MATHEMATICAL AND COMPUTATIONAL MODELS OF IMMUNE-RECEPTOR SIGNALLING

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The process of signalling through receptors of the immune system involves highly connected networks of interacting components. Understanding the often counter-intuitive behaviour of these networks requires the development of mathematical and computational models. Here, we focus on the application of these models to understand signalling through immune receptors that are involved in antigen recognition. Simple models, which ignore the details of the signalling machinery, have provided considerable insight into how ligand–receptor binding properties affect signalling outcomes. Detailed models, which include specific molecular components and interactions beyond the ligand and receptor, are difficult to develop but have already provided new mechanistic understanding and uncovered relationships that are difficult to detect by experimental observation alone. They offer hope that models might eventually predict the full spectrum of signalling behaviour.

MULTICHAIN IMMUNE-RECOGNITION RECEPTOR (MIRR). The prototypical members of the MIRR family are the B-cell receptor, the T-cell receptor and the high-affinity IgE receptor (FcεRI). Each of these cell-surface receptors is multimeric and involved in antigen recognition.

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The cells of the adaptive immune system use B-cell receptors (BCRs), T-cell receptors (TCRs) and Fc receptors to recognize antigens, peptide antigens bound to MHC molecules and antigen–antibody complexes, respectively. The BCR and TCR are members of the MULTICHAIN IMMUNE-RECOGNITION RECEPTOR (MIRR) family[1]. The subunits of these receptors can be divided into those that participate in antigen recognition and those that participate in intracellular signalling. All of the signalling subunits have at least one copy of a particular amino-acid sequence in their cytoplasmic domains; this is called the immunoreceptor tyrosine-based activation motif (ITAM). For the MIRRs, each ITAM is composed of a pair of Tyr-Xaa-Xaa-Leu/Ile sequences separated by a variable number of amino-acid residues[2,3]. The multichain Fc receptors — FcαR, FcεRI, FcγRI and FcγRIII — are also members of the MIRR family and have ITAM-containing cytoplasmic domains[4].

For a soluble ligand (such as an antigen, an immune complex or a peptide–MHC oligomer[5,6]) to activate a cell that expresses MIRRs, the ligand needs to induce the receptors to aggregate. The ligand achieves this through multivalency[7]. Aggregation of MIRRs is required for the activation of cellular responses and, at low concentrations of the ligand, this is crucial for enabling capture of the ligand. By forming multivalent attachments, the ligand holds the ITAM-containing cytoplasmic tails of receptors in proximity for a much longer period of time than when the cytoplasmic tails are juxtaposed through random encounters of receptors diffusing in the cell membrane[8]. A membrane-associated SRC-family kinase initiates the signalling cascade by phosphorylating canonical tyrosine residues in the ITAMs: through their tandem SRC HOMOLOGY 2 (SH2) DOMAINS, SYK (spleen tyrosine kinase), which is expressed by B cells and mast cells, and ZAP70 (ζ-chain-associated protein kinase 70 kDa isoform), which is expressed by T cells, are recruited from the cytosol to doubly phosphorylated ITAMs[9–13] (FIG. 1). The phosphorylated forms of the cytoplasmic domains of the receptor and other scaffolding proteins are sites for the coalescence of kinases, phosphatases and adaptor molecules, but the structures that form are temporary, with components associating and dissociating rapidly[14]. The success of their assembly depends on the stability of the ligand–receptor bond. If binding occurs

SRC HOMOLOGY 2 DOMAINS (SH2 domains). Protein domains that bind phosphorylated tyrosine residues and are present in many signalling proteins, including the kinases of the SRC and SYK (spleen tyrosine kinase) families.

too briefly, the structures are not completed and further signalling is prevented or complexes form that lead to inhibitory signals[15–18]. The nature of the signalling complex that forms following ligand–receptor engagement — stimulatory or inhibitory — also depends on many other parameters, such as the concentrations of other proteins that can bind to the complex, the strength of these interactions and the level of enzymatic activity of the kinases and phosphatases. A detailed model of signalling needs to incorporate these quantitative properties and make different predictions for different values of each parameter.

A mathematical model represents the essential characteristics of a system — the signalling machinery of a cell, in this case — as a set of mathematical equations that can either be solved generally (an analytical solution) or solved for specific numerical values of the parameters using a computer. Mathematical models present a well-controlled setting in which to test different hypotheses about how a system works. A useful model needs to make predictions that match a range of experimental observations. Often, the testing of a model against experimental data leads to the discovery of missing elements that are crucial for obtaining accurate results. Analysis of the model can then yield further insights into the underlying mechanisms, which leads to a new round of predictions and experiments.

Here, we review recent efforts to develop both simple and detailed models of signalling through antigen receptors and discuss what we have learned from them. First, we describe how two relatively simple models — serial engagement and kinetic proofreading — have been used to show, in general terms, how signalling is affected by the dissociation rate of ligand–receptor binding. Second, we look at how more-detailed models have considered membrane-proximal events (at the level of ITAM phosphorylation and the assembly of receptor-containing complexes), revealing some unexpected phenomena.

The ingredients of a mathematical model

Simple models. Many models attempt to capture some feature or features of cell signalling but make no attempt to describe realistically the signalling cascades that are activated. In these models, the actual signalling cascade is replaced by one or more arbitrary transitions. These models are simple in the sense that they have few components and a simple mathematical description. But 'simple' models are also intrinsically more abstract than detailed ones: their components and parameters often do not correspond directly to well-defined physical quantities. Nonetheless, they can provide insight into the behaviour of a system and drive experimental and detailed modelling efforts.

Detailed models. Building a detailed, but manageable, mathematical model of a cell-signalling cascade involves selecting for study a limited set of the protein components that participate in signalling. The role of the mathematical model is to improve our understanding

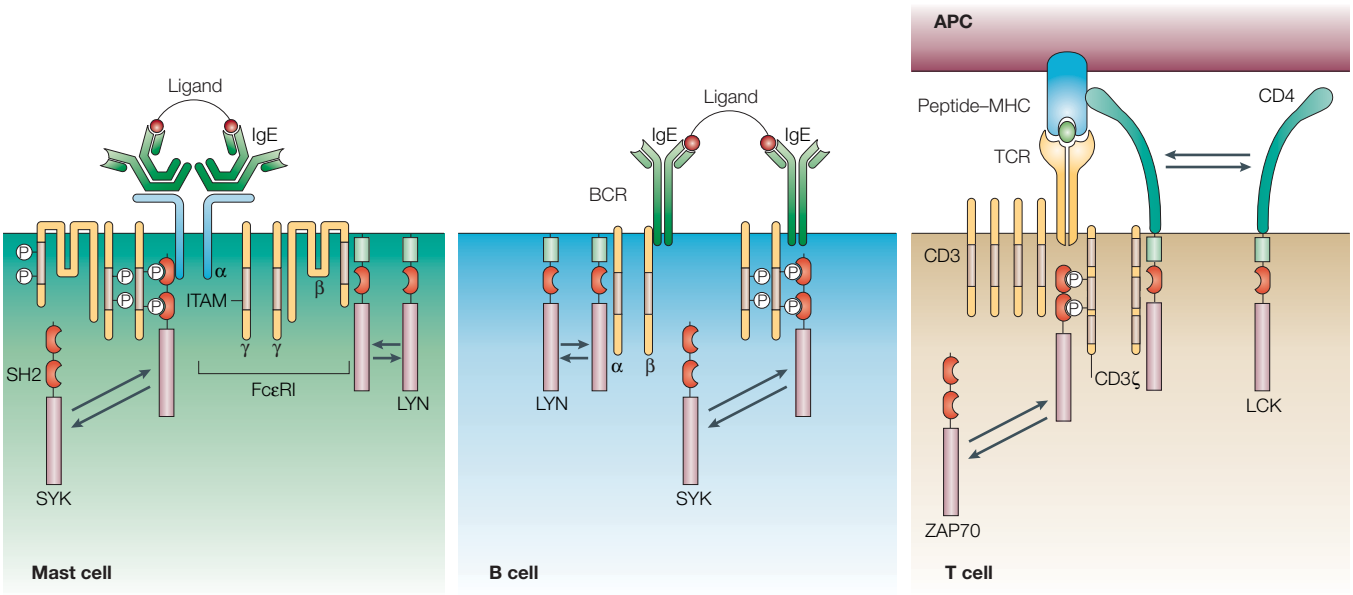


Figure 1 | **Initiation of immune-receptor signalling.** After B-cell receptor (BCR) or Fc receptor aggregation, or after the binding of a peptide–MHC complex to a T-cell receptor (TCR) (which occurs in the contact region between the T cell and an antigen-presenting cell, APC), a SRC-family kinase phosphorylates tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs). The SRC kinase LYN associates with the unphosphorylated α-chain (CD79a) of the BCR[83] and the unphosphorylated β-chain of the high-affinity IgE receptor (FcεRI)[84]. In T cells, the SRC kinase LCK associates with CD4 (or CD8); CD4 also interacts with MHC class II molecules, and LCK also interacts with phosphorylated CD3ζ, through its SRC homology 2 (SH2) domain. LYN and LCK can also bind with higher affinity to phosphorylated ITAMs (not shown). SYK (spleen tyrosine kinase) is recruited to the α- and β-chains of the BCR and the γ-chains of FcεRI when their ITAMs are doubly phosphorylated. Similarly, ZAP70 (ζ-chain-associated protein kinase 70 kDa isoform) is recruited to doubly phosphorylated ITAMs on the ζ-chains of the TCR–CD3 complex. All the binding reactions are reversible with relatively short half-lives (tens of seconds). There are also phosphatases present that can rapidly dephosphorylate phosphotyrosines that are not protected from dephosphorylation by being bound to SH2 domains (not shown). For a more detailed description of these early signalling events see REFS 85–88.

