Pairing computation with experimentation: a powerful coupling for understanding T cell signalling

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Abstract | T cells are activated when extracellular stimuli, such as a ligand binding to the T cell receptor, are converted into functional outputs by the T cell signalling network. T cell receptor signalling is a highly complex, stochastic and dynamic process involving many interacting proteins. This complexity often confounds intuition, making it difficult to develop mechanistic principles that underly experimental observations. In this Review, we describe how computational approaches can partner successfully with biological experimentation to help address this challenge, and we illustrate this paradigm by summarizing recent work that shows new aspects of the T cell signalling network.

T cell activation is important for orchestrating adaptive immune responses, and its aberrant regulation can lead to autoimmunity. The cost of infectious diseases and autoimmune disorders has motivated much experimental research aimed at understanding how T cell activation is regulated. These studies have led to important discoveries regarding how T cells develop in the thymus, how they recognize antigen-derived peptide–MHC complexes in the periphery, and the proteins that mediate T cell signalling[1–10]. In spite of these major advances, the mechanistic principles that govern T cell activation remain poorly defined.

T cell activation involves complex interactions between numerous proteins and spans a range of timescales. Molecular interactions between membrane-bound receptors and their ligands occur in seconds and initiate downstream signalling cascades which then determine gene transcription programmes. These processes, which occur on the membrane, in the cytoplasm and in the nucleus, are subject to feedback regulation, last minutes to hours and can induce the secretion of components into the surrounding tissue. In turn, the tissue environment (including the cytokine milieu) can influence molecular interactions in the signalling pathways. The complexity of these multi-scale cooperative processes often makes it difficult to intuitively develop an understanding of the mechanisms underlying experimental observations. This complexity is exacerbated by the stochastic nature of these processes, which can make the response of each cell unique.

A key challenge in this field of research is to understand how the biochemical reactions that define the complex T cell signalling networks occur stochastically, but in concert, to mediate specific functional outcomes. Recently, several studies of the T cell receptor (TCR) signalling pathway have confronted this challenge by combining theoretical and computational approaches (approaches that are established in the fields of statistical physics and engineering) with genetic, biochemical and imaging experiments. For such an interdisciplinary approach to be beneficial, computation and theory must generate mechanistic hypotheses and associated predictions that can be tested by experimental methods. The effectiveness of combining computational approaches with modern experimental methods is beginning to enable basic researchers in immunology to better define mechanistic principles that describe how the T cell signalling network is regulated.

Why use computational models?

Computational studies of cell signalling processes can be divided into two broad classes based on their goals (FIG. 1). The first type of study (type I) aims to convert a detailed conceptual knowledge of the signalling pathway and measured values of parameters (such as protein concentrations and rates of biochemical reactions) to a quantitative description. Such a computational model can then be used to obtain numerically accurate estimates of a change in cellular response when conditions are changed by a quantitatively specified amount. Examples of systems for which experimental and computational studies have

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been combined to provide such a detailed description are the cell cycle of budding yeast[11] and chemotaxis in *Escherichia coli*[12]. Our knowledge of the TCR signal transduction network has not reached this point of maturity, and accurate measurements of many relevant parameters (protein concentrations and rates of biochemical reactions) are not available. At present, computational models can provide numerically accurate descriptions of only small signalling modules present in T cells. This can motivate studies of how a particular signalling module may interact with other pathways, but by itself has limited value for aiding the discovery of new phenomena and mechanisms.

Computational research that aims to elucidate new mechanisms relevant to T cell signalling (a type II study) may be sparked by experimental observation of a new and

