows between \( Y \), \( P \), and an additional node \( X \) and four receptor states (Fig. 4E). A systematic analysis of the 26,069 networks with a connection between the ligand and \( P \) revealed 274 compatible networks (SI Appendix, Movie S2). However, examining these 274 networks showed that the basic mechanism underlying all compatible networks was KPL-IFF. For example, the incoherent feed-forward loop could involve indirect inhibition so long as inhibition saturates after activation as a function of ligand dose (Fig. 4F) or it could involve both direct activation and inhibition with the net effect being inhibition (Fig. 4G). As above, negative feedback could not produce bell-shaped dose–response curves (Fig. 4H).

In summary, the KPL-IFF signaling network (Fig. 3D) is sufficient to explain all phenotypic features. Given that the systematic analyses implicitly include simpler models (e.g., by allowing for the magnitude of reaction arrows to be negligible) we were able to further conclude that the KPL-IFF model is the simplest model able to explain all features.

The KPL-IFF model contains five parameters: kinetic proofreading (\( k_p \)), limited signaling (\( \delta \)), inhibition (\( \mu \)), activation (\( \delta \)), and amplification (\( \lambda \)). We used approximate Bayesian computations with sequential Monte Carlo to establish the set of these parameters that are able to reproduce the key features (44). We found that large variations of the parameters were possible provided that they...
obeyed certain relationships (SI Appendix, Fig. S6). For example, we found that $\mu$ and $\delta$ can vary by 1,000-fold provided that $\mu > \delta$ and that increases in $\phi$ can reproduce the phenotypic features provided that $k_\text{p}$ decreased proportionally. Using the provided applet (SI Appendix), we find that bell-shaped dose–response curves are less pronounced when the condition $\mu > \delta$ is not satisfied. A large variation in the parameters is tolerated because the phenotypic features are scale-free (SI Appendix).

The KPL-IFF Model Predicts the T-Cell Response to Copresentation of pMHC Ligands. T cells generally experience mixtures of pMHC ligands when becoming activated. Previous studies have shown that copresentation of an additional pMHC can modulate the T-cell response in various ways, including both enhancing and inhibiting T-cell activation (45).

To address the effects of pMHC copresentation, we extended the KPL-IFF model to include an additional pMHC ligand with different binding kinetics and concentrations (Fig. 5A). We used the extended KPL-IFF model to predict the T-cell response to a titration of a lower-affinity ligand in the presence of fixed concentrations of a higher-affinity ligand (Fig. 5B). As a result of the incoherent feed-forward loop the model predicted a sigmoidal dose–response when the concentration of the high-affinity ligand was left of its peak ($\leq 0.025$) and a constant response when the concentration of the high-affinity ligand was right of its peak ($\geq 0.025$). This is a direct result of the saturating activating pathway of the incoherent feed-forward. Surprisingly, the model predicted that T-cell activation cannot be inhibited by signals induced by the low-affinity ligand even when the high-affinity ligand is presented at concentrations that saturate the activation pathway of the incoherent feed-forward. We confirmed these predictions by stimulating T cells with a titration of the lower-affinity ligand, 5P, in the presence of fixed concentrations of the higher-affinity ligand, 4A (Fig. 5C). As predicted by the model, the dose–response curves appeared sigmoidal at lower doses of 4A and largely constant at higher doses, without any obvious inhibition of T-cell activation by 5P.

Discussion

We have measured the T-cell response to a 1 million-fold variation in antigen affinity and dose. We found bell-shaped dose–response curves with a different pMHC (and hence different affinity) producing the largest T-cell response at different doses. We show, without making prior biochemical assumptions and with the constraint of parsimony, that the KPL-IFF architecture is the only model identified able to explain all phenotypic features of the experimental data. We further confirmed predictions of the model concerning pMHC copresentation. Remarkably, the KPL-IFF model can explain the T-cell response to a 1 million-fold variation in antigen affinity and dose based on a simple pathway architecture despite the enormous molecular complexity in T-cell signaling.

The present work has uncovered two independent mechanisms that lead to an optimal pMHC affinity. At low doses (left of the peak) we find that limited signaling through the TCR allows a single intermediate affinity pMHC to dominate the dose–response curve, whereas at higher doses (right of the peak) a different pMHC affinity produces the most cytokine as a result of the bell-shaped dose–response curves.

Fig. 4. Systematic analyses of signaling models reveals that the KPL-IFF mechanism is unique. (A) To determine whether other models of equal (or lower) complexity to the KPL-IFF model (Fig. 3D) are able to produce all phenotypic features we performed a systematic search of 304 network architectures with three reaction arrows between the receptor states ($C_0$, $C_1$, and $C_2$), $Y$, and $P$. The only network architecture that is able to produce all phenotypic features is the KPL-IFF model (Movie S1). Conversely, (B) the mirrored KPL-IFF, (C) the redirected KPL-IFF, and (D) negative feedback network architectures are unable to produce the phenotypic features. (E) To determine whether more complex models can reproduce the phenotypic features using mechanisms different from those invoked in the KPL-IFF model, we performed a systematic analysis of 26,069 network architectures with four reaction arrows between four receptor states and $Y$, $P$, and an additional node $X$. Both activation and inhibition are considered but for clarity only activation arrows are depicted. We found 274 networks compatible with all phenotypic features but all of these networks relied on the KPL-IFF mechanism (SI Appendix, Movie S2). (F and G) Two representative compatible networks show that although the network is more complicated both rely on the KPL-IFF mechanism. (H) As before, negative feedback in the absence of incoherent feed-forward is unable to produce the phenotypic features. See SI Appendix for computational details.
A number of studies have implicated negative feedback in TCR signaling (6), but we find that negative feedback cannot explain the phenotypic features of T-cell activation. For example, negative feedback cannot produce bell-shaped dose–response curves. We note that our model does not preclude the existence of signaling proteins with negative effects, such as tyrosine phosphatases that can determine, for example, the net rate of TCR phosphorylation ($k_\text{on}$). Negative feedback may be more important for the short-timescale process of antigen discrimination rather than the longer timescale process of T-cell activation that has been the focus of the present study (1, 6).

The limited signaling mechanism is related to previous work showing that a trade-off between serial binding and kinetic proofreading leads to an optimal pMHC half-life (10, 46). Serial binding of a single pMHC to many TCRs can increase signaling when the pMHC concentration is low and individual TCRs signal for a limited period upon binding. Under these conditions longer binding half-lives can reduce the number of productive TCR engagements (9). We find that at low doses reduced signaling is only observed when the TCR/pMHC half-life measured in solution is longer than 1 min (e.g., low dose of 4A, 5Y, and 8S compared with 9V in SI Appendix, Fig. S2 B and C). It follows that although limited signaling (and hence serial binding) may not be critical for physiological TCR–pMHC interactions, which have half-lives that last seconds, it is likely to be important for the design of high-affinity therapeutic TCRs or CARs for T-cell adoptive transfer therapies (25).