RATE CONSTANTS
Parameters with a constant value in a mathematical expression for the rate of a chemical reaction. The rate of the elementary chemical reaction A→B is given by $k[A]$, where k is the rate constant and [A] is the concentration of species A.

PARTIAL DIFFERENTIAL EQUATIONS
(PDEs). Differential equations that involve more than one independent variable. Often the independent variables of interest are time and position in space.

of how the selected proteins interact with each other and how these interactions influence cell-signalling events. This requires the clear and detailed specification of all of the interactions that occur between the components and all of the rates at which they proceed. A three-part protocol for defining such a mathematical model follows.

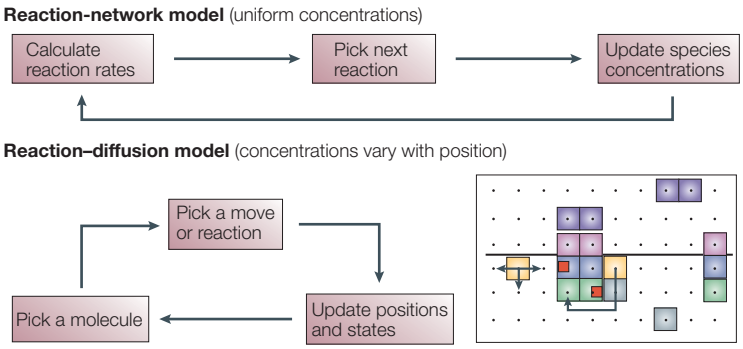
First, choose a set of components, and define their interactions based on what is known about the system. This information is typically gleaned from published studies, which are often ambiguous or contradictory, and incomplete. The choice of components and the level of detail used to describe them depend crucially on the questions to be asked, which are guided by the types of experiments that the model will be used to analyse. For example, investigating why mutation of a specific tyrosine residue has certain effects on signalling events requires detailed modelling of that residue and the effect of its phosphorylation on other interactions in the model. Comparison with experimental data often leads to revision of the set of components: those that do not affect properties of interest are removed and those originally deemed unnecessary might turn out to be crucial.

Second, select parameters that quantify the cellular concentrations of the components and the strength of the interactions between components (known as RATE CONSTANTS). Obtaining the values for these parameters, which often determine the behaviour of the model[19–23], is a considerable obstacle to developing new models. For example, in a given cell line, the absolute expression levels of most proteins are not known. The components and their interactions, together with these parameters, define a chemical-reaction network, which consists of a set of molecular species and the reactions that can take place between them. These species represent the different combinations and chemical-modification states of the components.

Third, choose a mathematical formulation or a method of simulation (BOX 1). Reaction-network models are based on the assumption that each species is uniformly distributed throughout the cell; this enables the construction of a set of ORDINARY DIFFERENTIAL EQUATIONS (ODEs) — one equation for each species in the model — the solution of which provides the average concentration of each species as a function of time. Reaction networks based on ODEs are the most commonly used approach for modelling the biochemistry of signal transduction, and a wide range of software is available to design and apply these models (see Systems Biology Markup Language and Kinetikit in online links box).

Reaction–diffusion models allow for the variation of species concentrations in different cellular compartments. Strong spatial patterning of signalling components occurs at cell–cell junctions, and also because the plasma membrane has regions with different lipid compositions (including those known as LIPID RAFTS)[24]. Such models have been used to simulate the formation of the IMMUNOLOGICAL SYNAPSE between a T cell and an antigen-presenting cell (APC)[25,26] and to simulate TCR-mediated signalling through the synapse[27,28].

Box 1 | Different ways of formulating mathematical models



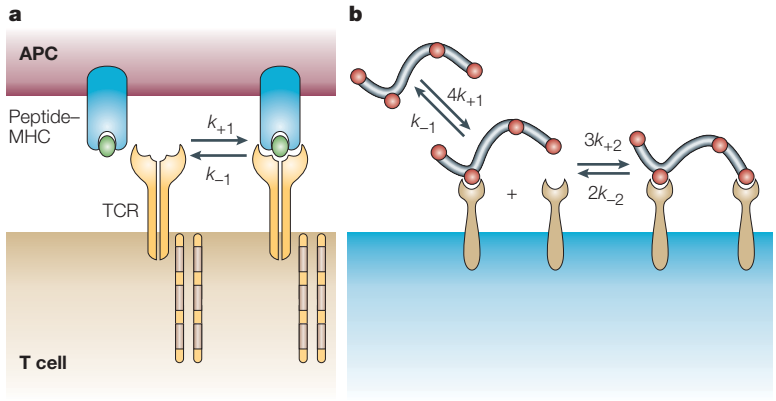
Reaction-network models are designed using a set of chemical species and the list of chemical reactions that can occur between these species, together with their associated rate constants; they are based on the assumption that each species is uniformly distributed throughout the cell. A reaction-network model can be formulated as a set of ordinary differential equations (ODEs): each species in the model generates a differential equation comprising a term from each reaction in which that species participates. The solution of these equations, which is usually obtained from a computer, provides the average concentration of each chemical species as a function of time. The same reaction network can also be simulated stochastically using the Gillespie algorithm[80,81], which is illustrated schematically. At each step of such a simulation, a random number is used to select the next reaction among the possible reactions that can occur, with the probability that a reaction is selected being proportional to its rate. At any given time, a large number of different reactions can occur because many different species are present and because each species can undergo many different reactions. Each time a reaction occurs, the concentrations of species affected by the reaction are updated, and the reaction rates affected by these species are recalculated. Stochastic simulations can be used to compute the fluctuations or noise in the concentration of each species, which becomes important when the concentrations are low — an example of which is shown in FIG. 3.

Reaction–diffusion models take into account the cellular location of components, as well as their capacity to participate in reactions. These models can be formulated as a set of PARTIAL DIFFERENTIAL EQUATIONS (PDEs), the solution of which gives the average concentrations of the species as a function of both time and space. Solving PDEs is more demanding, but software is available that allows the non-specialist to develop such models[82]. Reaction–diffusion networks can also be simulated stochastically, but there is not yet a standard algorithm. In the example illustrated, each molecule in the simulation is represented as a particle or set of particles that move on a lattice. Membrane proteins are restricted to movement along the dark line, which represents the centre of the plasma membrane. The simulation proceeds by choosing at random a protein to move, undergo a self-reaction or react with a neighbouring protein. This is similar to the scheme used by Lee *et al.*[28] in their simulation of signalling through the immunological synapse. A two-dimensional lattice model has also been used to simulate the effect of cooperative interactions between various molecules on the specificity of recognition of TCR ligands[49].

Serial engagement and serial triggering

The serial-triggering theory states that in the immunological synapse, a single peptide–MHC complex (present on an APC) can bind to a TCR for a length of time sufficient to induce an activation signal, dissociate from the TCR and sequentially engage other TCRs[29,30]. This theory was proposed to explain how an APC, with a low density of peptide–MHC complexes, could trigger activation signals and induce the internalization of thousands of TCRs over a few hours[29]. Valitutti *et al.*[29] estimated that when peptide–MHC complexes were displayed on an APC at a low density, the interactions

Box 2 | **Rate of serial engagement**



For the reaction shown in part a , the rate of encounters (also known as the hitting rate) between a peptide–MHC complex on an antigen-presenting cell (APC) and a T-cell receptor (TCR) in an immunological synapse can be described using equation 1 (REF. 33).

$$\text{hits per second} = k_{-1}\left[\frac{KR}{1+KR}\right] \quad (1)$$

where K is the two-dimensional equilibrium binding constant, R is the surface concentration of free TCRs and k_{-1} is the dissociation rate constant. When receptors are in large excess ($KR>>1$) — so that a peptide–MHC complex spends most of its time bound to TCR — the hitting rate becomes k_{-1}. Equation 1 assumes that receptor internalization does not influence the dissociation of the peptide–MHC–TCR complex. If internalization acts on TCRs that are bound to peptide–MHC by breaking the peptide–MHC–TCR bond and freeing the peptide–MHC, this will enhance the rate of serial engagement by providing an additional way for peptide–MHC to dissociate from the TCR. The form of the hitting rate depends on the model for internalization[27].

Replacing K with K_2 (where $K_2 = k_{+2}/k_{-2}$) and k_{-1} with k_{-2} in equation 1 gives the rate at which a binding site on a multivalent ligand (with a total of four binding sites, in this example) serially engages receptors (as shown in part b).

of a single peptide–MHC complex induced the internalization of approximately 200 TCRs, whereas Itoh *et al.*[31] observed that for each peptide–MHC complex, approximately 100 TCRs were internalized on human T-cell clones, and 10 TCRs on mouse T-cell clones.

An alternative explanation for the level of TCR internalization that occurs in the presence of a low density of peptide–MHC complexes was proposed by San José *et al.*[32], who showed that TCRs that had not been engaged by peptide–MHC complexes could still be internalized in a peptide-dependent manner. This observation raised the possibility that much of the TCR internalization resulted from a bystander effect, rather than serial triggering. To answer the question of whether peptide–MHC complexes undergo serial engagement, Wofsy *et al.*[33] developed a mathematical model of the interaction between a peptide–MHC complex and TCRs in the immunological synapse; this model enabled calculation of the number of TCRs a peptide–MHC complex would engage while it diffused in the immunological synapse, under conditions of a low density of peptide–MHC complexes and a large excess of TCRs. Using experimentally determined rate constants for binding and dissociation, the calculations showed that the rate of serial engagement is an increasing function of the dissociation rate constant (BOX 2), and peptide–MHC complexes — irrespective of whether the peptide that is bound to MHC is an agonist, weak agonist or antagonist — all undergo serial engagement. When TCRs are in large excess, the rate of serial engagement is determined by how rapidly the peptide–MHC complex dissociates from the TCR. The model did not address the question of whether serial engagement leads to serial triggering (discussed later).