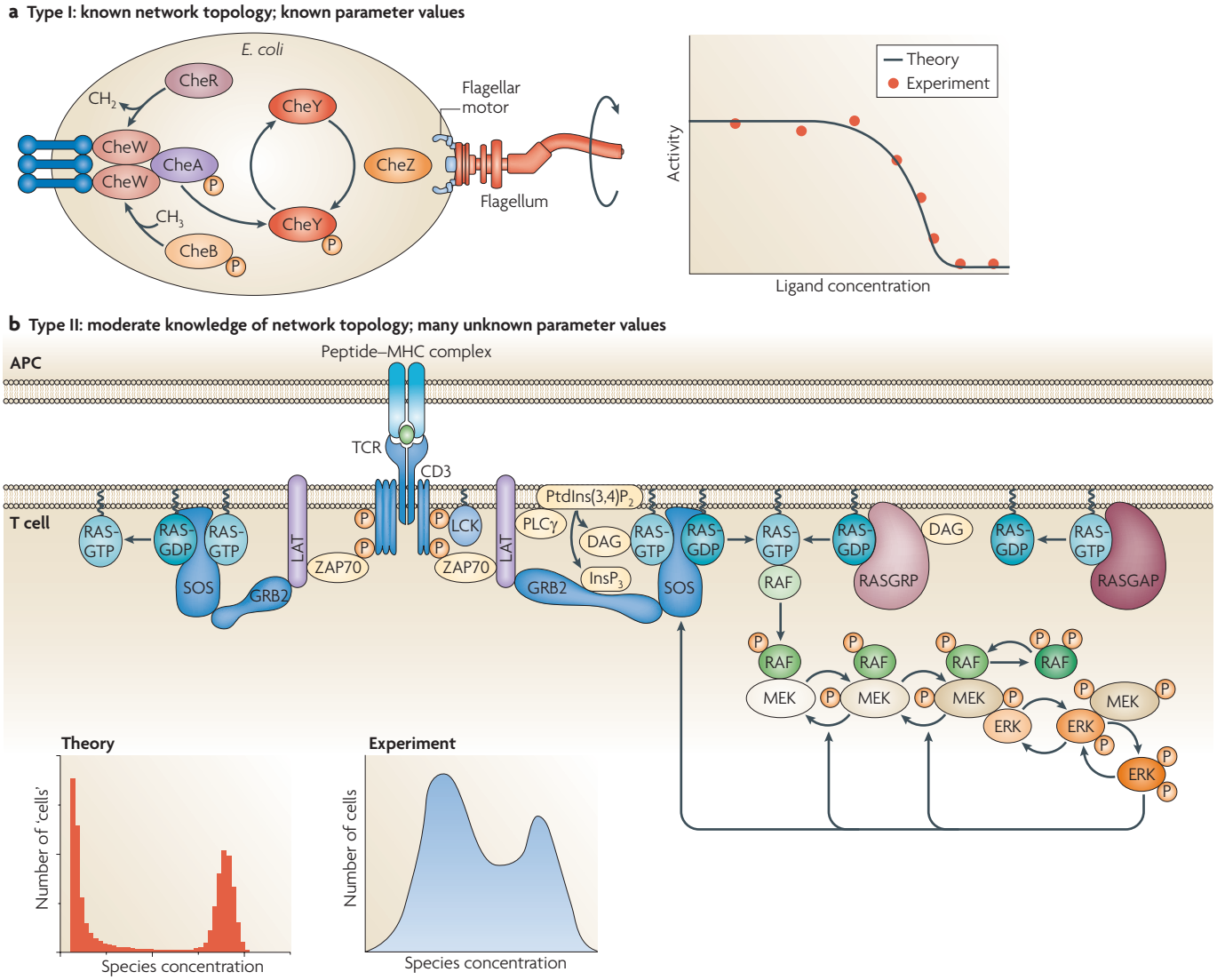


Figure 1 | **Types of computational and theoretical research methods.** Theoretical and computational research can be classified into two general types depending on their goals. **a** | Type I research constructs models that describe experimental observations in quantitative detail. Detailed knowledge of the signalling pathways and parameters (such as rates of biochemical reactions and numbers of signalling proteins) is required. The schematic shows an example of such a pathway: chemotaxis of *Escherichia coli*. **b** | Type II research complements experiments to aid the discovery of new aspects of the signalling machinery. Here, the emphasis is on generating hypotheses that describe a qualitative phenomenon robustly and helping the design of experiments that can test predictions to discriminate between viable hypotheses. A quantitatively accurate description is not the goal. The schematic shows the T cell receptor (TCR) signalling pathway as an example. Theoretical studies could make a qualitative prediction that the T cell signalling network contains motifs which result in cells expressing either high levels of active signalling proteins or very few, and experiments could test its veracity, thereby leading to new mechanistic insights. APC, antigen-presenting cell; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; GRB2, growth factor receptor-bound protein 2; $InsP_3$, inositol-1,4,5-trisphosphate; LAT, linker for activation of T cells; MEK, MAPK/ERK kinase; PLCγ, phospholipase Cγ; $PtdIns(3,4)P_2$, phosphatidylinositol-3,4-bisphosphate; RASGAP, RAS GTPase-activating protein; RASGRP, RAS guanyl-releasing protein; SOS, son of sevenless homologue; ZAP70, ζ-chain-associated protein kinase of 70 kDa.

unexplained phenomenon. Models can be formulated based on what is known about the signalling network and hypotheses that may explain the new observation. Feedback regulation and the many possible interactions between signalling components lead to combinatorial complexity, which often makes it difficult to parse the various implications of a hypothesis. Modern computers and computational methods can keep track of every possible event that could occur in keeping with a specified hypothesis and reveal how a particular event influences the qualitative nature of the cellular responses. For example, one could determine whether a certain hypothesis results in a graded or sharp dose–response curve. Results from these studies can be counter-intuitive because of the complexity of the signalling pathways and can show that some hypotheses that seemed plausible cannot explain the observed phenomena or imply cellular behaviour that is inconsistent with known facts. Using computational models to *a priori* dismiss such hypotheses can save fruitless experimental efforts to test them. Computational studies also reveal why a hypothesis does not explain a puzzling observation, and this helps to identify key missing areas of knowledge and generate new hypotheses.

Several parameters will be unknown when exploring new phenomena or pathways through computational approaches. Diverse biological effects are fairly robust to variations in most parameters[13–16] — that is, the qualitative response to a stimulus is stable despite variations in many parameters over wide ranges. It is likely that the 'wiring' of biological networks has evolved to exhibit such robustness. Therefore, if the outcome of a proposed signalling pathway fits with a new observation only when many parameters are in narrow ranges, it probably does not reflect the true biological network. However, biological networks are also sensitive to variations in a few key parameters or network characteristics[16]. For example, T cells can discriminate between numerous ligands with closely related affinities. Theoretical and computational approaches can be used to establish whether a proposed signalling network exhibits a response robustly and sensitively and identify key features and parameters that confer this property. This can be accomplished by 'brute force-' or Monte Carlo-based[17] calculations (BOX 1), in which parameters are varied, and by theoretical analyses.

Computational models can be used to mimic experiments with cells derived from animals with mutations that inhibit or enhance a particular pathway. In turn, results of such calculations can help to inform the design of experiments that will sensitively discriminate between viable hypotheses that are predicted to describe an observation of unknown origin equally well[18,19]. These results can also identify which key parameters need to be measured. Alternating iteration between experimental tests and further computations can potentially provide a mechanistic understanding of a new aspect of the T cell signalling network and its function. Most research that yields important results usually involves both type I and type II computational studies. For example, a quantitatively accurate model of a well-established signalling module may be generated first (a type I study). Theory, computation and complementary experiments would then be pursued to establish how this module functions when integrated in the complex T cell signalling network (a type II study).

Types of theoretical and computational models. The generation of a computational model begins by deciding how to describe the relevant signalling components. For example, as is done to aid protein structure determination, one could describe each protein in atomic detail (that is, each atom and its interactions with others are described). Despite the power of modern computers, it is not practical or necessary to provide such a detailed description of the many proteins engaged in dynamic processes during T cell signalling in order to study cellular responses. Rather, each protein is presumed to interact with others according to specified rules (for example, a TCR binds cognate peptide–MHC ligands) and parameters (for example, the on-rate of an interaction). The rules are either established facts or predictions to be tested.