The internalization of TCR is known to take place upon TCR triggering (1, 46) and it can be realized by different mechanisms. Intracellular signaling induced by activated TCR that leads to TCR internalization is a form of negative feedback and, as discussed above, negative feedback cannot explain the observed phenotypic features. Limited signaling may result from the tagging of TCR for internalization and explicitly including this internalization, without incoherent feed-forward, does not lead to bell-shaped dose–response curves in the steady state (SI Appendix, Fig. S8). The KPL-IFF model may implicitly be capturing TCR surface dynamics because directed movement (47) combined with polarized recycling (48, 49) of TCR into the immune synapse may balance with TCR internalization (46) to maintain the relatively constant TCR concentration at the immune synapse assumed by the KPL-IFF model, which is consistent with previous calculations (50). A recent study has shown that changing the pMHC affinity can induce a program that over a timescale of several days changes TCR levels (51). The KPL-IFF model can explain their observation that the higher-affinity ligand induced greater TCR down-regulation if TCR levels are determined by the output of the KPL-IFF model.

The systematic analyses revealed that a large number of more complex models can explain the phenotypic features (Fig. 4E). This illustrates the broad challenge of (i) formulating unique models based on the known biochemistry and (ii) relating the unique model we have formulated to the known biochemistry. Limited signaling may result from modification of the TCR signalosome, such as ubiquitination (52) and/or its movement into membrane environments incompatible with signaling (e.g., endosomes (46) or microvesicles (53)) (SI Appendix, Fig. S7B). Kinetic proofreading can be realized by a number of different molecular mechanisms, such as sequential or random phosphorylation of the TCR (54, 55) and/or the recruitment of Lck-associated coreceptors (56) (SI Appendix, Fig. S7A). The incoherent feed-forward loop may result from the fact that LAT can both activate (via Grb2 and SOS) and inhibit (via Dok1/Dok2 and RasGAP) Ras (7) (SI Appendix, Fig. S7C) or from the observation that the TCR signalosome, by virtue of being able to associate with both a tyrosine kinase (ZAP-70) and a tyrosine phosphatase (SHP-1), can produce incoherent signals (2). Additionally, the positive and negative arms of the incoherent feed-forward may represent two pathways that converge to regulate cytokine production. Future work is required to map the known biochemistry onto the KPL-IFF architecture.

Fig. 5. The KPL-IFF model predicts T-cell activation in response to copresentation of pMHC ligands. (A) Schematic of signal integration by two distinct populations of pMHC ligands in the context of the KPL-IFF model. The model predicts that a titration of a low-affinity ligand ($k_\text{off} = 1 \text{s}^{-1}$) in the presence of a fixed concentration of a high-affinity ligand ($k_\text{off} = 0.001 \text{s}^{-1}$) will be either sigmoidal or constant when the concentration of the high-affinity ligand is left of its peak (purple, cyan, and green) or right of its peak (orange, brown, and red), respectively. Appreciably inhibition by the low-affinity ligand is left of its peak (purple, cyan, and green) or right of its peak (orange, brown, and red). The limited signaling mechanism is related to previous work showing that a trade-off between serial binding and kinetic proofreading leads to an optimal pMHC half-life (10, 46). Serial binding of a single pMHC to many TCRs can increase signaling when the pMHC concentration is low and individual TCRs signal for a limited period upon binding. Under these conditions longer binding half-lives can reduce the number of productive TCR engagements (9). We find that at low doses reduced signaling is only observed when the TCR/pMHC half-life measured in solution is longer than 1 min (e.g., low dose of 4A, 5Y, and 8S compared with 9V in SI Appendix, Fig. S2 B and C). It follows that although limited signaling (and hence serial binding) may not be critical for physiological TCR–pMHC interactions, which have half-lives that last seconds, it is likely to be important for the design of high-affinity therapeutic TCRs or CARs for T-cell adoptive transfer therapies (25).

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The systematic search for parsimonious models that can reproduce phenotypic features of cellular activation, without prior biochemical assumptions, produces signaling pathways with tractable architectures. Just as subatomic details (e.g., nuclear structure) are not necessary for atomic molecular dynamics simulations, we argue that the correct description of signaling pathways may not require detailed biochemical knowledge of individual proteins. These predictive pathway models provide a mechanistic understanding of the modular network components required to integrate input signals from the cell surface into cellular activation outputs. Although they do not include full molecular detail, they offer an intuitive framework upon which biochemical information can be mapped, which has so far been elusive with reductionist approaches.

Materials and Methods

Production of Lentivirus for Transduction. HEK 293T cells were used to produce the lentiviral transfer vectors and packaging plasmids using a standard single-cycle kinetic mode but we found that these produced several interference factors. Populations expanded to adequate sizes.

Isolation and Transduction of Primary T Cells. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor blood by density gradient centrifugation; Blood collected in heparinized tubes was diluted 1:2 with PBS, carefully layered onto Ficoll-Paque in 50-ml tubes and spun without brake at 400 × g at room temperature for 30 min. The PBMCs were collected from the interphase, spun at 520 × g for 5 min, and washed once with PBS. CD8+ T cells were isolated from PBMCs using the Dynabeads Untouched Human CD8 T Cells Kit (Life Technologies) following the manufacturer’s instructions. Briefly, PBMCs were resuspended in isolation buffer (0.1% BSA and 0.01% EDTA in PBS), blocked with FBS, and unwanted cells were incubated with an antibody mix (containing biotinylated antibodies for human CD4, CD14, CD16, CD19, CD36, CD56, CDw123, and CD235a). Subsequently, the PBMCs were washed and incubated with streptavidin-coated Dynabeads. The suspension was resuspended thoroughly with isolation buffer before the tube was placed into a magnet. The contaminant containing “untouched” CD8+ T cells was collected. This process was repeated twice and the supernatants were combined.

The isolated CD8+ T cells were spun at 520 × g for 5 min and resuspended at a concentration of 10^6 cells per mL in completely reconstituted DMEM, supplemented with 50 units/mL IL-2 and 10^6 CD3/CD28-coated Human T-Activator Dynabeads (Life Technologies) per mL. Cells were cultured at 37 °C in 10% CO2 overnight.

T-Cell Stimulation. Strepavidin-coated 96-well plates (Sigma-Aldrich) were washed two times with PBS 0.05% Tween followed by one time with PBS. Plates were incubated at 37 °C with PBS and 1% BSA for 1 h. Serially diluted pMHCs (in PBS) were transferred to the plates and incubated at 4 °C for 90 min (volume of 100 μL per well). Plates were washed three times with PBS following incubation. Plates were always prepared in pairs so that one plate was touched.

The off-rate (koff) was determined by fitting a one-phase exponential decay to the dissociation trace,

\[ Y = Y_0 - \frac{Y_0 - Y_{\infty}}{1 + e^{-k_{\text{on}}t}} \]

where \( Y_0 \) and \( Y_{\infty} \) are the initial and long-time asymptotic RU, respectively. The mean \( k_{\text{on}} \) across concentrations was used to determine \( k_{\text{on}} \). The kinetics were such that the association phase could be resolved in time (i.e., sufficiently slow \( k_{\text{off}} \)) we fit the following one-phase exponential association to the association trace:

\[ Y = \frac{T_{\text{CR}}B_{\text{MHC}}}{T_{\text{CR}} + K_{\text{off}}} \]

where \( k_{\text{on}} = k_{\text{on}}[T_{\text{CR}}] + k_{\text{off}} \). We note that for high-affinity interactions where the dissociation trace lifetime was >15 min only a single concentration of TCR was used. Injection of multiple TCR concentrations is possible using the single-cycle kinetic model but we found that these produced several incomplete association traces resulting in larger variability in \( k_{\text{on}} \) between experiments. We note that multiple analyte concentrations are particularly critical to determine the stoichiometry of the interaction. When the kinetics were such that the association phase could not be resolved in time (i.e., fast \( k_{\text{off}} \)) we fit the following Langmuir binding equation to the steady-state response units to obtain an estimate for \( k_{\text{on}} \):

\[ Y_{\infty} = \frac{T_{\text{CR}}X_{\text{MHC}}}{K_{\text{on}} + T_{\text{CR}}} \]

where \( Y_{\infty} \) is the steady-state RU. The on-rate is determined using \( k_{\text{on}} = k_{\text{off}}/k_{\text{on}} \). All data fitting was performed in Prism (GraphPad).