A peptide–MHC molecule displayed on an APC can serially engage receptors because it is held near the T-cell surface through interactions that maintain the immunological synapse. During the hours an immunological synapse remains intact, a single peptide–MHC complex participates in many rounds of serial engagement as it diffuses in and out of the contact region. However, serial engagement is not restricted to cell-surface molecules present in the contact regions between cells. After a soluble multivalent ligand, such as an antigen or immune complex, binds to receptors that are mobile on the cell surface, the ligand can serially engage numerous receptors. A model in which mobile receptors reversibly bind to sites on multivalent ligands predicts that the time a ligand remains at the surface (from when it initially binds to a receptor) is much longer than the lifetime of a single bond between a receptor and a binding site[34]. This 'avidity effect' is a result of serial engagement of receptors by the various binding sites present on a single ligand.

Kinetic proofreading

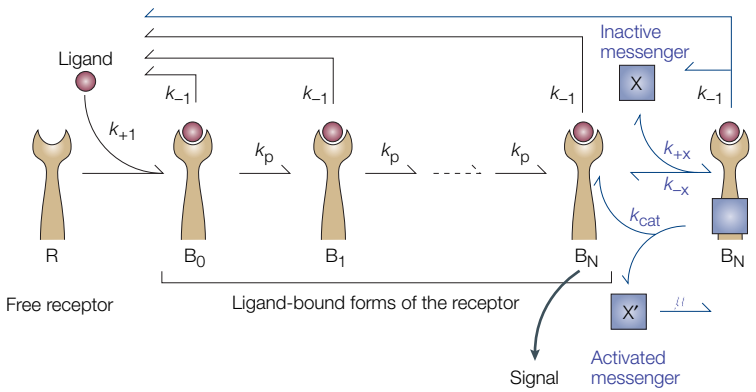
McKeithan[35] was the first person to use the concept of 'kinetic proofreading' to explain how a T cell could discriminate (with high specificity) between ligands on the basis of the lifetime of the ligand–receptor bond. He introduced a simple model (BOX 3), in which receptor engagement initiates a sequence of receptor modifications that involve energy-consuming reactions, such as phosphorylation of ITAM tyrosine residues. Receptor modifications are immediately reversed if the ligand and receptor dissociate, and signals are generated only by receptors that reach the terminal state of modification. This activation sequence sets a threshold time that a TCR needs to remain bound to a peptide before it can become activated. Slowly dissociating ligands generate stronger signals than rapidly dissociating ligands, because they have a greater chance of remaining bound long enough to reach the fully modified state. The duration of receptor engagement (also known as the dwell time) can therefore determine whether interaction with a particular ligand induces activation signals.

Experimental studies[36–39] of T-cell activation generally support the predictions of the kinetic proofreading model: that activation increases with the dwell time, and that small differences in the dwell time produce large changes in cellular responses. Some reports[40–43], however, seem to be inconsistent with the kinetic proofreading model. But, although other properties of the ligand–TCR bond might affect signalling[44], the dwell time is often the dominant one[36–39]. Extra support for the model is provided by the observation that

ORDINARY DIFFERENTIAL EQUATIONS (ODEs). Differential equations that involve only one independent variable, such as $d[A]/dt = -k[A]$, where the independent variable is time (t), and the concentration of the species [A] is a dependent variable that depends on t. k is the rate constant. A system of ODEs involves multiple dependent variables, all of which are functions of the same independent variable.

LIPID RAFTS
Lipid rafts are microdomains of the cell membrane that are enriched in sphingolipids. Several membrane-associated signalling molecules, such as LYN, are concentrated in these rafts.

Box 3 | **Kinetic proofreading extended to include a messenger**



The figure shows McKeithan's[35] original model (black). Elements have also been added to consider a cytosolic second messenger (blue), as in the studies of Hlavacek *et al.*[52] Ligand–receptor binding is monovalent and characterized by the on-rate k_{+1}, and the off-rate k_{-1}. A bound receptor can undergo a series of N sequential modifications, each step of which is energy driven and is characterized by the rate constant k_p. R is the number of unbound receptors; B_0 is the number of bound unmodified receptors; B_1 is the number of bound receptors modified once; B_N is the number of bound receptors modified N times. A fully modified receptor generates a signal (as in the original model) and/or catalyses the activation of a cytosolic messenger through a Michaelis–Menten mechanism, in which the enzyme is a fully modified receptor, the substrate is an inactive messenger and the product is an activated messenger. The parameters of messenger activation are the rate constants k_{+x} and k_{-x}, which describe enzyme–substrate binding, and k_{cat}, which describes the catalytic conversion of substrate to product. The activated form of the messenger (X′) returns to the inactive form (X) with rate constant μ. If the ligand and receptor dissociate, it is assumed that receptor modifications are immediately reversed. Similarly, if the ligand dissociates from a messenger-associated receptor, it is assumed that the messenger dissociates simultaneously.

ligands with different dwell times induce different phosphoforms of the ζ-chain of CD3 (CD3ζ), the signalling subunit that is associated with the TCR[45].

Modellers have considered several revisions of the original kinetic proofreading model to further understand the distinction between agonist and antagonist peptides. Rabinowitz *et al.*[46] proposed a two-step kinetic proofreading model, in which singly modified TCRs generate a negative signal and doubly modified TCRs generate a positive signal. In this 'kinetic discrimination' model, the response depends on the ratio of positive to negative signals, because strong negative signals can suppress positive ones. Lord *et al.*[47] introduced a model in which bound TCRs can activate two separate pathways that lead to positive or negative signals. These models indicate two possible ways that the dwell time could allow the cell to discriminate between agonist and antagonist peptides.

Specificity versus sensitivity. TCR signalling shows both high sensitivity (to ligands at low concentrations) and high specificity (of discrimination between ligands with different dwell times). Although McKeithan[35] found that his kinetic proofreading model required a trade-off between these two properties, Chan *et al.*[48] showed that the model could provide high specificity without a loss of sensitivity, as long as the number of modifications and the rate of modification were increased simultaneously (to keep the threshold time for activation fixed). That is, specific kinetic proofreading could be accomplished while maintaining sensitivity by 'a long chain of fast reactions'[48]. The question remains whether this description captures the essential features of the biochemical cascade. Chan *et al.* have suggested that robust specificity might require other mechanisms, such as cooperativity between neighbouring receptors[49] or feedback regulation[48]. However, a detailed model of FcεRI signalling[22] that lacks these additional mechanisms (discussed later) has demonstrated that SYK activation shows high specificity through the kinetic proofreading of early phosphorylation and binding events.

FcεRI-mediated signalling. The effects of kinetic proofreading have been experimentally detected not only during signalling through TCRs but also for signalling through FcεRI[15,50,51], and extended forms of McKeithan's model that incorporate features of FcεRI signalling have been used to study this system[52,53]. Torigoe *et al.*[15] found that doses of rapidly and slowly dissociating ligands that induced similar levels of receptor phosphorylation generated distinct levels of downstream activation, in terms of SYK phosphorylation. In agreement with the extended models[52,53], the slowly dissociating ligand generated stronger downstream responses than the rapidly dissociating ligand, and the differences increased with the distance downstream[15].

Escape from kinetic proofreading. Although initial results showed that the strength of FcεRI signalling depends on the dwell time[15], subsequent studies[50,51] have shown that some responses escape kinetic proofreading. In these studies, a delayed response to FcεRI signalling (transcription of the gene encoding monocyte chemotactic protein 1) was observed to escape the effect of kinetic proofreading, whereas other responses, such as transcription of *FOS*, remained sensitive to the dwell time.

In an attempt to understand how some downstream responses could escape kinetic proofreading, Hlavacek *et al.*[52,53] proposed an extension of McKeithan's model to include a cytosolic messenger that is activated by fully modified receptors (BOX 3). If two ligands with different dwell times both induce saturation of messenger activation, then responses controlled by the messenger can escape kinetic proofreading (FIG. 2). It has been suggested that calcium ions could be this messenger[50,51].

Escape from kinetic proofreading has also been reported for TCRs[54]. Exposure of T cells to a low-affinity tetrameric peptide–MHC ligand was observed to generate a committed T-cell response (as determined by the upregulation of expression of CD69), even though the ligand did not generate detectable proximal T-cell responses, such as the phosphorylation of CD3ζ or the mobilization of calcium ions. To explain these results, Rosette *et al.*[54] proposed that activated TCRs might generate long-lived counter molecules (analogous to the messenger in the model of Hlavacek *et al.*[52,53]), which

IMMUNOLOGICAL SYNAPSE
A stable region of contact between a T cell and an antigen-presenting cell that forms through cell–cell interaction of adhesion molecules. The mature immunological synapse contains two distinct, stable membrane domains: a central cluster of TCRs, known as the central supramolecular activation cluster (cSMAC) and a surrounding ring of adhesion molecules known as the peripheral supramolecular activation cluster (pSMAC).