There are several commonly used computational methods that have varying degrees of complexity and that require differing amounts of computational time (BOX 1). Importantly, a particular computational method is chosen according to its suitability for answering the question of interest. For example, if stochastic effects arising from the intrinsically random nature of biochemical reactions (intrinsic noise)[20] or cell–cell variations in protein expression (extrinsic noise)[21] are thought to affect a phenomenon of interest, one should not use a computational method that ignores stochastic fluctuations.

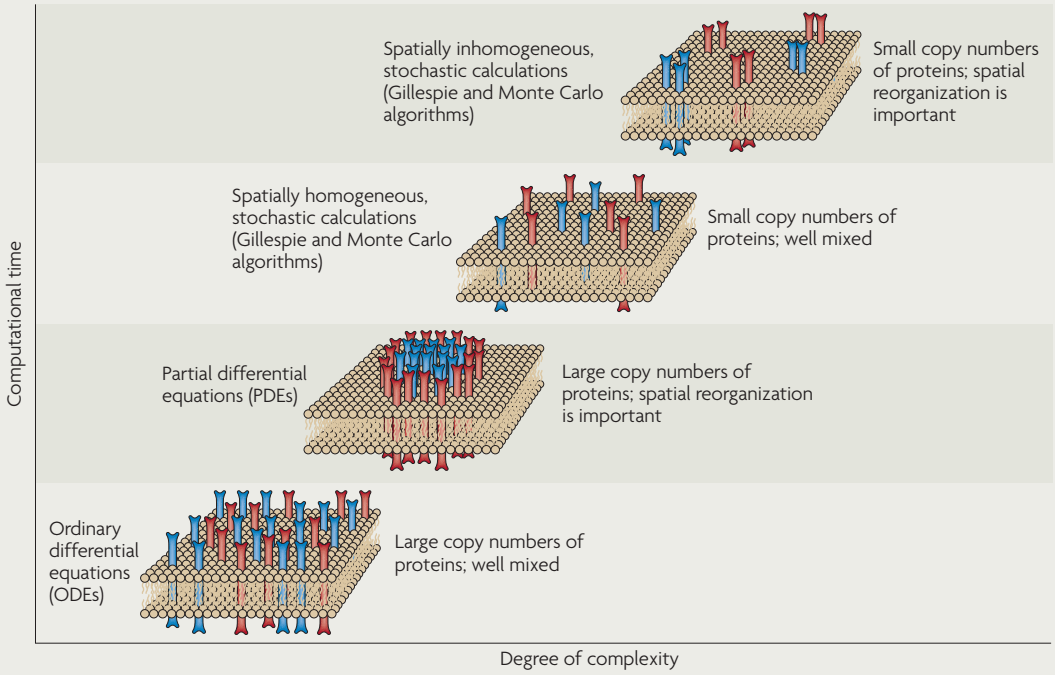
Finally, we note that theory and computation are not synonyms. Results from computational studies show how a biological system would behave if an input hypothesis is correct. Theoretical analyses and calculations of simplified versions of complex models can help the investigator to understand why a hypothesis worked or failed and to identify the key elements and parameters of a viable hypothesis that should be subjected to experimental tests. Here, we describe a small subset of studies that have successfully used complementary theoretical, computational and experimental approaches to elucidate the complex TCR signalling network.

Membrane-proximal feedback regulation

Stimulation of the TCR and/or co-stimulatory and cytokine receptors with different ligands (inputs) leads to diverse biological outcomes (such as the development and differentiation of thymocytes[4,6] and the proliferation, differentiation[22] and anergy[23] of mature T cells). A specific outcome is reached by inputs that are within a certain range of quality and quantity, whereas other outcomes are reached by inputs in a different range. Feedback regulation can mediate this type of stimulus–response behaviour. The TCR signalling pathway is replete with modules that are subject to feedback regulation[24,25]. Two recent studies[19,26] show how complementary *in silico* and *in vitro* investigations can be used to determine how feedback loops enable T cells to translate certain types of input into decisions.

Digital signalling and hysteresis during RAS activation in lymphocytes. When cell surface receptors are weakly stimulated — for example, by just a few cognate ligands — the basal level of active downstream signalling molecules is maintained. A continuous (analogue) increase in the number of stimulatory ligands could lead to two types of response (FIG. 2). The population of cells could increase the number of active downstream signalling molecules in a continuous manner (this is referred to as an analogue response). Alternatively, beyond a threshold

Box 1 | **Types of theoretical and computational approaches**



Ordinary differential equations (ODEs)
ODEs can be used to study phenomena for which the intrinsic stochastic nature of the biochemical reactions is unimportant and signalling components are assumed to be uniformly distributed in the cell membrane or the cytoplasm. ODE-based calculations[97] are appropriate if the relevant proteins are expressed in large amounts and the spatial organization of signalling components is irrelevant. These calculations yield average concentrations of active signalling molecules in a population of cells, similar to results obtained from a western blot assay. Effects of cell–cell variations in protein expression (extrinsic stochastic effects) can be described by ODEs by carrying out several calculations using experimentally determined distributions of expression levels. Of the commonly used methods, such computations require the least amount of computer time (see the figure).

Partial differential equations (PDEs)
PDEs can be used to study phenomena for which the spatial organization and reorganization of signalling components, mediated by unknown clustering mechanisms, protein diffusion or active transport of molecules, are important[77,98] but stochastic effects are not. Imaging experiments that observe the spatio-temporal evolution of signalling components complement PDE-based calculations as they indicate how mean protein concentrations vary with time in different locations. Such computations require more computer time than ODE-based calculations.

Monte Carlo algorithms
These methods are used to study phenomena for which intrinsic stochastic fluctuations are important, and they require the most computer time. The Gillespie method[38,99,100] is a widely used algorithm among the various Monte Carlo techniques developed recently[100,101]. Assays that can analyse single cells in a population (for example, flow cytometry) are experimental counterparts. Such experiments reflect both intrinsic and extrinsic stochastic effects. Variants of these methods can be used to investigate issues in which the spatial patterning of signalling components is unimportant or significant. Experimental imaging of single cells and spatially inhomogeneous, stochastic calculations provide the most detailed information but also require large amounts of computer time.