Production of Lentivirus for Transduction. HEK 293T cells were seeded into 175-cc flasks 24 h before transfection to achieve 50-80% confluency on the day of transfection. Cells were cotransfected with the respective third-generation lentiviral transfer vectors and packaging plasmids using a standard PEI (polyethyleneimine) transfection protocol as follows. The medium was replaced with serum-free DMEM. Transfer vector and the packaging plasmid mix (17.5 μL of pRSV-rev and pMDLg/pRRE as well as 6.8 μL of pVSV-G) were diluted in 400 μL of serum-free DMEM and a dilution of 112 μg PEI in serum-free DMEM was prepared in another tube. Both were mixed vigorously and incubated at room temperature for 20 min. The mixture was added dropwise to the cells, which were then incubated at 37 °C in 10% CO2 for 4-5 h. Afterward, the medium was replaced with complete medium. The supernatant was harvested and filtered through a 0.45-μm cellulose acetate filter 24 h later. Lentiviral particles were concentrated using Lentipac Lentivirus concentrator (GeneCopeia) according to the manufacturer’s protocol.

Transduction of Jurkat T Cells. The Jurkat E6.1 T-cell line expressing the NFAF/ -AP-1 luciferase reporter and CD8α (57) were transduced with the cS861 TCR. To do this, 3 million cells were resuspended in 2 mL of concentrated virus followed by centrifugation at 2,095 × g for 1-2 h. The cells were incubated at 32 °C for 3.5-6 h and then cultured at 37 °C in 10% CO2 in DMEM supplemented with 10% (vol/vol) FBS, 100 μM penicillin, and 100 μg/mL streptomycin.
Flow Cytometry Assays. **Intracellular cytokine staining.** T-cell stimulation was performed as described above except that 50 μL of medium supplemented with Brefeldin A (2.5 μg/mL final concentration) was added to the respective samples followed by spinning at 520 × g for 5 min before returning the cells to the incubator for another 2 h (primary T cells, 4-h total stimulation) or for another 3 h (Jurkat T cells, 8-h total stimulation).

After stimulation, cells were spun at 520 × g for 5 min and resuspended with 2 mM EDTA in PBS. After an additional spin, the cell pellets were fixed by resuspension in 500 μL/well 4% (vol/vol) formaldehyde in cold PBS at 4 °C (10 min). Fixed cells were washed with PBS, spun down at 520 × g for 10 min, and resuspended in 100 μL per well permeabilization buffer (PBS with 2% (wt/vol) BSA and 0.1% Triton X-100). After 10 min at 4 °C, cells were spun at 520 × g for 10 min and resuspended in permeabilization buffer containing the respective antibody (EBN1 APC-conjugated IL-8 antibody or 45. B.A. Abendroth, R.D. Chen, J. IFNγ (antibody-dependent) for 20 min at 4 °C. After washing twice with PBS (250 μL for 10 min), cells were resuspended in 100–150 μL PBS per sample and transferred into FACS tubes for analysis. Jurkat cells had to be spun before the transfer to mitigate cell losses.

**Annexin V assay.** Jurkat T cells were stimulated as described above except that 100,000 cells were used per well. After 16 h of stimulation, cells were removed first by gently pipetting them out of each well and second by washing each well with 1 mL of PBS and transferred to 1.5-mL tubes and washed two times with PBS. Cells were resuspended in annexin-V buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2) at a concentration of 1–5 × 10^6 cells per mL.

Cells were stained with PE-Annexin-V (556421; BD Biosciences) at a concentration of 1 μL per 100 μL cells and incubated at room temperature in the dark for 15 min. After washing twice with PBS, samples were ready for flow cytometry.

**586241; BD Biosciences** at a concentration of 5 μL per 100 μL cells and incubated at room temperature in the dark for 15 min. After washing twice with PBS, samples were ready for flow cytometry.

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C5621-T-cell receptor expression. 5 × 10^5 T cells per sample were washed with PBS in FACS tubes (3 mL, 5 min at 520 × g) and stained with high-affinity 9V pMHC (7 μg/mL; 200 μL per sample) for 30 min. Subsequently, they were washed with PBS and stained with R-PE-conjugated streptavidin (STAR4A, 1:100; 200 μL per sample; AbD Serotec) for another 30 min. After washing twice with PBS, samples were ready for flow cytometry.

All flow cytometry was performed using a FACSalibur (BD Biosciences) with at least 10,000 cells. All analysis was performed using the software FlowJo (TreeStar).

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Movie S1. Systematic analysis of 304 network architectures (Fig. 4A). Each 1-s frame shows the network architecture (Left) and the network output (Right). The network architecture displays the three receptor states ($C_0$, $C_1$, and $C_2$) together with $Y$ and $P$, where green arrows indicate activation and red arrows indicate inhibition (magnitudes of inhibition arrows are in italics). The network output displays $P$ (y axis) over the ligand concentration (x axis) for ligands with decreasing $k_{off}$ (blue to red). Networks appear in ascending order of the sum of squared residuals (SSR) so that networks that are better able to produce the phenotypic features appear first. See SI Appendix for computational details.

Other Supporting Information Files

SI Appendix (PDF)
Supplementary Information Appendix:
Architecture of a minimal signalling pathway explains the T cell response to a 1,000,000-fold variation in antigen affinity and dose

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Supplementary Text

Phenotypic model equations (KPL-IFF)

The ordinary-differential-equations (ODEs) corresponding to kinetic proofreading with limited signalling coupled to incoherent feedforward (KPL-IFF, Figure 3d) are,

\[
\begin{align*}
\frac{dL}{dt} &= -k_{on}LR + k_{off}(C_0 + C_1 + C_2) \\
\frac{dR}{dt} &= -k_{on}LR + k_{off}(C_0 + C_1 + C_2) \\
\frac{dC_0}{dt} &= k_{on}LR - (k_{off} + k_p)C_0 \\
\frac{dC_1}{dt} &= k_pC_0 - (k_{off} + \phi k_p)C_1 \\
\frac{dC_2}{dt} &= \phi k_p C_1 - k_{off}C_2 \\
\frac{dY}{dt} &= \gamma^Y_+(Y_T - Y) - \gamma^Y_- Y + \lambda C_1(Y_T - Y) \\
\frac{dP}{dt} &= \gamma^P_+(P_T - P) - \gamma^P_- P + \delta Y(P_T - P) - \mu C_1 P
\end{align*}
\]

where \(k_{on}\) and \(k_{off}\) are the TCR-pMHC kinetic rate constants, \(k_p\) is the kinetic proofreading rate, \(\phi\) is the limited signalling parameter, \(\lambda\) is the amplification parameter, \(\mu\) is the inhibition parameter, and \(\delta\) is the activation
We solve the system in the steady-state to obtain the following,