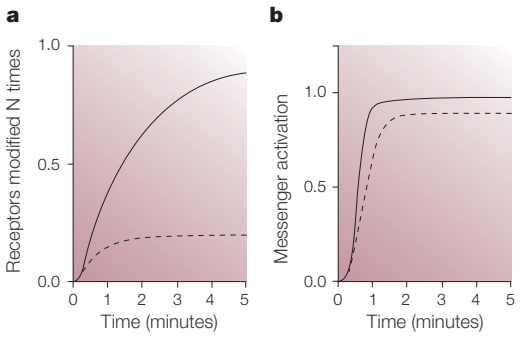


Figure 2 | **Escape from kinetic proofreading.** Cellular responses controlled by a cytosolic messenger can be insensitive to the kinetic quality of ligand-induced activation, even if other responses (such as those controlled by receptor modification) depend on the kinetics of ligand–receptor binding. **a** | The number of fully activated receptors stimulated by a slowly dissociating ligand (solid line) and a rapidly dissociating ligand (dashed line) is plotted as a function of time after ligand activation. **b** | The number of activated messengers activated by the same two ligands is plotted as a function of time. Calculations are based on the model of Hlavacek *et al.*[53], in the form of a system of ordinary differential equations, which is similar to that shown in BOX 3. These images are reproduced with permission from REF. 53 © (2002) Elsevier.

could trigger a response above a certain threshold concentration. They suggested that this counter molecule could be phosphorylated JUN, a transcription factor[54]. Other studies[55–57] also indicate that T cells have the ability to 'add up' TCR signals, through the incremental accumulation of long-lived signalling intermediates.

A model known as temporal summation might explain how successive TCR signals accumulate[58]. According to this model, when a TCR is triggered, signals — which could be in the form of activated counter molecules — are generated and then begin to decay. Before the signals decay completely, another TCR is triggered and more signals are generated. The result is an oscillating rise in the number of signals over time. A cellular response then occurs when the number of signals exceeds a threshold value. As illustrated in FIG. 3, fluctuations in the level of activated messenger in a single cell might seem to be additive (to follow the temporal summation model). The fluctuations arise only when sufficiently small numbers of receptors are involved in signalling, because when larger numbers are involved, activated receptors activate messengers at (effectively) a continuous rate. Because T-cell activation can be induced by an APC displaying small numbers of TCR-specific peptide–MHC ligands[59,60], noticeable fluctuations of second messengers are expected to occur during TCR signalling.

Serial engagement versus kinetic proofreading

Kinetic proofreading and serial engagement have competing effects. Kinetic proofreading works at the level of a single receptor; for a T cell to become activated, a TCR needs to remain bound to a peptide–MHC complex for sufficient time to undergo a series of modifications. Serial engagement works at the level of the cell; when there are only a few peptide–MHC complexes present, dissociation from the TCR needs to be rapid enough to allow a single complex to engage several TCRs, as is required for T-cell activation. This indicates that for T-cell activation there should be an optimal range of half-lives for the binding of a peptide–MHC complex to the TCR — these need to be long enough to allow an engaged TCR to progress through the signalling pathway (kinetic proofreading) and short enough to allow a peptide–MHC complex to engage many TCRs (serial engagement)[27,61,62]. When TCRs are in large excess, the rate of serial engagement is proportional to the dissociation rate constant of the bond between the TCR and the peptide–MHC complex (BOX 2). In this situation, the initial rate of T-cell activation is maximal when the dwell time is equal to the mean time required for a TCR to be fully modified[27]. A panel of cytotoxic T lymphocytes (CTLs) with mutated TCRs, which bind peptide–MHC complexes with different dwell times, showed an optimal range of dwell times for T-cell activation[63]. As the dwell time increased, T-cell activation also increased then reached a maximum and decreased.

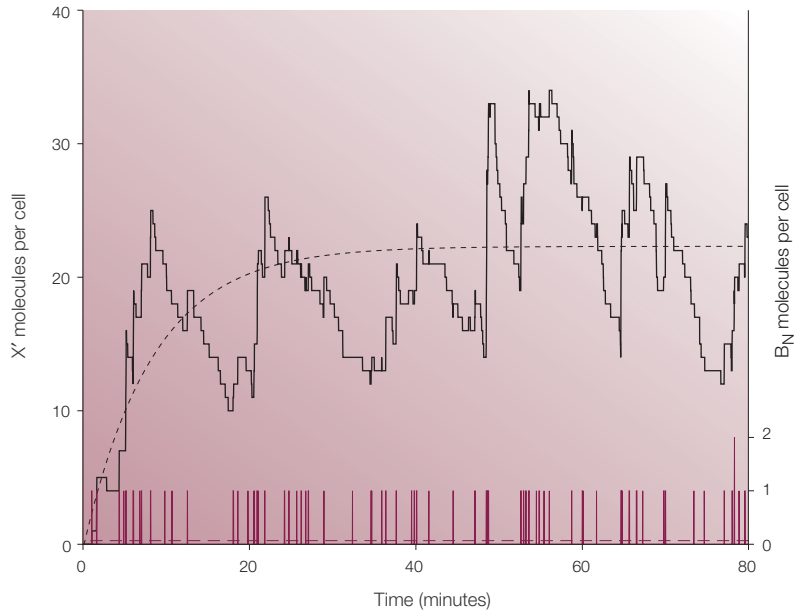
Although the trade-off between serial engagement and kinetic proofreading is predicted to lead to a peptide–MHC–TCR dwell time that is optimal for T-cell activation, this might not be the case for the downregulation of cell-surface expression of TCRs. If an activated TCR is subject to downregulation only when bound to a peptide–MHC complex and reverts to its basal state upon dissociation, then the rate of TCR downregulation increases as the dwell time increases and never reaches a maximum. In this case, long-lived bonds are always better at triggering the downregulation of TCRs than short-lived bonds[27]. When a bound TCR is internalized, the peptide–MHC complex is freed to engage other receptors. So internalization increases the rate of dissociation and therefore the rate of serial engagement. However, if the internalization of a TCR can occur after the peptide–MHC–TCR bond has been broken — that is, the TCR remains 'marked' for internalization for some time after dissociation — then the competition between serial engagement and kinetic proofreading produces an optimal window of peptide–MHC–TCR dwell times for TCR internalization. Using the same panel of CTLs (with mutated TCRs) that was used to study T-cell activation[63], Coombs *et al.*[27] found that the internalization of TCRs also showed an optimal window of peptide–MHC–TCR dwell times, indicating that activated TCRs remain marked for internalization after dissociation from peptide–MHC complexes. Sousa and Carneiro[64] reached a similar conclusion using a series of models to analyse the TCR downregulation experiments of Valitutti *et al.*[29]

Detailed models of immunoreceptor signalling

The serial engagement and kinetic proofreading models associate the measured properties of ligand–receptor interactions with the amplitude of signalling responses, but they do not describe molecular interactions — beyond ligand–receptor binding — in a realistic way.

Understanding how downstream components can affect the signalling cascade requires more-detailed models that include these components, together with their known activities and interactions. The aim of such models is to predict how the components of a signalling cascade work together to decode signals that arrive in the form of binding events at the cell surface.

Combinatorial complexity. A detailed model[22,65] of the membrane-proximal events that occur in signalling through FcεRI is described in FIG. 4. The model includes a bivalent ligand, the receptor, and the two kinases LYN and SYK. The most surprising aspect of this model is that 4 components and 9 interactions (FIG. 4a,b) give rise to a biochemical network with 354 species and 3,680 reactions. This outcome — that such a small number of components and interactions yields a large number of distinct combinations of the components — is a property of signalling networks that has been called combinatorial complexity[66]. Despite the complexity of this network, it is possible to develop a quantitative model that requires a much smaller number of input parameters to be determined from experimental data. The parameters of the FcεRI model shown here include 21 rate constants (FIG. 4c); this design is based on the assumption that only 1 or 2 interactions within a complex affect the rate of a particular reaction. For example, the 2 reactions shown in FIG. 4c are assumed to occur with the same rate constant as 22 other reactions that involve the transphosphorylation of the γ-ITAM of FcεRI by LYN, when LYN is bound (through its SH2 domain) to the phosphorylated β-ITAM. It is therefore possible to build models able to account for combinatorial complexity that are based on currently available data and that can incorporate more detailed information about interactions and reaction rates as it becomes available.



Model parameters and rate constants

L_T	Agonist peptide–MHCs per APC–T-cell interface		30
R_T	TCRs per APC–T-cell interface		900
X_T	Messengers per T cell		10,000
N	Maximum number of receptor modifications		6
k_p	0.25 s^{-1}	k_{+x}	1.2×10^{-3} $molecules^{-1}s^{-1}$
k_{+1}	6.7×10^{-3} $molecules^{-1}s^{-1}$	k_{-x}	0.01 s^{-1}
k_{-1}	0.5 s^{-1}	k_{cat}	100 s^{-1}
		k_{-p}	0.002 s^{-1}

Figure 3 | **Production of activated messengers and fully modified receptors in a single T cell, as a function of time after ligand stimulation.** Over the time course shown, the fully modified receptor (B_N) state (red) is occupied intermittently by a single T-cell receptor (TCR) or in one instance two TCRs. While a TCR is in this state, the receptor catalyses messenger activation. As illustrated, the number of activated messengers (X′) fluctuates. Calculations are based on the model illustrated in BOX 2. The jagged solid curve and the pulses (red) represent the results of a single stochastic simulation using the Gillespie algorithm[80,81] (BOX 1) and predict the response of a single cell. The dashed curve, calculated using ordinary differential equations, predicts the average number of activated messengers per cell for a population of cells. Parameter values for ligand–receptor binding correspond to the case of an interaction between an antigen-presenting cell (APC) and a T cell (through a single cell–cell contact region) and are based in part on estimates by Coombs *et al.*[27] See BOXES 2,3 for definitions of model parameters and rate constants. Red pulses represent the number of B_N molecules per cell.