Informatics and logic-based methods
Analysing high-throughput data on signalling responses using principal component analysis and partial least square regression has yielded important information on other cell types[102,103] and more studies of this type are required to further investigate lymphocytes. These methods provide 'smart' ways to correlate complex data. The correlations are interpolated or extrapolated to predict outcomes as inputs are varied. However, these methods rarely provide mechanistic insights; so, given the focus of this Review, we do not discuss them further. Similarly, models based on Boolean[104,105] and Fuzzy logic[106] that have been used recently to analyse cell signalling networks and provide some mechanistic insights are also not discussed as these models are distinct from the physical molecular models discussed in this Review.

stimulus level, the population of cells could split into two subpopulations — one that maintains basal signalling, and another that turns on a large proportion of a downstream signalling molecule (this is referred to as a digital response).

Early markers of TCR-initiated signalling (such as activation of members of the extracellular signal-regulated kinase (ERK) family) exhibit digital responses[19,26]. Active RAS family proteins[27,28] target many downstream signalling pathways, and a recent study suggests that digital signalling in lymphocytes is controlled by the way in which RAS is activated[19]. In lymphocytes, RAS is activated by two families of guanine nucleotide exchange factors (GEFs), RAS guanyl releasing proteins (RASGRPs) (RASGRP1 and RASGRP2)[29,30] and son of sevenless homologue (SOS) proteins (SOS1 and SOS2)[31–33], which convert inactive RASGDP to active RASGTP.

After receptor stimulation, SOS is targeted to the membrane[34] and inhibition of its catalytic domain is removed[33,35]. The binding of RASGTP to an allosteric site in SOS leads to a large increase in the enzymatic activity of SOS[32]. Thus, the GEF activity of SOS is subject to positive feedback regulation, which can give rise to sharp dose–response curves[32,36] but does not necessarily lead to this result. Using mostly known rate constants of RAS activation by SOS (FIG. 3a), calculations showed how the amount of active RASGTP varies with the amount of uninhibited SOS (or stimulus) if the SOS module functioned in isolation[19]. The amount of RASGTP produced in a given time depends on the number of times each type of relevant biochemical reaction occurs during this period (FIG. 3a). Biochemical reactions are stochastic events and so the number of times each reaction occurs, and the corresponding amount of RASGTP produced, is different for each process (intrinsic stochastic cell–cell variations). For initial exploration of the SOS module, these stochastic effects were not studied and, therefore, ordinary differential equations (ODEs; see BOX 1) were used to calculate the average concentration of RASGTP. For low or high levels of stimulus, levels of active RAS are low or high, respectively (FIG. 3b).

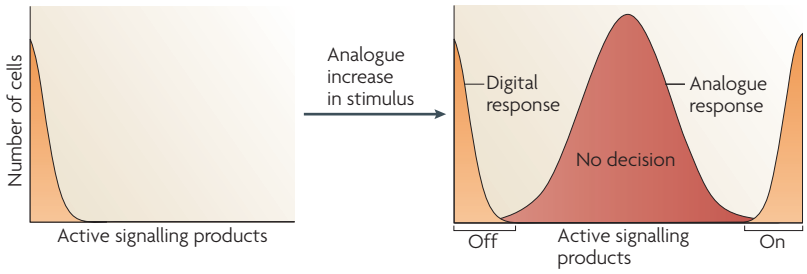


Figure 2 | **Digital and analogue responses of signalling networks stimulated by continuous increases in stimulus.** Digital and analogue responses of cells to a continuous (or analogue) increase in stimulus level (for example, ligand concentration). If the population of cells continuously increases the level of active signalling molecules in response to continuous increases in stimulus, the response is termed analogue. If, when above a sharply defined threshold stimulus level, the population of cells splits into two subpopulations, one with high levels of active signalling molecules and the other with low levels, the response is termed digital. Analogue responses may not enable decisive functional outcomes.

But, for intermediate values of the stimulus, two possible stable states of RAS activity were predicted for the same stimulus level (bistability). One state corresponds to high GEF activity because many SOS proteins have RASGTP bound to the allosteric site, and the other state corresponds to one in which the activity of SOS is low because RASGTP is not bound to this site. Thus, feedback regulation of the enzymatic activity of SOS leads to the bistable behaviour. The prediction of two possible states of RAS activity above a threshold stimulus (FIG. 3b) suggested that positive feedback regulation of SOS might cause the digital signalling in lymphocytes. As most parameters were known, this hypothesis emerged from a computational study similar to one with a goal of a type I study (FIG. 1a). Testing this hypothesis, however, required a type II study.

T cells can be stimulated by few TCR–ligand interactions[37] and several T cell signalling proteins are present in small copy numbers. Therefore, the number of biochemical reactions that can occur in a given time is small, making stochastic cell–cell variations important. Computer simulations of part of the T cell signalling network (FIG. 1b), including the interplay of RAS activation by RASGRP and SOS, were carried out using the Gillespie algorithm[38], which accounts for stochastic effects (BOX 1). Several replicate simulations were carried out with the same parameters. Each simulation mimics the behaviour of a single cell, and results for the amount of active RASGTP in all the *in silico* 'cells' were represented as a histogram. This type of analysis yields results analogous to those from studies using flow cytometry in which a particular active protein is assayed in individual cells in a population.

Many parameters (for example, reaction rates and protein copy numbers) required for computer simulations of the network under study are unknown (FIG. 1b). As noted previously, it is important to use the computational model to establish whether the qualitative outcome of a hypothesis is robust to variations in unknown parameters before subjecting it to experimental tests. Using theoretical analyses to complement brute-force parameter sensitivity studies, it was shown that the qualitative results summarized below are robust if the following conditions are met: first, the enzymatic activity of SOS is subject to positive feedback regulation; second, RASGRP activity is not so high that all RAS proteins are activated before the SOS feedback loop can be engaged; finally, RAS GTPase-activating proteins (RASGAPs) enzymatically deactivate active RAS.