\[
\begin{align*}
\dot{\hat{C}}_0 &= \left( \frac{k_{\text{off}}}{k_{\text{off}} + k_p} \right) \hat{C}_T \\
\dot{\hat{C}}_1 &= \left( \frac{k_{\text{off}}}{k_{\text{off}} + k_p} \right) \left( \frac{k_p}{k_{\text{off}} + \phi k_p} \right) \hat{C}_T \\
\dot{\hat{C}}_2 &= \left( \frac{\phi k_p}{k_{\text{off}} + k_p} \right) \left( \frac{k_p}{k_{\text{off}} + \phi k_p} \right) \hat{C}_T \\
\hat{Y} &= \frac{1 + \hat{\lambda} \hat{C}_1}{1 + \lambda \hat{C}_1 + \hat{\gamma}^y} \\
\hat{P} &= \frac{1 + \hat{\delta} \hat{Y}}{1 + \hat{\delta} \hat{Y} + \hat{\gamma}^p + \mu \hat{C}_1}
\end{align*}
\]

with

\[
\hat{C}_T = \left( \frac{(L_T / R_T + 1 + k_{\text{off}} / k_{\text{on}} R_T) - \sqrt{(L_T / R_T + 1 + k_{\text{off}} / k_{\text{on}} R_T)^2 - 4L_T / R_T}}{2} \right)
\]

where we have used ‘hat’ quantities to represent nondimensionalized concentrations and parameters: \( \hat{C}_T = C_T / R_T \), \( \hat{\hat{C}}_0 = C_0 / R_T \), \( \hat{\hat{C}}_1 = C_1 / R_T \), \( \hat{\hat{C}}_2 = C_2 / R_T \), \( \hat{\hat{Y}} = Y / Y_T \), \( \hat{\hat{P}} = P / P_T \), \( \hat{\lambda} = \lambda R_T / \gamma^y_+ \), \( \hat{\gamma}^y = \gamma^-_y / \gamma^y_+ \), \( \hat{\delta} = \delta Y_T / \gamma^p_+ \), \( \hat{\gamma}^p = \gamma^-_y / \gamma^p_+ \) and \( \hat{\mu} = \mu R_T / \gamma^p_+ \).

The model calculations in Figure 3d were generated using \( k_p = 0.01 \text{ s}^{-1}, \phi = 0.1, \gamma^p = \gamma^y = 500, k_{\text{on}} R_T = 1, \lambda = 10000, \delta = 5000, \) and \( \hat{\mu} = 50000 \) with the indicated variation of \( L_T / R_T \) (x-axis) and a variation of \( k_{\text{off}} \) from \( 10^{-4} \) to \( 10^2 \text{ s}^{-1} \) (coloured lines). We provide a web applet that can be used to examine the predicted dose-response from this phenotypic model for any parameter values (Applet S1).

### Systematic analysis of phenotypic model network architectures

The key objective of the systematic analysis is to determine whether other models can also produce the key features that the kinetic proofreading with limited signalling coupled to incoherent feedforward (KPL-IFF) model is able to produce (Figure 3d, Table 1).

We performed two systematic analyses of phenotypic models: a simpler systematic analysis looking at 304 networks (Figure 4a) and a more complex analysis looking at 26,069 networks (Figure 4e). Given that the methodology is identical for both analyses and that the simpler analysis is nested within the more complex analysis, this section will focus on describing the more complex analysis. The methodology extends previous efforts to systematically study pre-defined network architectures that can produce specific phenotypes (1, 2).

**Defining the set of network architectures.** The set network architectures that we examine are defined based on the possible reactions that we consider. Each network has a receptor that can undergo kinetic proofreading with 4 steps \( (C_0, C_1, C_2, \) and \( C_3) \) and 3 additional nodes \( (X, Y, \) and \( P) \). We consider all possible networks that have 4 reaction arrows (either activating or inhibiting) between the receptor, \( X, Y, \) and \( P \) (Figure 4d). Note that every network contains the 4 kinetic proofreading states. The total number of reaction arrows is 36 and therefore the total number of networks is 58,905 (36 choose 4). Without loss of generality we identify \( P \) as the output and remove all networks where there is no connection between any of the kinetic proofreading states and \( P \) (either directly or indirectly), which reduces the number of networks to 26,069. We omit networks where \( X, Y, \) or \( P \) modulate kinetic proofreading to maintain computational feasibility. Each network contains a total of 6 free parameters; 2 kinetic proofreading parameters \( (k_p \) and \( \phi) \) and 1 parameter for each of the 4 reaction arrows.
A scale-free comparison measure. The large number of networks that are to be systematically examined means that it is not feasible to manually identify (i.e. by eye) whether a specific proposed network can also produce the output of the KPL-IFF network (Figure 3d). To automate identification we constructed a quantitative measure that can compare the output of the KPL-IFF network to a specific proposed network.

First, we define four elementary numbers for any dose-response curve (Figure S4a): the value of $P$ at the lowest concentration ($W_1$), the maximum value of $P$ ($W_2$), the value of $P$ at the largest concentration ($W_3$), and the concentration of ligand producing half-maximal response ($EC_{50}$). In cases where two values of $EC_{50}$ are possible the lower value is used (i.e. to the left of the peak in a bell-shaped dose-response).

Second, we define four metrics that capture the key qualitative features of the KPL-IFF output,

$$
F_1 = \frac{W_1}{W_2} \\
F_2 = \frac{(W_3/W_2)/(W_3^*/W_2^*)}{(W_3/W_2)/(W_3^*/W_2^*)} \\
F_3 = \frac{W_2}{W_2^*} \\
F_4 = \frac{EC_{50}}{EC_{50}^*}
$$

where the superscript * refers to the values for an index ligand, which we take to be the highest affinity ligand. These four metrics can be calculated for each ligand and plotted as a function of the ligand $k_{off}$ value for the KPL-IFF network (Figure S4b-g). The first measure ($F_1$) ensures that the dose-response curve exhibits an increase. The second measure ($F_2$) ensures that a bell-shaped dose-response is observed. The third measure ($F_3$) ensures that the peak height is similar for high affinity ligands but decreases for low affinity ligands. The fourth measure ($F_4$) ensures that ligands of intermediate affinity are first to increase as a function of dose. The normalisation to an index ligand means that these measures are largely independent of the absolute values of $L(dose)$ and $P(response)$. Note that these measures rely on fold-changes and therefore are independent of the absolute scale of the response (value of $P$).

Lastly, we define a single measure that can compare the similarity between $F_1$, $F_2$, $F_3$, and $F_4$ (across a wide range of $k_{off}$ values) for any specific network and the KPL-IFF network,

$$
SSR = \sum_{i=1}^{N} (F_1^i - \hat{F}_1^i)^2 + (F_2^i - \hat{F}_2^i)^2 + (F_3^i - \hat{F}_3^i)^2 + (F_4^i - \hat{F}_4^i)^2
$$

where the index $i$ represents each ligand ($i = 1$ to $i = N$ ligands with $N = 12$ in our example). The values of $F$ with a hat represent those for the KPL-IFF network whereas the non-hat values are for the specific network being tested.

In summary, the output of any proposed network are the values of $P$ as a function of ligand concentrations for 12 ligands with different values of $k_{off}$. This output is used to calculate $F_1$, $F_2$, $F_3$, and $F_4$ for each ligand which can then be used to compute SSR. Proposed networks with small SSR values are more likely to be compatible with the output of the KPL-IFF network and therefore the key features.