FcεRI-mediated signalling. An early model of the events that immediately follow ligand–receptor binding described LYN associating with and phosphorylating FcεRI dimers, which had formed as a result of the binding of covalently crosslinked dimers of IgE[19]. This model highlighted an important difference between signalling through growth-factor receptors, which possess intrinsic kinase activity, and through MIRRs, which must first associate with an extrinsic SRC-family kinase (LYN in the case of the FcεRI and the BCR, and LCK or FYN in the case of the TCR) to become phosphorylated. The requirement for an extrinsic kinase means that the level of active kinase available can limit the maximum level of receptor activation. Indeed, the measured time course of receptor phosphorylation that is induced by the binding of IgE dimers reaches a plateau when only approximately 10–20% of the receptors have been aggregated[67]. The model could explain this saturation of receptor phosphorylation if the supply of active LYN was small compared with the number of receptors. This hypothesis was confirmed by experiments in which phosphorylation induced by irreversible crosslinking of a subset of the available FcεRIs was decreased by the crosslinking of a second population of receptors that could compete for LYN[19]. Subsequent experiments showed that the process was reversible: breaking up the crosslinks in the second population of receptors returned the phosphorylation level within the first group to its original value[68].

The model also predicted that at low LYN concentrations, the level of receptor phosphorylation should vary linearly with the concentration of available LYN, providing the impetus for an experimental study[69] in which stably transfected cells (containing FcεRI) with varying levels of LYN expression showed the predicted linear variation in response to stimulation. An alternative model that required activation of LYN through co-clustering within an aggregate failed to predict the

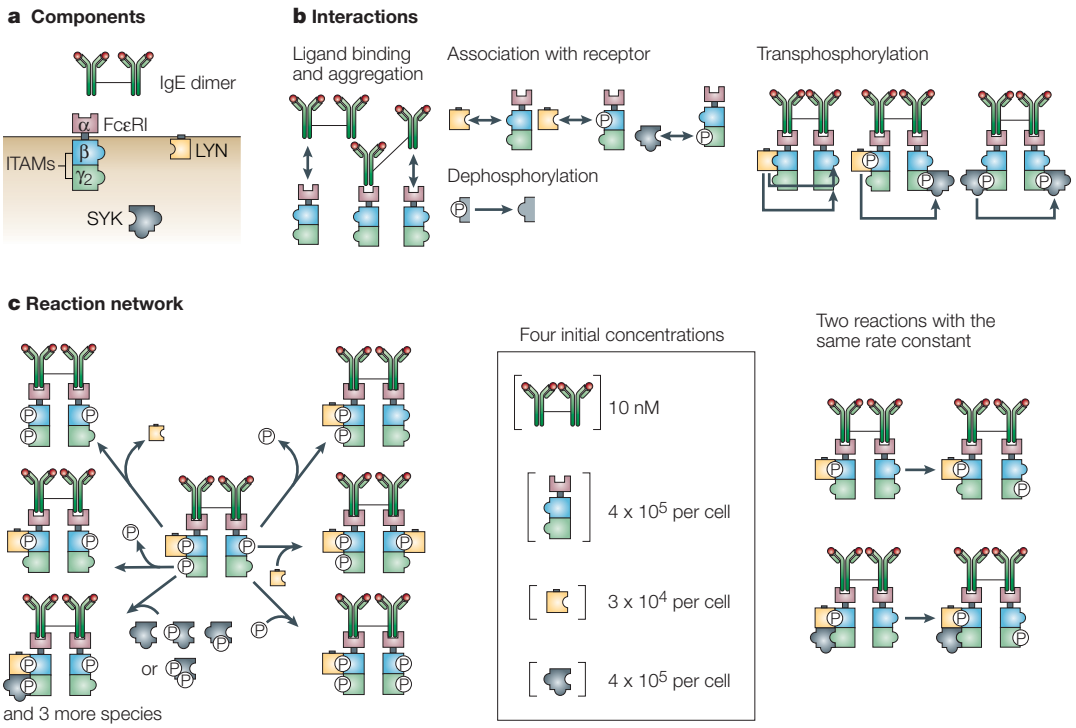


Figure 4 | **Detailed model of early events in FcεRI signalling. a** | The four components in the model are the ligand (IgE dimer), the receptor (FcεRI) and the two kinases LYN and SYK (spleen tyrosine kinase). The receptor is represented as an extracellular domain that binds the ligand and two cytoplasmic domains each containing an immunoreceptor tyrosine-based activation motif (ITAM). SYK also contains two regions that can be phosphorylated. **b** | Nine basic interactions are included: five for association or dissociation of molecular components, three for catalysis of phosphorylation and one for dephosphorylation, which can occur at any phosphorylated site that is not protected by binding another protein. **c** | The interactions give rise to a large number of complexes and phosphorylation states (354 states), each of which is tracked as a separate species. One typical species is illustrated along with the nine different reactions it can undergo. The species are connected by a large biochemical reaction network (composed of 3,680 reactions). A small number of parameters define this network — the initial concentrations of the 4 proteins and 21 rate constants — because the same rate constant can be used for many similar reactions. The figure depicts 2 of the 24 reactions in which LYN transphosphorylates the γ-ITAM (subsequent to its activation through binding the phosphorylated β-ITAM).

observed behaviour, thereby supporting the conclusion that a single LYN molecule binding to a receptor aggregate was sufficient to induce phosphorylation[69].

The finding that the available pool of LYN kinase is much smaller than the number of FcεRI molecules is surprising, because biochemical measurements indicate that the total level of LYN is much higher[69], indicating that most of the LYN is unavailable to bind FcεRI. The model therefore implies that LYN activity (for binding and phosphorylating FcεRI) is negatively regulated *in vivo*, but it provides no mechanism for this. One possibility is that LYN needs to be pre-activated to interact with FcεRI. Measurements in rat basophilic leukaemia (RBL) cells indicate that only a small fraction of LYN is pre-activated through phosphorylation of its kinase domain and that this level does not change substantially upon stimulation of the cell[70]. LYN could also be actively held in an inactive state through the activity of CSK (carboxy-terminal SRC kinase), which is recruited to the plasma membrane (after the activation of FcεRI) through its association with phosphorylated CSK-binding protein, a putative LYN substrate[71,72]. Future models will need to include the regulation of LYN activity to help resolve which mechanisms are important.

This model of FcεRI signalling was extended to include SYK[22,65] (FIG. 4), the activation of which is crucial for downstream responses, including the degranulation of mast cells[73]. The inclusion of SYK provides a good demonstration of combinatorial complexity — the number of species increases from 20 to 354 and the number of reactions from 72 to 3,680. The model increases this much because LYN and SYK are allowed to bind independently to the phosphorylated β- and γ-subunits of the receptor, respectively (FIG. 4). In the model, the independent binding of LYN and SYK produces different relative levels of phosphorylation of FcεRI subunits, with the ratio depending on the concentrations of both LYN and SYK[22]. The predictions of the model were consistent with experimental measurements of the ratio of γ- to β-phosphorylation only when the cellular concentration of SYK was comparable to the concentration of FcεRI, which was confirmed by directly measuring the SYK concentration[22]. The model also showed that to compete with the rebinding of kinases, the catalytic rate of dephosphorylation must be much higher than the observed rate at which receptors are dephosphorylated following disaggregation[74]. The high rate of receptor

dephosphorylation effectively prevents the spread of the signal to receptors that are not contained in aggregates, and it also limits the level of SYK activation to a small fraction of the total number of receptors.

TCR signalling. There has been a considerable effort to develop mathematical models of the physical processes that lead to the formation of an immunological synapse, to identify forces that drive synapse formation and to determine the conditions under which synapses form[25,26,75,76]. Recently, an agent-based modelling approach (BOX 1) has been used to investigate how spatial organization within the synapse might affect signalling[28,77]. This approach allowed complex physical and biochemical phenomena to be integrated into a single model, which predicts the signalling events initiated by the binding of peptide–MHC complexes to TCRs. The parameters and outputs of this model do not correspond directly to measurable properties of the system in the same way that the biochemical network models described above do (for example, time is reported in number of steps and the correspondence to the actual time is ill-defined), but this model considers a broader range of factors influencing the dynamics of the system. The main question investigated by Lee *et al.*[28] was the role of the accumulation of TCRs in the central supramolecular activation cluster (cSMAC) in signalling. Given that increasing the density of receptors is a well-known mechanism for signal initiation, it is not surprising that the simulations showed increased TCR activation and phosphorylation in the cSMAC.

It was surprising, however, that because in the simulation only fully phosphorylated receptors could be degraded following internalization, the formation of the cSMAC was also essential for turning off signalling through the TCR. The model was crucial in interpreting the potentially misleading experimental finding that T cells from CD2-associated-protein (CD2AP)-deficient mice, which do not form cSMACs under normal conditions[78], produce delayed but sustained activation when stimulated by APCs. One possible explanation for this result is that the cSMAC is not an important site of TCR activation. But the model indicated an alternative explanation — TCRs are not effectively degraded in the CD2AP-deficient cells. Subsequent experiments confirmed that TCR degradation was defective in CD2AP-deficient cells, even when these cells were coaxed into forming a cSMAC using planar lipid bilayers that contained adhesion molecules and peptide–MHC ligands. These experiments confirmed the prediction of the model that in the absence of receptor degradation, the cSMAC region shows the strongest signalling, as measured by staining with phosphotyrosine-specific antibodies. By contrast, because rapid TCR degradation follows receptor triggering, the cSMAC region of wild-type cells shows decreased signalling. The model therefore provides a comprehensive functional description of the cSMAC, a region in which signalling is initially enhanced by high TCR density and is then rapidly shut off as a result of the efficient degradation of fully activated receptors, thereby avoiding antigen-induced apoptosis[28]. The authors describe this function as an adaptive controller, which amplifies weak signals but also limits the strength and duration of activation — a hypothesis that can be tested by further experiments and modelling.