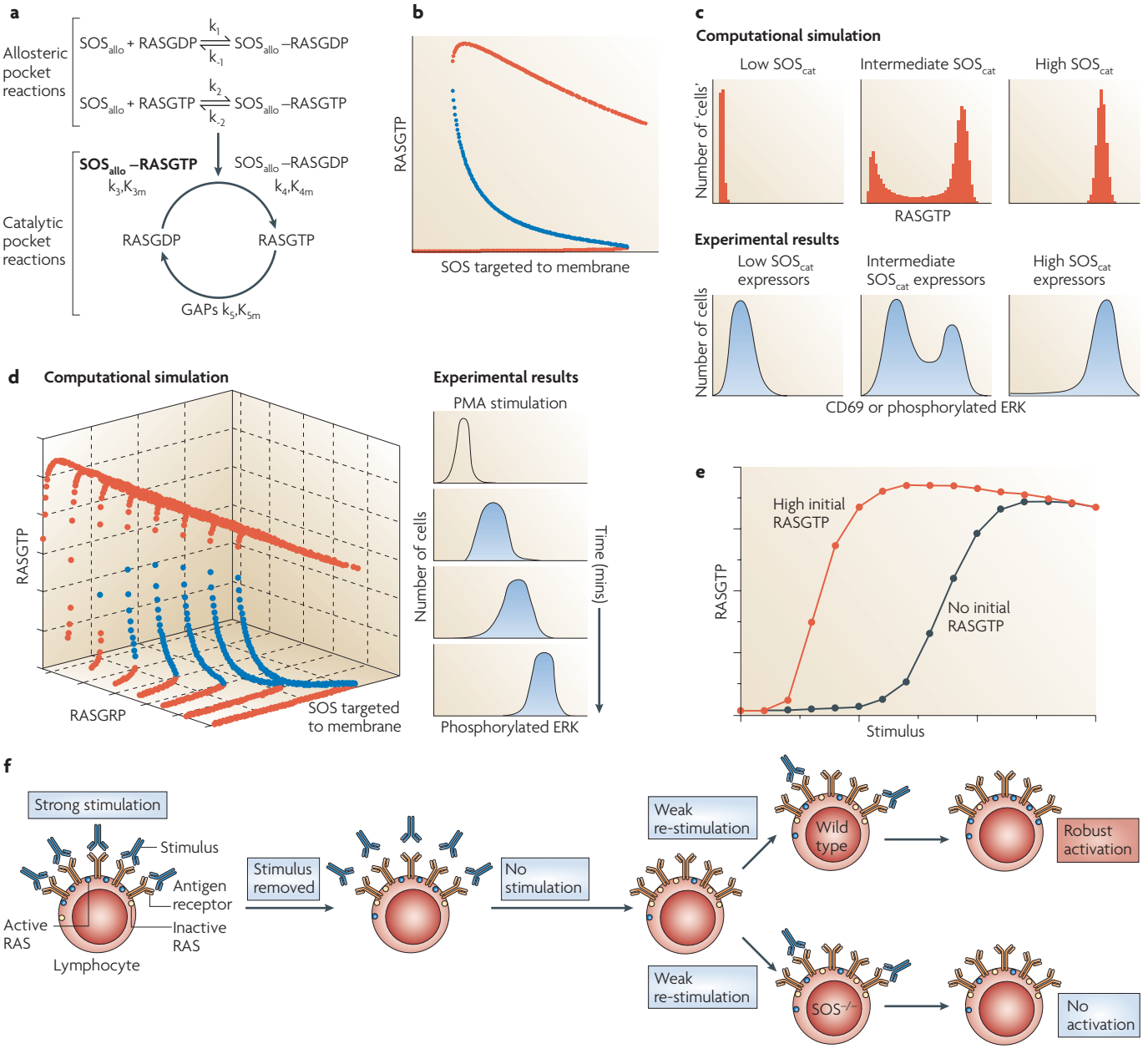
Computer simulations showed that, beyond a threshold amount of SOS targeted to the membrane, the population of cells split into two subpopulations with one population exhibiting high levels of active RAS and the other not (FIG. 3c). This digital response was dependent on positive feedback regulation of SOS. Experiments with different amounts of the catalytic domain of SOS transfected into Jurkat T cells (FIG. 3c), and stimulation of receptors of T and B cell lines, as well as primary T cells, supported these predictions[19]. But, because it is not feasible to measure RAS activation in single cells, these experiments assayed RAS-dependent downstream

Hysteresis
A biological system's memory of its recent history. For example, a cell may respond to a certain stimulus weakly if it is subject to stimulation for the first time, but it may respond strongly to the same stimulus if it has recently been robustly stimulated.

markers (such as ERK phosphorylation or the expression of the early activation molecule CD69). One could therefore argue that the observed digital signalling was due to signalling modules downstream of RAS.

A combination of theoretical predictions and experimental tests provided evidence indicating that feedback regulation of the enzymatic activity of SOS is a dominant factor in enabling digital signalling in lymphocytes. It was shown that digital responses, leading to a population of cells that exhibit high RAS activity, occur at a higher stimulus threshold for RASGRP-deficient systems (FIG. 3d) because RASGRP produces the first RAS molecules that prime the feedback loop that regulates SOS. Computations predicted that, although intermediate levels of RASGRP activity led to optimal digital responses, RAS signalling is analogue in nature if RASGRP activity dominates RAS activation[19] (FIG. 3d). So, if high levels of RAS activity were to be stimulated by RASGRP alone and downstream markers (such as ERK phosphorylation) exhibited analogue responses, this would indicate that digital RAS activation was important for digital responses in lymphocytes. Experiments using T and B cells stimulated with phorbol 12-myristate 13-acetate (PMA) (which causes RAS activation through RASGRP only) or using SOS-deficient B cells stimulated through the B cell receptor showed purely analogue responses at all stimulus doses[19] (FIG. 3d).