Workflow. The workflow for the systematic analysis is shown in Figure S4h. The first step is to select one of the 26,069 networks to analyse. The next two steps aim to determine the values of the 6 model parameters that produce the smallest value of the SSR for the selected network. First, the method performs 1 million evaluations of the network where the 6 model parameters for each evaluation are randomly sampled (uniform distribution in log-space) and the SSR for each evaluation is determined. Second, the values of the 6 parameters for the 15 network evaluations that produced the smallest values of SSR (from the 1 million that were sampled) are then used as initial conditions for a non-linear optimisation algorithm ($fminsearch$ in Matlab) that uses a modified simplex method to further minimize the SSR. Finally, the 6 parameter values that produced the smallest SSR (among the 15 optimised parameter values) are recorded along with the associated SSR for the network. The procedure is repeated for all 26,069 networks.
Given that the analysis relies on two steps that have a stochastic element (random parameter sampling and further optimisation) we repeated the analysis 3 times for both the simpler and complex analysis but found no difference to the results (see below) suggesting that the search algorithm had sufficient coverage of parameter space.

**Results.** The output of the analysis is a list of the 26,069 networks (or the 304 networks for the simpler analysis) ordered from the smallest to the largest SSR. We summarise this output in Movie S2 (or Movie S1 for the simpler analysis) where each 1 second frame corresponds to a network architecture evaluated with the 6 optimised parameter values. By examining the movies we find that 274 of the 26,069 networks in the complex analysis (or 1 of the 304 networks in the simpler analysis) are compatible with the key features as they are able to reproduce the output of the KPL-IFF network. Importantly, all 274 compatible networks contained the same underlying KPL-IFF mechanism. In many cases these more complex networks were reduced to the same KPL-IFF model shown in Figure 3d (e.g. networks 2, 4, 7 to 21, etc) whilst in other cases the incoherent feedforward exhibited indirect inhibition (e.g. networks 1, 3, 5, 6, etc) but operated in a parameter regime where inhibition saturated after activation.

**Mathematical model.** The systematic analysis relies on evaluating many network architectures. To do this, we have implemented a single general mathematical model that can be reduced to all 26,069 networks by setting appropriate reactions to zero.

The ordinary-differential-equations (ODEs) corresponding to the most general mathematical model are,

\[
\begin{align*}
\frac{dL}{dt} &= -k_{on} LR + k_{off} CT \\
\frac{dR}{dt} &= -k_{on} LR + k_{off} CT \\
\frac{dC_0}{dt} &= k_{on} LR - (k_{off} + k_p)C_0 \\
\frac{dC_i}{dt} &= k_p C_{i-1} - (k_p + k_{off}) C_i & 1 \leq i < N - 1 \\
\frac{dC_{N}}{dt} &= k_p C_{N-1} - (k_{off} + \phi k_p) C_{N} \\
\frac{dC_{N+1}}{dt} &= \phi k_p C_{N} - k_{off} C_{N+1} \\
\frac{dX}{dt} &= \gamma^x_+ (X_T - X) - \gamma^x_- X + (\tilde{X}^x \cdot \tilde{C})(X_T - X) - (\tilde{P}^x \cdot \tilde{C})X \\
&+ (\eta^x_I Y + \eta^x_P P)(X_T - X) - (\eta^y_I Y + \eta^y_P P) X \\
\frac{dY}{dt} &= \gamma^y_+ (Y_T - Y) - \gamma^y_- Y + (\tilde{X}^y \cdot \tilde{C})(Y_T - Y) - (\tilde{P}^y \cdot \tilde{C}) Y \\
&+ (\beta^x_+ X + \beta^x_P P)(Y_T - Y) - (\beta^y_+ X + \beta^y_P P) Y \\
\frac{dP}{dt} &= \gamma^p_+ (P_T - P) - \gamma^p_- P + (\tilde{X}^p \cdot \tilde{C})(P_T - P) - (\tilde{P}^p \cdot \tilde{C}) P \\
&+ (\delta^x_+ X + \delta^x_P Y)(P_T - P) - (\delta^y_+ X + \delta^y_P Y) P
\end{align*}
\]

where \( C_T = \sum_{i=0}^{N+1} C_i \) and \( \tilde{X}^x \cdot \tilde{C} \) is the vector dot product between the parameter vector \( (\tilde{X}^x = [\lambda_0^x, \lambda_1^x, ..., \lambda_{N+1}^x]) \) and the vector of complexes \( \tilde{C} = [C_0, C_1, ..., C_{N-1}, C_N, C_{N+1}] \). We solve the ODE system in the steady-state.
to obtain,
\[ C_0 = \alpha^0(1 - \alpha)C_T \]
\[ C_1 = \alpha^1(1 - \alpha)C_T \]
\[ \vdots \]
\[ C_{N-1} = \alpha^{(N-1)}(1 - \alpha)C_T \]
\[ C_N = \alpha^N \left( \frac{k_{off}}{k_{off} + \phi k_p} \right) C_T \]
\[ C_{N+1} = \alpha^N \left( \frac{\phi k_p}{k_{off} + \phi k_p} \right) C_T \]

where
\[ \frac{C_T}{R_T} = \left[ \frac{L_T}{R_T} + 1 + \frac{k_{off}}{k_{on}R_T} - \sqrt{\left( \frac{L_T}{R_T} + 1 + \frac{k_{off}}{k_{on}R_T} \right)^2 - 4 \frac{L_T}{R_T}} \right] / 2, \]
\[ \alpha = \frac{k_p}{k_{off} + k_p}, \]

and the equations for \( X, Y, \) and \( P \) simplify to,
\[ 0 = (1 - X) - \gamma_m^x X + (\tilde{\lambda}_m^x \cdot \tilde{C}) (1 - X) - (\mu_m^x \cdot \tilde{C}) X + (\alpha_m^x P + \alpha_m^x P)(1 - X) - (\eta_m^y Y + \eta_m^y P) X \]
\[ 0 = (1 - Y) - \gamma_m^y Y + (\tilde{\lambda}_m^y \cdot \tilde{C}) (1 - Y) - (\mu_m^y \cdot \tilde{C}) Y + (\beta_m^y X + \beta_m^y P)(1 - Y) - (\beta_m^y X + \beta_m^y P)Y \]
\[ 0 = (1 - P) - \gamma_m^p P + (\tilde{\lambda}_m^p \cdot \tilde{C}) (1 - P) - (\mu_m^p \cdot \tilde{C}) P + (\delta_m^p X + \delta_m^p Y)(1 - P) - (\delta_m^p X + \delta_m^p Y)P \]

where we have nondimensionalized concentrations \( (R \text{ by } R_T, \ C_i \text{ by } R_T, \ X \text{ by } X_T, \ Y \text{ by } Y_T, \ P \text{ by } P_T) \) but retained original notation for clarity. The subscript \( m \) indicates that the parameter has been modified as a result of the nondimensionalization process: \( \gamma_m^x = \gamma_m^x / \gamma_{+,x}, \ \gamma_m^y = \gamma_m^y / \gamma_{+,y}, \ \gamma_m^p = \gamma_m^p / \gamma_{+,p}, \ \tilde{\lambda}_m^x = \tilde{\lambda}_m^x R_T / \gamma_{+,x}, \ \tilde{\lambda}_m^y = \tilde{\lambda}_m^y R_T / \gamma_{+,y}, \ \mu_m^x = \mu_m^x / \gamma_{+,x}, \ \mu_m^y = \mu_m^y / \gamma_{+,y}, \ \beta_m^x = \beta_m^x / \gamma_{+,x}, \ \beta_m^y = \beta_m^y / \gamma_{+,y}, \ \beta_m^p = \beta_m^p / \gamma_{+,p}. \)

In the case of the more complex analysis (4 nodes, 4 reaction arrows) the number of kinetic proofreading steps was 4 \((N = 3)\). The ratio of background inhibition to background activation of \( X, Y, \) and \( P \) were fixed at 500 so that without any ligand the concentrations of \( X, Y, \) and \( P \) were near zero \((\gamma_m^x = \gamma_m^y = \gamma_m^p = 500)\). The remaining parameters were set to 0 with the exception of \( k_p, \phi, \) and 4 other parameters that defined the network architecture. The same procedure was carried out for the simpler analysis (3 nodes, 3 reaction arrows) except that the number of kinetic proofreading steps was 3 \((N = 2)\) and all reactions to and from \( X \) were set to zero.