Kinetic proofreading and serial engagement. Kinetic proofreading and serial engagement are emergent properties of both of the detailed signalling models we have discussed. Phosphorylation profiles of FcεRI signalling components as a function of the ligand–receptor off-rate (the inverse of the dwell time) show complex behaviour (FIG. 5a), because the balance between kinetic proofreading and serial engagement alters, moving down the signalling cascade. Because serial engagement increases with the off-rate (BOX 2), an increase in phosphorylation with off-rate indicates that serial engagement is the dominant effect, whereas a decrease indicates that kinetic proofreading is dominant. Profiles of the earliest and latest phosphorylation events, β-ITAM and SYK phosphorylation (FIG. 5a) are similar and reflect the dominance of kinetic proofreading. The profile of the intermediate event, γ-ITAM phosphorylation, passes through a maximum similar to that of TCR activation and internalization, indicating a transition between control by serial engagement and control by kinetic proofreading. Furthermore, whereas SYK phosphorylation decreases to zero at high off-rates, as predicted by simple models of kinetic proofreading[35,53], both β- and γ-ITAM phosphorylation plateau at high off-rates (to values only about twofold lower than maximal), which indicates that receptor phosphorylation is not subject to strong kinetic proofreading in the model. In agreement with experiments[15], the model predicts that SYK activation shows substantial kinetic proofreading of ligand–receptor interactions.

The detailed model of TCR signalling also produces curves of ZAP70 activity as a function of the dwell time (FIG. 5b) that are similar to the experimental curves for TCR activation[63] and internalization[27], again demonstrating the competition between serial engagement and kinetic proofreading. Therefore, both detailed models show substantial kinetic proofreading of ligand–receptor interactions that arises from phosphorylation and binding events, leading to the activation of SYK or ZAP70, although feedback from downstream events also contributes to the drop in ZAP70 activation in the TCR model[28] (FIG. 5b). It remains to be seen whether the magnitude of this proofreading is strong enough to account for the observed specificity of ligand recognition in immune-receptor signalling.

Concluding remarks

Much of the experimental effort towards unravelling what occurs inside a cell after a ligand binds to a receptor, and initiates a cellular response, has focused on identifying the components that participate in the process and the interactions between them. This is no small task because many of the components are complex 'machines' that are regulated in numerous ways.

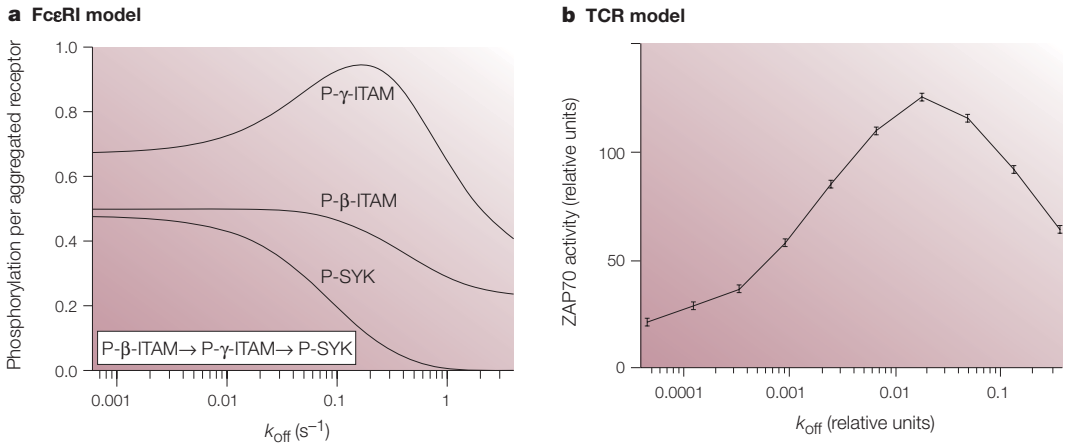


Figure 5 | **Effect on signalling events of varying the ligand–receptor off-rate in two detailed models. a** | The model depicted is that reported in REF. 22 for signalling through the high-affinity IgE receptor (FcεRI). The curves show the level of phosphorylated β-immunoreceptor tyrosine-based activation motif (P-β-ITAM), phosphorylated γ-ITAM (P-γ-ITAM) and phosphorylated SYK (P-SYK) per aggregated receptor, under steady-state conditions, at ligand concentrations in which fewer than 1% of receptors are aggregated. Phosphorylation levels are normalized in terms of the total number of receptors in aggregates, to control for the decrease in ligand affinity as the off-rate (k_{off}) is increased. The inset box shows the order of phosphorylation events in the model; this is only approximate because LYN can associate with the unphosphorylated receptor, and therefore β-ITAM phosphorylation is not absolutely required to precede γ-ITAM phosphorylation[22]. SYK phosphorylation shows strong kinetic proofreading, decreasing to nearly zero at higher off-rates. Phosphorylation of the β-ITAM, which is upstream of SYK phosphorylation, decreases more slowly (as predicted by the kinetic proofreading model) but does not decrease to zero (in contrast to the kinetic proofreading model)[53]. Intermediate between these two, γ-ITAM phosphorylation increases with increasing off-rate (a signature of serial engagement) and passes through a maximum before declining at higher off-rates (kinetic proofreading). **b** | The model depicted is the full network model reported in REF. 28 for signalling through the T-cell receptor (TCR). The curve shows the relative level of ZAP70 (ζ-chain-associated protein kinase 70 kDa isoform) activity at a fixed number of steps after the stimulation of 120 TCRs with 12 peptide–MHC complexes. The error bars represent the standard error of the mean for 10 trials. Up to an off-rate of approximately 0.01, ZAP70 activation increases with an increasing off-rate, which demonstrates serial engagement and triggering of TCRs. Although serial engagement continues to increase at higher off-rates, kinetic proofreading makes ligand–receptor encounters ineffective at generating a downstream signal, which leads to an overall reduction in ZAP70 activation. Normalization of ZAP70 activation (as shown in part **a**) is not required, because even at the highest off-rates, essentially all peptide–MHC complexes are bound as a result of the effectively high concentration of TCRs. This image is reproduced with permission from REF. 28 © (2003) American Association for the Advancement of Science.

However, the ultimate goal is to understand how the components in a signalling cascade work in concert to direct cellular responses to changes in the extracellular environment. This understanding will be achieved when we can accurately predict how a signalling cascade will behave for any set of conditions. The tool for making such predictions is mathematical modelling.

Both simple and detailed mathematical models have contributed to our understanding of signalling through immune receptors. Serial engagement and kinetic proofreading have given us a framework to judge whether a peptide–MHC complex controls T-cell activation mainly through the lifetime of the bond it forms with the TCR or whether other properties of the binding significantly influence T-cell activation[44]. A combination of experimental results and prediction using models has indicated that TCRs in the immunological synapse remain marked for degradation after they are activated and no longer bound to peptide–MHC complexes[27]. Combined with experimental data, modelling has also helped to define the conditions under which immunological synapses form[25,26,75,76] and the role they have in TCR signalling and degradation[28,77]. In the mast-cell system, modelling in combination with experiments has enabled several conclusions to be made: that a single LYN molecule associated with an FcεRI in an aggregate is sufficient to induce receptor phosphorylation[69]; that the amount of LYN available to the receptor is limiting[19]; that the amount of SYK is not limiting[22]; and that the rate of dephosphorylation of receptor ITAMs is much faster than observed[22].

Despite successes, at present no model can accurately predict the behaviour of more than a few proteins that become activated after a ligand induces receptor ITAM phosphorylation. Many technical challenges must be overcome if such models for immune-receptor signalling are to be constructed. Probably the most formidable one is the problem of combinatorial complexity[66], which raises the question of how to incorporate in a model the enormous number of possible complexes that can arise in a signalling cascade as a result of the numerous ways signalling proteins can be modified and combined.

The challenges that building mathematical models of signalling cascades present to the experimental community are as considerable as those faced by the modellers. Mathematical models of the scope and detail we envision demand various quantitative data that are not routinely reported in published studies. Technical breakthroughs

are anticipated to change this, but at present, the lack of such data is a major impediment to the development and testing of these types of model. In some ways, little has changed since 1995, when Henry Metzger (of the National Academy of Sciences, United States) pointed out that collecting quantitative data "…is time-consuming, frustrating and generally unappreciated by one's peers! Establishing an atmosphere in our scientific community where such work will be encouraged is a not inconsiderable challenge!" (Dyer Lecture, National Institutes of Health, Bethesda, Maryland, United States, 1995). However, because cell signalling is so complex, and the need to understand its workings so crucial, we expect that mathematical modelling will become an integral part of the cell-signalling endeavour, and the data such modelling requires will be forthcoming.