It was also reported that digital RAS activation was accompanied by a phenomenon known as hysteresis[19], which may enable lymphocytes to integrate signals from a series of individual encounters with antigen-presenting cells (APCs) *in vivo*[39]. Work resulting in this



finding also exemplifies how calculations can suggest ways to experimentally test for phenomena that cannot be directly assessed owing to technical limitations (for example, the inability to assay single cells for RAS activation). Hysteresis is indicated by the finding that the same stimulus dose can induce a higher level of active RAS in a population of cells that has previously been stimulated robustly than in cells that have not (FIG. 3e). When a cell is weakly stimulated for the first time, most SOS molecules do not have RASGTP bound to the allosteric sites, and resulting active RAS levels are low. For a finite period of time, a previously stimulated cell will have more SOS molecules with RASGTP bound to the allosteric sites. The resulting higher enzymatic activity of SOS can lead to RAS activation after weak stimulation. Digital signalling (bistability) owing to feedback regulation of SOS implies hysteresis. The predicted hysteresis in RAS activity was tested experimentally by measuring RAS levels in populations of cells (using western blots). In this way, digital RAS activation was tested by inference.

◀ Figure 3 | **An example of complementary *in silico* and *in vitro* studies elucidating the function of a feedback loop in the membrane-proximal T cell receptor signalling pathway. a** | The reactions show activation of RAS at the catalytic site of son of sevenless homologue (SOS) and feedback regulation of this activity owing to nucleotide-associated RAS binding to the allosteric site (allo) of SOS. The rate constants (k) of the reactions in this small network are known from *in vitro* experiments. SOS with RASGTP bound in the allosteric pocket has 75-fold higher catalytic activity than SOS with RASGDP bound. **b** | Steady states of the mean-field kinetic rate equations show production of low and high concentrations of RASGTP (characterized by stable fixed points shown in red) at low and high values of total SOS concentration (a measure of signal strength), respectively. At the intermediate levels of SOS concentrations three states arise; but the states shown in blue are unstable, so cells would not be observed in these states. **c** | Distributions of RASGTP (red histograms) calculated from stochastic simulations at low, intermediate or high levels of SOS (twofold increments) in *in silico* 'cells'. At intermediate levels of SOS, a bimodal RASGTP pattern arises. In experiments (blue histograms) Jurkat T cells expressing low, intermediate and high levels of SOS_{cat} (the catalytic domain of SOS without inhibitory domains) were analysed for activation of RAS by measuring levels of CD69 or phosphorylated extracellular signal-regulated kinase (ERK) expression by flow cytometry. Similar to the computational simulation, upregulation of CD69 and phosphorylated ERK expression at intermediate levels of SOS_{cat} was bimodal. **d** | Computational calculations showing RAS activation as a function of SOS and RAS guanyl-releasing protein (RASGRP) expression. The results show that RASGRP alone activates RAS in an analogue manner. Stimulating Jurkat T cells using phorbol 12-myristate 13-acetate (PMA), which activates RAS through RASGRP alone, results in analogue ERK activation, regardless of stimulus dose. The blue histograms show how phosphorylated ERK is gradually activated, in unimodal distributions, in flow cytometry experiments. **e** | Prediction of hysteresis from stochastic simulations. Points in black and red denote RAS activation when *in silico* cells had either no or high amounts of RASGTP initially, respectively. Each pair of these points is calculated as the SOS concentration, a measure of the signal strength, is varied. The red and black points do not coincide for intermediate levels of SOS concentrations (hysteresis). **f** | The experiments that test these results are based on the following effect of hysteresis in RAS activation. The RAS molecules in a lymphocyte reach maximal activation within minutes after a strong stimulus is delivered. If the stimulus is withdrawn at this point, RAS activation will start to decrease owing to the effect of RAS GTPase-activating proteins (RASGAPs). If a suboptimal second stimulus (that is incapable of producing significant RAS activation in a resting lymphocyte, such as a low antigen dose) is then applied shortly afterwards, RAS activation returns to the maximal level because of hysteresis in RAS activation. However, if the second stimulus is given after a long period of time, such that the RAS activation has reduced almost to the basal level, the stimulus would fail to produce any significant RAS activation (not shown). Because SOS is responsible for the hysteresis in RAS activation, the suboptimal second stimulus applied soon after the first will not cause maximal RAS activation in SOS-deficient cells. Figure is modified, with permission, from REF. 19 © (2009) Elsevier Science.

Interestingly, it was shown that SOS-dependent hysteresis allowed cells to exhibit short-term molecular memory of past encounters with antigen, enabling previously stimulated cells to respond robustly to weak signals that are not stimulatory for cells not previously exposed to the antigen (FIG. 3f).

Effects of positive and negative feedback regulation on T cell sensitivity to antigen. T cells can discriminate between agonist, antagonist and non-cognate peptide–MHC ligands. Germain and co-workers[26,40] have suggested that an interesting interplay of feedback loops during TCR signalling may underlie this ability. Based on experimental results[41], they proposed that the proto-oncogene tyrosine kinase LCK mediates activation of its own inhibitor, SH2-domain-containing protein tyrosine phosphatase 1 (SHP1; also known as PTPN6). But this inhibitory interaction is prevented if active ERK molecules, generated by the activation of the mitogen-activated protein kinase pathway, phosphorylate serine 59 of LCK. The consequences of these positive and negative feedback loops were explored using a computational model[26]. In addition to these feedback loops, a simplified model of signal propagation from the TCR to RAS activation and a model for the mitogen-activated protein kinase pathway leading to ERK activation were included. The system was presumed to be spatially homogeneous, stochastic effects were ignored and the average concentration of various species in a population of cells was calculated using ODEs (BOX 1). Altan-Bonnet *et al.*[26] made careful estimates of various parameters with the aim of obtaining numerically accurate results that could be compared with experimental measurements (type I study). They also used the computational model to predict the consequences of feedback regulation of LCK (type II study).