The mathematical model was numerically solved using fzero in Matlab (Mathworks, MA), which allowed the code to be translated to C++ by the Matlab Coder toolbox. We found that the solution converged rapidly when using 0 as the initial guess.

**KPL-IFF model parameters compatible with phenotypic features**

To determine the set of parameters in the KPL-IFF model \((\lambda, \delta, \mu, k_p, \phi)\) compatible with the key features we utilised Approximate Bayesian Computations with Sequential Monte Carlo (ABC-SMC) \((3, 4)\). We used the SSR (described above) as the summary statistic and terminated the algorithm when \(> 10,000 \) particles were found with \( SSR < 1 \). Distributions of the 5 parameters along with their pairwise correlations are shown in Figure S6.
Negative feedback cannot produce a bell-shaped dose-response

The systematic analysis described in the previous section (see also main text and Fig. 4) has revealed that negative feedback, although well known to produce oscillations in time, cannot produce a bell-shaped dose-response in the steady-state. In this section we provide further evidence for this numerical result by providing an intuitive explanation together with an analytical proof.

First, consider a two component negative feedback (Figure S5a) whereby P activates Y and Y in turn inhibits P. As the activator of P increases (\(\hat{C}_1\) in this example), P will increase. In order to decrease P (to produce a bell-shaped dose-response) P needs to inhibit Y more strongly at larger values of P. But a larger value of active Y (needed for stronger inhibition of P) requires larger value of active P. Therefore, P and Y are subjected to two conflicting requirements which cannot be satisfied simultaneously. It follows that a bell-shaped dose-response for P cannot be achieved in the steady-state.

In what follows we provide a mathematical proof that a bell-shaped dose-response cannot be produced with negative feedback in the steady-state. To do this, we derive an implicit expression for P and show that the first derivative cannot be zero for any positive reaction rate constants. The non-linear coupled system of ODEs for the two component negative feedback can be written as follows,

\[
\begin{align*}
\frac{d\hat{Y}}{dt} &= \frac{\eta^P}{1 + \left(\frac{\hat{P}}{\hat{P}_c}\right)^n} + \gamma^\pm \left[ \frac{\eta^P}{1 + \left(\frac{\hat{P}}{\hat{P}_c}\right)^n} + \gamma^+ + \gamma^- \right] \hat{Y} \\
\frac{d\hat{P}}{dt} &= (\lambda \hat{C}_1 + \gamma^P) (1 - \hat{P}) - \beta^\pm \hat{Y} \hat{P} - \gamma^P \hat{P}
\end{align*}
\]

where \(\hat{Y}\) and \(\hat{P}\) are non-dimensional (\(\hat{Y} = Y/Y_T\), \(\hat{P} = P/P_T\)) with \(\hat{\rho} = \rho/P_T\), and \(\hat{\beta}^\pm = \beta^\pm Y_T\). Note that all reactions considered in the main text are based on non-saturating mass action but in this analysis we have included a more general saturating inhibition, which can be reduced to mass action in the limit of large \(\hat{\rho}\) with \(n = 1\). In the steady-state we find,

\[
\hat{Y} = \frac{(\lambda \hat{C}_1 + \gamma^P)(\frac{1}{\hat{P}} - 1) - \gamma^P}{\beta^\pm}
\]

which, we substitute into the steady-state of equation (2) to obtain,

\[
f = 1 - \left[ 1 + \frac{\gamma^Y}{\frac{\eta^P}{1 + \left(\frac{\hat{P}}{\hat{P}_c}\right)^n} + \gamma^+} \right] \left[ \frac{\kappa(\frac{1}{\hat{P}} - 1) - \gamma^P}{\beta^\pm} \right] = 0
\]

where \(\kappa = \lambda \hat{C}_1 + \gamma^P\) contains the input from \(\hat{C}_1\). To determine if P can exhibit a bell-shaped dose-response as a function of \(\hat{C}_1\) we determine the value of the parameters where the first derivative of \(\hat{P}\) is zero. To do this, we differentiate \(P\) with respect to \(\kappa\),

\[
\frac{df}{d\kappa} = - \left[ \frac{-n \gamma^Y \eta^P \left( \frac{\hat{P}}{\hat{P}_c} \right)^n \frac{d\hat{P}}{d\kappa}}{\hat{P} \left( \frac{\eta^P}{1 + \left(\frac{\hat{P}}{\hat{P}_c}\right)^n} + \gamma^+ \right)^2 \left[ 1 + \left(\frac{\hat{P}}{\hat{P}_c}\right)^n \right]^2} \right] \left[ \frac{\kappa(\frac{1}{\hat{P}} - 1) - \gamma^P}{\beta^\pm} \right] \\
- \left[ 1 + \frac{\gamma^Y}{\frac{\eta^P}{1 + \left(\frac{\hat{P}}{\hat{P}_c}\right)^n} + \gamma^+} \right] \left[ \frac{1}{\beta^-} \left( \frac{\hat{P} - \kappa \frac{d\hat{P}}{d\kappa}}{\hat{P}^2} - 1 \right) \right] = 0
\]
At \( \frac{d\hat{P}}{dx} = 0 \), equation (5) reduces to

\[
- \left[ 1 + \frac{\gamma_Y^+}{\eta_P^+} \right] \left[ \frac{1}{\beta_-^Y} \left( \frac{1}{\hat{P}} - 1 \right) \right] = 0
\]

which can be satisfied when

\[
1 + \frac{\gamma_Y^+}{\eta_P^+} = 0 \tag{6}
\]

and/or when

\[
\frac{1}{\beta_-^Y} \left( \frac{1}{\hat{P}} - 1 \right) = 0 \tag{7}
\]

Given that all reaction rate constants and concentrations must be positive it is clear that there are no values of \( \hat{P} \) and reaction rate constants that satisfy equation (6). We do find that equation (7) can be satisfied when \( \hat{P} = 1 \) (i.e. \( \hat{P} \) is maximally active) but at this value of \( \hat{P} \) we find that the equation for \( f \),

\[
f = 1 + \frac{\gamma_P^+}{\beta_-^Y} \left[ 1 + \frac{\gamma_Y^+}{\eta_P^+} + \gamma_Y^+ \right] = 0
\]

which, as above, can never be realised because all reaction rates must be equal or greater than zero. Therefore, we conclude that a simple negative feedback motif cannot explain the observed optimum in the dose-response curve.