1. Keegan, A. D. & Paul, W. E. Multichain immune recognition receptors: similarities in structure and signaling pathways. *Immunol. Today* **13**, 63–68 (1992).
2. Reth, M. Antigen receptor tail clue. *Nature* **338**, 383–384 (1989).
3. Cambier, J. C. Antigen and Fc receptor signaling: the awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). *J. Immunol.* **155**, 3281–3285 (1995).
4. Daeron, M. Fc receptor biology. *Annu. Rev. Immunol.* **15**, 203–234 (1997).
5. Boniface, J. J. *et al*. Initiation of signal transduction through the T cell receptor requires the multivalent engagement of peptide/MHC ligands. *Immunity* **9**, 459–466 (1998).
6. Cochran, J., Cameron, T. & Stern, L. The relationship of MHC–peptide binding and T cell activation probed using chemically defined MHC class II oligomers. *Immunity* **12**, 241–250 (2000).
7. Metzger, H. Transmembrane signaling: the joy of aggregation. *J. Immunol.* **149**, 1477–1487 (1992).
8. Delisi, C. The magnitude of signal amplification by ligand-induced receptor clustering. *Nature* **289**, 322–323 (1981).
9. Irving, B. A., Chan, A. C. & Weiss, A. Functional characterization of a signal transducing motif present in the T cell antigen receptor ζ chain. *J. Exp. Med.* **177**, 1093–1103 (1993).
10. Wange, R. L., Malek, S. N., Desiderio, S. & Samelson, L. E. Tandem SH2 domains of ZAP-70 bind to TCRζ and CD3ε from activated Jurkat T cells. *J. Biol. Chem.* **268**, 19797–19801 (1993).
11. Kihara, H. & Siraganian, R. P. Src homology-2 domains of Syk and Lyn bind to tyrosine-phosphorylated subunits of the high-affinity IgE receptor. *J. Biol. Chem.* **269**, 22427–22432 (1994).
12. Bu, J.-Y., Shaw, A. S. & Chan, A. C. Analysis of the interaction of Zap-70 and Syk protein-tyrosine kinases with the T-cell antigen receptor by plasmon resonance. *Proc. Natl Acad. Sci. USA* **92**, 5106–5110 (1995).
13. Kumaran, S., Grucza, R. A. & Waksman, G. The tandem Src homology 2 domain of the Syk kinase: a molecular device that adapts to interphosphotyrosine distances. *Proc. Natl Acad. Sci. USA* **100**, 14828–14833 (2003).
14. Bunnell, S. C. *et al*. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J. Cell Biol.* **158**, 1263–1275 (2002).
15. Torigoe, C., Inman, J. K. & Metzger, H. An unusual mechanism for ligand antagonism. *Science* **281**, 568–572 (1998).
Showed that the FcεRI-initiated signalling cascade is subject to kinetic proofreading, and proposed a mechanism for ligand antagonism, in which antagonists bind receptors and sequester the crucial initiating kinase, LYN.
16. Dittel, B. N., Stefanova, I., Germain, R. N. & Janeway, C. A. Cross-antagonism of a T cell clone expressing two distinct T cell receptors. *Immunity* **11**, 289–298 (1999).
17. Robertson, J. M. & Evavold, B. D. Cutting edge: Dueling TCRs: peptide antagonism of CD4$^+$ T cells with dual antigen specificities. *J. Immunol.* **163**, 1750–1754 (1999).
18. Stefanova, I. *et al*. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nature Immunol.* **4**, 248–254 (2003).
19. Wofsy, C., Torigoe, C., Kent, U. M., Metzger, H. & Goldstein, B. Exploiting the difference between intrinsic and extrinsic kinases: implications for regulation of signaling by immunoreceptors. *J. Immunol.* **159**, 5984–5992 (1997).
20. Burack, W. R. & Shaw, A. S. Signal transduction: hanging on a scaffold. *Curr. Opin. Cell Biol.* **12**, 211–216 (2000).
21. Levchenko, A., Bruck, J. & Sternberg, P. W. Scaffold proteins may biphasically affect the levels of mitogen-activated protein kinase signaling and reduce its threshold properties. *Proc. Natl Acad. Sci. USA* **97**, 5818–5823 (2000).
22. Faeder, J. R. *et al*. Investigation of early events in FcεRI-mediated signaling using a detailed mathematical model. *J. Immunol.* **170**, 3769–3781 (2003).
Presented a detailed model of the network of early phosphorylation events that occur during signalling through FcεRI and lead to SYK activation, and used this model to analyse various data on the phosphorylation of the β- and γ-receptor subunit ITAMs.
23. Markevich, N. I., Hoek, J. B. & Kholodenko, B. N. Signaling switches and bistability arising from multisite phosphorylation in protein kinase cascades. *J. Cell Biol.* **164**, 353–359 (2004).
24. Simons, K. & Ikonen, E. Functional rafts in cell membranes. *Nature* **387**, 569–572 (1997).
25. Qi, S. Y., Groves, J. T. & Chakraborty, A. K. Synaptic pattern formation during cellular recognition. *Proc. Natl Acad. Sci. USA* **98**, 6548–6553 (2001).
Used a spatial-kinetic model of immunological-synapse formation to show that receptor proteins at the synapse have a strong tendency to self-organize in the presence of a stimulating ligand, which might be reinforced by active cellular processes but does not require them.
26. Burroughs, N. J. & Wulfing, C. Differential segregation in a cell–cell contact interface: the dynamics of the immunological synapse. *Biophys. J.* **83**, 1784–1796 (2002).
27. Coombs, D., Kalergis, A. M., Nathenson, S. G., Wofsy, C. & Goldstein, B. Activated TCRs remain marked for internalization after dissociation from pMHC. *Nature Immunol.* **3**, 926–931 (2002).
28. Lee, K. H. *et al*. The immunological synapse balances T cell receptor signaling and degradation. *Science* **302**, 1218–1222 (2003).
Combined modelling and experimental study of the role of the cSMAC in T-cell activation. Showed that enhanced TCR signalling occurs at the cSMAC initially, but later it becomes the site of rapid TCR degradation and diminution of signalling.
29. Valitutti, S., Muller, S., Cella, M., Padovan, E. & Lanzavecchia, A. Serial triggering of many T-cell receptors by a few peptide–MHC complexes. *Nature* **375**, 148–151 (1995).
Introduced the idea of serial triggering of TCRs. This paper and reference 31 present data showing that when there are only a few cognate peptide–MHC complexes on an APC, a single peptide–MHC complex can trigger the downregulation of numerous TCRs.
30. Valitutti, S. & Lanzavecchia, A. Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition. *Immunol. Today* **18**, 299–304 (1997).
31. Itoh, Y., Hemmer, B., Martin, R. & Germain, R. N. Serial TCR engagement and down-modulation by peptide–MHC molecule ligands: relationship to the quality of individual TCR signaling events. *J. Immunol.* **162**, 2073–2080 (1999).
32. San José, E., Borroto, A., Niedergang, F., Alcover, A. & Alarcon, B. Triggering the TCR complex causes the downregulation of nonengaged receptors by a signal transduction-dependent mechanism. *Immunity* **12**, 161–170 (2000).
33. Wofsy, C., Coombs, D. & Goldstein, B. Calculations show substantial serial engagement of T cell receptors. *Biophys. J.* **80**, 606–612 (2001).
34. Hlavacek, W. S., Percus, J. K., Percus, O. E., Perelson, A. S. & Wofsy, C. Retention of antigen on follicular dendritic cells and B lymphocytes through complement-mediated multivalent ligand–receptor interactions: theory and application to HIV treatment. *Math. Biosci.* **176**, 185–202 (2002).
35. McKeithan, T. W. Kinetic proofreading in T-cell receptor signal-transduction. *Proc. Natl Acad. Sci. USA* **92**, 5042–5046 (1995).
Introduced the idea of kinetic proofreading in the context of receptor signalling and presented a mathematical model of kinetic proofreading to explain how T cells can discriminate between ligands with moderately different dissociation rates.
36. Matsui, K., Boniface, J. J., Steffner, P., Reay, P. A. & Davis, M. M. Kinetics of T-cell receptor binding to peptide/I–E^k complexes: correlation of the dissociation rate with T-cell responsiveness. *Proc. Natl Acad. Sci. USA* **91**, 12862–12866 (1994).
37. Lyons, D. S. *et al*. A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity* **5**, 53–61 (1996).
38. Kersh, G. J., Kersh, E. N., Fremont, D. H. & Allen, P. M. High- and low-potency ligands with similar affinities for the TCR: the importance of kinetics in TCR signaling. *Immunity* **9**, 817–826 (1998).
39. Ding, Y. H., Baker, B. M., Garboczi, D. N., Biddison, W. E. & Wiley, D. C. Four A6-TCR/peptide/HLA-A2 structures that generate very different T cell signals are nearly identical. *Immunity* **11**, 45–56 (1999).
40. Holler, P. D. & Kranz, D. M. Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation. *Immunity* **18**, 255–264 (2003).
41. Sykulev, Y., Vugmeyster, Y., Brunmark, A., Ploegh, H. L. & Eisen, H. N. Peptide antagonism and T cell receptor interactions with peptide–MHC complexes. *Immunity* **9**, 475–483 (1998).
42. Alam, S. M. *et al*. T-cell-receptor affinity and thymocyte positive selection. *Nature* **381**, 616–620 (1996).
43. Baker, B. M., Gagnon, S. J., Biddison, W. E. & Wiley, D. C. Conversion of a T cell antagonist into an agonist by repairing a defect in the TCR/peptide/MHC interface: implications for TCR signaling. *Immunity* **13**, 475–484 (2000).
44. Krogsgaard, M. *et al*. Evidence that structural rearrangements and/or flexibility during TCR binding can contribute to T cell activation. *Mol. Cell* **12**, 1367–1378 (2003).
45. Kersh, E. N., Shaw, A. S. & Allen, P. M. Fidelity of T cell activation through multistep T cell receptor ζ phosphorylation. *Science* **281**, 572–575 (1998).
46. Rabinowitz, J. D., Beeson, C., Lyons, D. S., Davis, M. M. & McConnell, H. M. Kinetic discrimination in T-cell activation. *Proc. Natl Acad. Sci. USA* **93**, 1401–1405 (1996).
47. Lord, G. M., Lechler, R. I. & George, A. J. T. A kinetic differentiation model for the action of altered TCR ligands. *Immunol. Today* **20**, 33–39 (1999).
48. Chan, C., George, A. J. T. & Stark, J. T cell sensitivity and specificity — kinetic proofreading revisited. *Discrete Contin. Dyn. Syst. Ser. B* **3**, 343–360 (2003).
49. Chan, C., George, A. J. T. & Stark, J. Cooperative enhancement of specificity in a lattice of T cell receptors. *Proc. Natl Acad. Sci. USA* **98**, 5758–5763 (2001).
50. Liu, Z. J., Haleem-Smith, H., Chen, H. X. & Metzger, H. Unexpected signals in a system subject to kinetic proofreading. *Proc. Natl Acad. Sci. USA* **98**, 7289–7294 (2001).
Showed that many FcεRI-mediated mast-cell responses follow kinetic proofreading, as described in reference 15, but also found an exception — a rapidly dissociating ligand that activated a late response but failed to activate earlier responses, similar to the findings for T cells in reference 54.
51. Eglite, S., Morin, J. M. & Metzger, H. Synthesis and secretion of monocyte chemotactic protein-1 stimulated by the high affinity receptor for IgE. *J. Immunol.* **170**, 2680–2687 (2003).
52. Hlavacek, W. S., Redondo, A., Metzger, H., Wofsy, C. & Goldstein, B. Kinetic proofreading models for cell signaling predict ways to escape kinetic proofreading. *Proc. Natl Acad. Sci. USA* **98**, 7295–7300 (2001).
Extended McKeithan's kinetic proofreading model to aggregating receptor systems and to receptor systems in which signalling depends on an external initiating kinase. Considered the kinetics of signalling molecules that are generated by aggregated receptors but do not remain associated with the receptor complex.