The Germain group's findings suggest that, whereas weak TCR stimulation can lead to LCK recruitment and SHP1 activation, ERK activation and concomitant protection of LCK from the action of SHP1 requires strongly stimulatory TCR ligands. This is presumably because ζ-chain-associated protein kinase of 70 kDa (ZAP70) recruitment and activation (which is a prerequisite for ERK activation) is favoured by full phosphorylation of TCR chains, a state that is more likely to be reached following stimulation with ligands that bind to the TCR strongly. Results from experiments with $CD8^+$ OT-I T cells (which are specific for an ovalbumin peptide) were consistent with predictions that the transition from antagonism to agonism occurred when the number of agonists presented exceeded a sharply defined threshold (FIG. 4a). More generally, Altan-Bonnet and Germain suggested that the ability of T cells to discriminate between different ligands (FIG. 4b) was dependent on the competing effects of feedback regulation of LCK by SHP1 and activated ERK[26]. They also noted that model predictions matched the qualitative trends of the experimental results only if the model was tuned such that ERK activation was digital. Digital ERK activation in lymphocytes has now been suggested to originate in RAS activation characteristics[19].

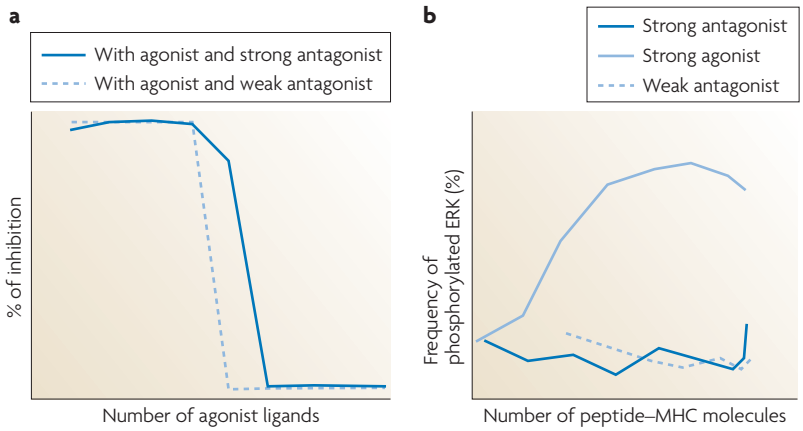


Figure 4 | **An example of complementary *in silico* and *in vitro* studies showing the effects of positive and negative feedback regulation of LCK on T cell activation.** **a** | Results from computer simulations show activation in T cells following exposure to a range of agonist and antagonist ligands. Percentage of inhibition is defined as the ratio of fully phosphorylated extracellular signal-regulated kinase (ERK) following stimulation by both agonist and antagonist ligands to that stimulated by agonists alone. The degree of inhibition decreases sharply as the number of agonist ligands in the mixture is increased. These predictions are supported by experiments using OT-I T cells. **b** | Dose responses for ERK phosphorylation in naive OT-I T cells after activation by an agonist peptide and its antagonistic variants. The computational model has been used to suggest a mechanism for the sensitivity of T cells to these ligands of varying potency. Figure is modified from REF. 26.

MicroRNA
Small (~21–23 nucleotides in length), single-stranded RNA molecules that regulate the expression of genes by binding to the 3′ untranslated regions of homologous target mRNAs.

Immunological synapse
A large junctional structure that is formed at the cell surface between a T cell that is interacting with an antigen-presenting cell. Important molecules involved in T cell activation, including the T cell receptor, numerous signal-transduction molecules and molecular adaptors, accumulate in an orderly manner at this site.

Kinetic proofreading
A model of T cell activation in which a series of sequential modifications (such as phosphorylation) of the TCR needs to be completed for the triggering of downstream signalling events. TCR modifications are reversed if the ligand and TCR dissociate prior to reaching the terminal state of modification. The model, therefore, suggests that there is a threshold time that a TCR needs to remain bound to a peptide–MHC complex for T cell activation.

The results described above suggest that SHP1 has an important role in regulating LCK activity *in vitro*[26]. Elegant experiments have been carried out using the microRNA miR-181a, which suppresses the expression of phosphatases such as SHP2 (also known as PTPN11), dual-specificity protein phosphatase 5 (DUSP5), DUSP6 and protein tyrosine phosphatase, non-receptor type 22 (PTPN22), but does not affect SHP1 expression. It was found that miR-181a dramatically changed the sensitivity of T cells to ligands and converted an antagonist into a weak agonist[42]. These results indicate that SHP1 may not be the only negative regulator that influences T cell sensitivity.

In addition to the intrinsic stochastic nature of biochemical reactions that mediate T cell signalling, cell–cell differences could be further accentuated by stochastic variations in the expression of proteins in each cell. Extrinsic stochastic effects have been observed to have an important role in other signalling networks regulating cell fate decisions[43,44]. Recently, one group has examined these extrinsic stochastic effects in OT-I T cells[40]. They found that levels of CD8 and SHP1 expression varied between individual cells, but these fluctuations in each cell correlated: high SHP1 expression was compensated by high CD8 levels. Based on calculations, they proposed that this effect minimized extrinsic stochastic noise, leading to a narrow antigen sensitivity threshold for a population of cells. Intrinsic stochastic effects, however, can still be important in this system, as shown by a stochastic simulation of a simplified model of the phosphorylated ERK-mediated positive feedback regulation of LCK activation[45].

Spatial organization of signalling components

The influence of the spatial organization of cellular components on T cell signalling is emerging as an important area of research owing to the observations that TCRs are pre-clustered on resting T cells[46,47], that TCR signalling is initiated in microclusters of TCRs and peptide–MHC ligands[48,49] and that large-scale spatial reorganization of proteins occurs at the immunological synapse[50,51]. We summarize recent work that uses complementary experimentation and computation to examine this issue.

Initiation of TCR signalling. Several studies of the earliest events in TCR signalling have investigated the effects of spatial organization of proteins on T cell activation. It was proposed that one peptide–MHC complex could serially trigger many TCRs, thus enabling T cells to detect minute amounts of agonists[52]. Computational approaches have been used to study this hypothesis. One study[53] divided the T cell–APC junction into two zones and examined how serial triggering and kinetic proofreading[54,55] influenced TCR internalization (a marker of phosphorylated receptors). The computations led to the prediction that an optimal value of the peptide–MHC–TCR off-rate maximizes TCR internalization if both free and ligand-bound TCRs can be internalized. Complementary experiments seemed to support this suggestion[53], but other data do not indicate an optimum off-rate[56]. So, it remains unclear whether the optimal off-rate of the most stimulatory TCR ligands is reached by balancing the need to enhance serial triggering with the need to bind long enough to allow kinetic proofreading. The influence of ligand–TCR concentration on serial triggering[57] and that of second messengers on kinetic proofreading have also been studied[58], and recently summarized in detail[59–61].