We have also performed this analysis on a 3 node network with non-saturating mass action (Figure S5d-f). As before, \( C_1 \) activates \( P \) which in this network indirectly activates the inhibitor \( X \) by the activation of \( Y \). In this architecture we observe oscillations in time (Figure S5e) but a bell-shaped dose-response in the steady state was not possible (Figure S5f). In what follows we show that this model is a special case of the model presented above and in this way we show that it cannot produce bell-shaped dose-response curves.

The system of ODEs for this model is,

\[
\frac{d\hat{X}}{dt} = \eta_P^+ \hat{Y} (1 - \hat{X}) + \gamma_+^X (1 - \hat{X}) - \gamma_-^X \hat{X} \tag{8}
\]

\[
\frac{d\hat{P}}{dt} = (\lambda \hat{C}_1 + \gamma_+^P)(1 - \hat{P}) - \gamma_+^P \hat{P} - \beta_-^X \hat{X} \hat{P} \tag{9}
\]

\[
\frac{d\hat{Y}}{dt} = (\delta_+^P \hat{P} + \gamma_+^Y)(1 - \hat{Y}) - \gamma_-^Y \hat{Y} \tag{10}
\]

where \( \hat{X} = \frac{X}{X_T}, \hat{P} = \frac{P}{P_T}, \hat{Y} = \frac{Y}{Y_T}, \eta_+^Y = \eta_+^Y Y_T, \beta_-^X = \beta_-^X X_T \) and \( \delta_+^P = \delta_+^P P_T \). In the steady-state we find,

\[
\hat{X} = \frac{\lambda \hat{C}_1 + \gamma_+^P (\frac{1}{\hat{P}} - 1)}{\eta_P^+ + \gamma_+^P + \beta_-^X X_T} \tag{11}
\]

\[
\hat{Y} = \frac{\gamma_+^Y}{\eta_P^+ + \gamma_+^P + \beta_-^X X_T} \tag{12}
\]

which, as above, can never be realised because all reaction rates must be equal or greater than zero. Therefore, we conclude that a simple negative feedback motif cannot explain the observed optimum in the dose-response curve.
which can be substituted into the steady-state expression for $\frac{d\hat{X}}{dt}$ to obtain an implicit equation for $\hat{P}$ in terms of the parameters of the system,

$$g = 1 - \left[ 1 + \frac{\gamma X}{\eta^1_\gamma + \gamma^X} \right] \frac{\kappa \left( \frac{1}{\rho} - 1 \right) - \gamma_\rho P}{\beta^X} = 0$$

(11)

where again, $\kappa = \lambda \hat{C}_1 + \gamma_\rho P$. When $n = 1$ the equation for $f$ (derived above) is identical to the equation of $g$ with the exception of a constant. Given that the conclusions above are independent of $n$ it follows that we can conclude that this 3 node network with negative feedback cannot exhibit bell-shaped dose-response curves in the steady-state. This is consistent with results from the systematic network analysis that did not find any negative feedback networks compatible with the phenotypic features.

**Predicted T cell activation by co-presentation of pMHC ligands in the KPL-IFF model**

The KPL-IFF model was extended to include an additional pMHC by first calculating the fraction of TCR bound to each ligand at steady-state using the following set of coupled ODEs,

$$\frac{dL_1}{dt} = -k_{on}^1 L_1 R + k_{off}^1 C^1$$
$$\frac{dL_2}{dt} = -k_{on}^2 L_2 R + k_{off}^2 C^2$$
$$\frac{dR}{dt} = -k_{on}^1 L_1 R + k_{on}^1 C^1 - k_{on}^2 L_2 R + k_{off}^2 C^2$$
$$\frac{dC^1}{dt} = k_{on}^1 L_1 R - k_{off}^1 C^1$$
$$\frac{dC^2}{dt} = k_{on}^2 L_2 R - k_{off}^2 C^2$$

where $C^1$ and $C^2$ are, respectively, the concentration of TCR bound to the first and second pMHC ligands. These ODEs were integrated to the steady-state to determine $C^1$ and $C^2$, which were used to calculate $P$ as follows,

$$\hat{C}_1 = \left( \frac{k_{off}^1}{k_{off}^1 + k_p} \right) \frac{k_p}{k_{off}^1 + \phi k_p} \hat{C}^1$$
$$\hat{C}_2 = \left( \frac{k_{off}^2}{k_{off}^2 + k_p} \right) \frac{k_p}{k_{off}^2 + \phi k_p} \hat{C}^2$$
$$\hat{Y} = \frac{1 + \hat{Y}(\hat{C}^1_1 + \hat{C}^2_1)}{1 + \hat{Y}(\hat{C}^1_1 + \hat{C}^2_1) + \gamma_\rho}$$
$$\hat{P} = \frac{1 + \hat{Y}}{1 + \hat{Y} + \gamma_\rho + \hat{P}(\hat{C}^1_1 + \hat{C}^2_1)}$$

where superscripts indicate ligand identity (1 or 2). Parameter values are identical to those used to generate Figure 3d with the ligand concentration and kinetic parameters as indicated in Figure 5.
Model of kinetic proofreading with limited signalling followed by TCR internalisation

The following ODEs were used to model kinetic proofreading with limited signalling with constitutive and induced TCR internalisation,

\[
\begin{align*}
\frac{dL}{dt} &= -k_{on}LR + k_{off}(C_0 + C_1 + C_2) + \beta(C_0 + C_1 + C_2) \\
\frac{dR}{dt} &= -k_{on}LR + k_{off}(C_0 + C_1) + \alpha - \beta R \\
\frac{dC_0}{dt} &= k_{on}LR - (k_{off} + k_p + \beta)C_0 \\
\frac{dC_1}{dt} &= k_pC_0 - (k_{off} + \phi k_p + \beta)C_1 \\
\frac{dC_2}{dt} &= \phi k_pC_1 - (k_{off} + \beta)C_2
\end{align*}
\]

where \(\alpha\) and \(\beta\) are the constitutive receptor recycling rates. In this model, it is assumed that \(C_2\) represents a state where the receptor is tagged for induced internalisation so that immediately upon pMHC unbinding it is internalised (i.e. \(k_{off}C_2\) does not appear in the equation for \(R\)). The model is integrated to the steady-state using the Matlab (Mathworks, MA) function ode23s with the parameters indicated in Figure S8.
Supplementary Movies

Movie S1. Systematic analysis of 304 network architectures (Figure 4a). Each 1 second frame shows the network architecture (left) and the network output (right). The network architecture displays the 3 receptor states ($C_0$, $C_1$, and $C_2$) together with $Y$ and $P$ where green arrows indicate activation and red arrows indicate inhibition (magnitudes of inhibition arrows are in italics). The network output displays $P$ (y-axis) over the ligand concentration (x-axis) for ligands with decreasing $k_{off}$ (blue to red). Networks appear in ascending order of the SSR with networks that are better able to produce the phenotypic features appearing first. See Supplementary Information for computational details.