53. Hlavacek, W. S., Redondo, A., Wofsy, C. & Goldstein, B. Kinetic proofreading in receptor-mediated transduction of cellular signals: receptor aggregation, partially activated receptors, and cytosolic messengers. *Bull. Math. Biol.* **64**, 887–911 (2002).
54. Rosette, C. *et al*. The impact of duration versus extent of TCR occupancy on T cell activation: a revision of the kinetic proofreading model. *Immunity* **15**, 59–70 (2001). **Showed that a low-affinity tetrameric peptide–MHC complex generated a late T-cell response but not early responses. Proposed that T-cell responses are triggered by a cumulative signal.**
55. Faroudi, M., Zaru, R., Paulet, P., Muller, S. & Valitutti, S. Cutting edge: T lymphocyte activation by repeated immunological synapse formation and intermittent signaling. *J. Immunol.* **171**, 1128–1132 (2003).
56. Muller, S., Demotz, S., Bulliard, C. & Valitutti, S. Kinetics and extent of protein tyrosine kinase activation in individual T cells upon antigenic stimulation. *Immunology* **97**, 287–293 (1999).
57. Borovsky, Z., Mishan-Eisenberg, G., Yaniv, E. & Rachmilewitz, J. Serial triggering of T cell receptors results in incremental accumulation of signaling intermediates. *J. Biol. Chem.* **277**, 21529–21536 (2002).
58. Rachmilewitz, J. & Lanzavecchia, A. A temporal and spatial summation model for T-cell activation: signal integration and antigen decoding. *Trends Immunol.* **23**, 592–595 (2002).
59. Sykulev, Y., Joo, M., Vturina, I., Tsomides, T. J. & Eisen, H. N. Evidence that a single peptide–MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity* **4**, 565–571 (1996).
60. Irvine, D. J., Purbhoo, M. A., Krogsgaard, M. & Davis, M. M. Direct observation of ligand recognition by T cells. *Nature* **419**, 845–849 (2002).
61. Lanzavecchia, A., Iezzi, G. & Viola, A. From TCR engagement to T cell activation: a kinetic view of T cell behavior. *Cell* **96**, 1–4 (1999).
62. Van Den Berg, H. A., Rand, D. A. & Burroughs, N. J. A reliable and safe T cell repertoire based on low-affinity T cell receptors. *J. Theor. Biol.* **209**, 465–486 (2001).
63. Kalergis, A. M. *et al*. Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex. *Nature Immunol.* **2**, 229–234 (2001). **Showed that TCR activation increases to a maximum value and then declines as a function of dwell-time, as predicted by competition between serial engagement and kinetic proofreading.**
64. Sousa, J. & Carneiro, J. A mathematical analysis of TCR serial triggering and down-regulation. *Eur. J. Immunol.* **30**, 3219–3227 (2000).
65. Goldstein, B. *et al*. Modeling the early signaling events mediated by FcεRI. *Mol. Immunol.* **38**, 1213–1219 (2002).
66. Hlavacek, W. S., Faeder, J. R., Blinov, M. L., Perelson, A. S. & Goldstein, B. The complexity of complexes in signal transduction. *Biotechnol. Bioeng.* **84**, 783–794 (2003).
67. Wofsy, C., Kent, U. M., Mao, S. Y., Metzger, H. & Goldstein, B. Kinetics of tyrosine phosphorylation when IgE dimers bind to Fcε receptors on rat basophilic leukemia cells. *J. Biol. Chem.* **270**, 20264–20272 (1995).
68. Torigoe, C., Goldstein, B., Wofsy, C. & Metzger, H. Shuttling of initiating kinase between discrete aggregates of the high affinity receptor for IgE regulates the cellular response. *Proc. Natl Acad. Sci. USA* **94**, 1372–1377 (1997).
69. Wofsy, C., Vonakis, B. M., Metzger, H. & Goldstein, B. One Lyn molecule is sufficient to initiate phosphorylation of aggregated high-affinity IgE receptors. *Proc. Natl Acad. Sci. USA* **96**, 8615–8620 (1999).
70. Young, R. M., Holowka, D. & Baird, B. A lipid raft environment enhances Lyn kinase activity by protecting the active site tyrosine from dephosphorylation. *J. Biol. Chem.* **278**, 20746–20752 (2003).
71. Rafnar, T. *et al*. Stimulation of the high-affinity IgE receptor results in the tyrosine phosphorylation of a 60 kD protein which is associated with the protein-tyrosine kinase Csk. *Mol. Immunol.* **35**, 249–257 (1998).
72. Ohtake, H., Ichikawa, N., Okada, M. & Yamashita, T. Cutting edge: Transmembrane phosphoprotein Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains as a negative feedback regulator of mast cell signaling through the FcεRI. *J. Immunol.* **168**, 2087–2090 (2002).
73. Siraganian, R. P. Mast cell signal transduction from the high-affinity IgE receptor. *Curr. Opin. Immunol.* **15**, 639–646 (2003).
74. Mao, S. Y. & Metzger, H. Characterization of protein-tyrosine phosphatases that dephosphorylate the high affinity IgE receptor. *J. Biol. Chem.* **272**, 14067–14073 (1997).
75. Lee, S. J., Hori, Y., Groves, J. T., Dustin, M. L. & Chakraborty, A. K. The synapse assembly model. *Trends Immunol.* **23**, 500–502 (2002).
76. Coombs, D., Dembo, M., Wofsy, C. & Goldstein, B. Equilibrium thermodynamics of cell–cell adhesion mediated by multiple ligand–receptor pairs. *Biophys. J.* **86**, 1408–1423 (2004).
77. Chakraborty, A. K., Dustin, M. L. & Shaw, A. S. *In silico* models for cellular and molecular immunology: successes, promises and challenges. *Nature Immunol.* **4**, 933–936 (2003).
78. Dustin, M. L. *et al*. A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell* **94**, 667–677 (1998).
79. Hopfield, J. J. Kinetic proofreading: a new mechanism for reducing errors in biosynthetic processes requiring high specificity. *Proc. Natl Acad. Sci. USA* **71**, 4135–4139 (1974).
80. Gillespie, D. T. A general method for numerically simulating the stochastic time evolution of coupled chemical reactions. *J. Comp. Phys.* **22**, 403–434 (1976).
81. Gibson, M. A. & Bruck, J. Efficient exact stochastic simulation of chemical systems with many species and many channels. *J. Phys. Chem. A* **104**, 1876–1889 (2000).
82. Slepchenko, B. M., Schaff, J. C., Macara, I. & Loew, L. M. Quantitative cell biology with the virtual cell. *Trends Cell Biol.* **13**, 570–576 (2003).
83. Pleiman, C. M. *et al*. Distinct p53/56lyn and p59fyn domains associate with nonphosphorylated and phosphorylated Ig-α. *Proc. Natl Acad. Sci. USA* **91**, 4268–4272 (1994).
84. Vonakis, B. M., Chen, H. X., Haleemsmith, H. & Metzger, H. The unique domain as the site on Lyn kinase for its constitutive association with the high affinity receptor for IgE. *J. Biol. Chem.* **272**, 24072–24080 (1997).
85. Turner, H. & Kinet, J. P. Signalling through the high-affinity IgE receptor FcεRI. *Nature* **402**, B24–B30 (1999).
86. Kurosaki, T. Molecular mechanisms in B cell antigen receptor signaling. *Curr. Opin. Immunol.* **9**, 309–318 (1997).
87. Kane, L. P., Lin, J. & Weiss, A. Signal transduction by the TCR for antigen. *Curr. Opin. Immunol.* **12**, 242–249 (2000).
88. Davis, S. J. *et al*. The nature of molecular recognition by T cells. *Nature Immunol.* **4**, 217–224 (2003).

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Competing interests statement

The authors declare that they have no competing financial interests.

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