Movie S2. Systematic analysis of 26,069 network architectures (Figure 4e). Movie generated as described (see Movie S1) except that one additional receptor state and one additional node were included (see main text and Figure 4e for details). This movie can be found at this link: https://dx.doi.org/10.6084/m9.figshare.3491792.v1

Supplementary Applet

Applet S1. A Javascript applet that can be used to examine how the 5 model parameters ($k_p$, $\phi$, $\mu$, $\lambda$, and $\delta$) modulate the predicted dose-response for ligands of varying affinities for the KPL-IFF model (Figure 3d). Default parameter values in the applet are the same as those used to generate Fig. 3d. This applet can be found at this link: https://dx.doi.org/10.6084/m9.figshare.3491807.v1

Supplementary Table

Table S1: Measured binding properties for the c58c61 TCR for the indicated peptide in complex with HLA-A*02:01 (pMHC ligand). Results are averages of at least 3 measurements.
Figure S1: Representative surface plasmon resonance (SPR) measurements of TCR-pMHC interactions (Table S1). All experiments are performed by injecting recombinant c58c61 TCR over immobilised recombinant pMHC (see Materials & Methods). The precise protocol for determining the kinetic rate constants ($k_{on}$ and $k_{off}$) was dependent upon the affinity regime of the TCR-pMHC interaction. a-b) In the case of TCR-pMHC interaction with small $k_{off}$, a single concentration of TCR is injected over the surface. The value of $k_{off}$ is determined from the dissociation curve (right) which is used to fit the association curve (middle) with exponential rate of $k_{on}[TCR]+k_{off}$. c-d) In the case of TCR-pMHC interactions with a larger $k_{off}$, multiple concentrations of the TCR were injected over the surface. The value of $k_{off}$ was determined from the dissociation curve and the value of $k_{on}$ was either determined from fitting the association curves (as in c) or by first determining the $K_D$ ($k_{on} = k_{off}/K_D$, as in d). When kinetics were too rapid to be resolved we only report the value of $K_D$. See Materials & Methods for a detailed protocol and mathematical analysis of the data.
Figure S2: a) Expression of the c58c61 TCR on primary CD8\(^+\) T cells and Jurkat T cells using the biotinylated high affinity pMHC (9V) with R-PE-conjugated streptavidin (red line) or only R-PE-conjugated streptavidin as a staining control (grey line). The Jurkat T cells were sorted following transduction. b,c) Functional assays (top) together with pMHC immobilisation controls (bottom) for b) primary CD8\(^+\) T cells and c) Jurkat T cells with a larger number of higher affinity ligands. Functional assays were corrected for differences in pMHC immobilisation (see Materials & Methods). d) The transcriptional activity of NFAT in Jurkat T cells was determined at 16 hours for the indicated pMHC ligands revealing qualitatively similar behaviour to IL-8. This reporter of T cell activation was not used routinely because of the poor signal-to-noise ratio of the assay. e) Activation assays with primary T cells were performed as described in the main text for the indicated pMHC except that brefeldin A was added to block cytokine secretion for the last 2 hours of the assay (reducing the amount of supernatant cytokine in the cell population assay, left column) at which point intracellular cytokine levels were determined by flow cytometry (right column). The supernatant concentration of cytokine (cell population assay) compares favorably with the mean intensity of cytokine in the positive population (single cell assay). All data are representative of at least 3 independent experiments.
Figure S3: The concentration of TCR-pMHC complexes that regulate the activation of $P$ for the phenotypic model calculations in Figure 3. The concentration of a) $C_0$ in the occupancy model, b) $C_0$ in the occupancy coupled to incoherent feedforward model, c) $C_1$ in the kinetic proofreading coupled to incoherent feedforward model, and d) $C_1$ in the KPL-IFF model. The maximum concentration of signalling competent TCR-pMHC complexes for the occupancy models (a,b) is independent of the TCR-pMHC off-rate ($k_{off}$) whereas for the kinetic proofreading models (c,d) the maximum concentration of signalling competent TCR-pMHC complexes is dependent on the off-rate.

Figure S4: Quantitative metrics for comparing signalling network outputs. a) Schematic of a bell-shaped dose-response showing the definition of $W_1$, $W_2$, $W_3$, and $EC_{50}$. b) Schematic of the network architecture of the KPL-IFF phenotypic model where green arrows indicate activation and red arrows indicate inhibition with the magnitude of the arrow indicated (compare to Figure 3d). c) Output of the KPL-IFF phenotypic model shown in B with 12 pMHC ligands whose $k_{off}$ varies from $10^{-4}$ (red) to 10 (blue). d-g) The values of the metrics $F_1$, $F_2$, $F_3$, and $F_4$ as a function of $k_{off}$ for the output in panel c. h) Workflow of the systematic network search algorithm.
Figure S5: Negative feedback can produce oscillations in time but not bell-shaped dose-response curves in the steady-state. a) A two node negative feedback, whereby $P$ activates its inhibitor $Y$ (see Figure 4d) can b) produce oscillations in $P$ over time but c) not bell-shaped dose-response in the steady-state. d) A three node negative feedback, where $P$ activates $Y$ which in turn activates $X$ that is able to inhibit $P$ can (as for the two component negative feedback) e) produce damped oscillations in $P$ over time but f) not bell-shaped dose-response in the steady-state. See Supplementary Information for calculation details.
Figure S6: KPL-IFF model parameters compatible with phenotypic features. The probability density of the indicated parameter is shown along the diagonal along with pairwise correlations in the off-diagonals with yellow indicating a high frequency of occurrence (scale bar indicates number of occurrences). Vertical dashed lines in the probability densities indicate the default parameter used in Figure 3d. We find a broad range of parameter values compatible with phenotypic features but with certain relationships amongst them. For example, we find that $\mu > \delta$ but both parameters can vary more than 1000-fold provided this relationship is maintained. See Supplementary Information for calculation details.
Figure S7: Possible relationships between the phenotypic model modules and known T cell signalling components. 

a) Kinetic proofreading can be realised by the sequential phosphorylation of the T cell receptor ITAMs and/or the recruitment of Lck associated coreceptors (5) (not shown) followed by the recruitment and subsequent activation of ZAP-70 (6). b) Limited signalling of the T cell receptor may occur as a result of ubiquintination (7) and/or the receptor entering membrane environments that are incompatible with signalling (8). c) Incoherent feedforward in the signalling cascade initiated by the T cell receptor may occur between LAT and Ras (9). Phosphorylated LAT provides docking sites for Grb2 which recruits the guanine nucleotide exchange factor (GEF) SOS that promotes the formation of the active form of Ras (RasGTP) that promotes downstream signalling. However, phosphorylated LAT also provides docking sites for DOK1/DOK2 which recruits the GTPase activating protein (GAP) RasGAP that promotes the formation of RasGDP and hence reduces the active form of Ras. Although not depicted in the schematic, incoherent feedforward may also arise from the TCR signalosome because it is able to associate with both a tyrosine kinase (ZAP-70) and a tyrosine phosphatase (SHP-1) (10).
Figure S8: Internalisation of TCR following signalling does not produce bell-shaped dose-responses in the steady-state. a) Schematic of internalisation model that includes kinetic proofreading with limited signalling followed by TCR internalisation (from state $C_2$) with basal recycling of receptor at the cell surface. b) Concentration of signalling TCR and c) total surface TCR over ligand concentrations for different ligand affinities at steady-state. Parameters: $k_p = 0.01 \text{ s}^{-1}$, $\phi = 0.1$, $\alpha = 0.01 \mu\text{m}^{-2}\text{s}^{-1}$, $\beta = 0.001 \text{ s}^{-1}$ with a variation of $k_{off}$ from $10^{-4}$ to $10^2 \text{ s}^{-1}$ (coloured lines).
References