The observation that T cells are sensitive to minute numbers of agonist peptide–MHC ligands among numerous endogenous ones[37,62] has motivated complementary experimental and computational studies using the Gillespie algorithm (BOX 1). In particular, the possible role that endogenous peptide–MHC ligands have in cooperating with agonist ligands to amplify signalling has been studied in detail[63–66]. One view emerging from these studies is that LCK that is recruited and activated following the binding of agonist ligands to the TCR can subsequently phosphorylate neighbouring receptors that bind transiently to endogenous ligands. However, how the spatial organization of proteins influences the cooperative interactions between TCR, LCK and endogenous and agonist ligands has yet to be studied carefully. Spatially resolved stochastic computational methods will be important complements to imaging experiments in unravelling these mechanisms.

The influence of the spatial organization of proteins on potentially altering the basal phosphorylation and dephosphorylation of the cytoplasmic tails of the TCR–CD3 complex has been studied using complementary experimental and computational techniques[67]. It was proposed that TCR triggering is a consequence of the spatial segregation of phosphatases (CD45 and CD148 (also known as RPTPη) and kinases (LCK) in the T cell plasma membrane (the

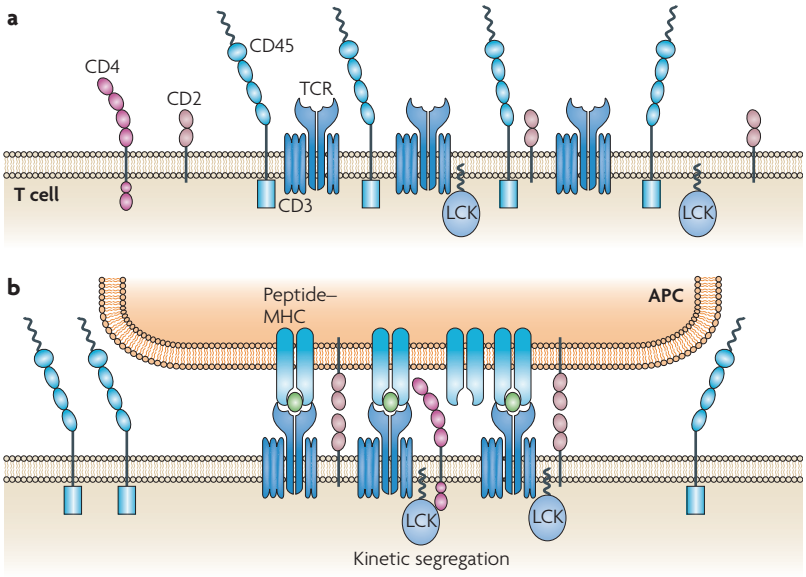


Figure 5 | **The kinetic segregation model.** In the absence of ligation between a T cell and an antigen-presenting cell (APC), T cell receptor (TCR)-associated CD3 molecules are continuously phosphorylated and dephosphorylated by kinases (such as LCK) and phosphatases (such as CD45), respectively. However, these two competing processes balance each other and do not initiate signalling in a resting T cell. When peptide–MHC molecules interact with TCRs, large CD45 molecules are pushed outside the close contact regions, owing to steric interactions, where the membranes of the conjugated APC and the T cell are separated by a distance of approximately the size of the peptide–MHC–TCR complex. This shifts the balance of TCR phosphorylation in favour of kinases, thereby initiating downstream signalling. Figure is reproduced, with permission, from *Nature Immunology* REF. 67 © (2006) Macmillan publishers Ltd. All rights reserved.

kinetic segregation model, FIG. 5). Phosphorylation (by LCK) and dephosphorylation (by phosphatases) of the TCR complex is balanced in unstimulated cells, thereby preventing TCR triggering. T cell–APC interactions result in regions where the inter-membrane separation is approximately the size of the peptide–MHC–TCR complex (15 nm). CD45 and CD148 molecules, which are larger (25–40 nm) than the peptide–MHC–TCR complex, are excluded from these regions, thereby enabling the kinases to dominate in the balance between phosphorylation and dephosphorylation. Experiments with modified CD45 molecules with smaller ectodomains and peptide–MHC complexes with larger ectodomains support this model[68]. A stochastic Monte Carlo simulation (BOX 1) of a patterned surface in which kinase-rich regions are embedded in a phosphatase-rich background also show that fully phosphorylated TCRs are generated in the kinase-rich region[69,70] owing to the longer residence time of the bound peptide–MHC–TCR complexes and increased kinase activity of LCK molecules in the kinase-rich domains. Although mechanistically appealing, this model does not account for the many ways in which LCK is regulated by kinases and phosphatases (such as carboxy-terminal SRC kinase (CSK) and CD45) and so further study is warranted.

Signalling in the immunological synapse. A few minutes after receptor–ligand engagement, proteins at the T cell–APC junction organize into a characteristic pattern[50,51,71]. For example, TCR and peptide–MHC molecules (along with other proteins) accumulate in a central region 3–5 μm in diameter. This region, known as the central supramolecular activation cluster (cSMAC), is surrounded by a peripheral ring of adhesion molecules known as the peripheral supramolecular activation cluster (pSMAC), and together they form the immunological synapse. The immunological synapse has many functions: it can direct the secretion of toxic molecules at juxtaposed cells, thereby minimizing bystander damage[72], and serve as a niche for asymmetrical T cell division[73]. We focus on its possible role in regulating TCR signalling.

Originally, it was proposed that the cSMAC enhances and sustains TCR signalling[50,51]. Consistent with this view, calculations[57] showed that, if TCRs were present at low levels, the accumulation of ligands (as in the cSMAC) would enhance the rate of peptide–MHC–TCR encounters, which presumably would increase serial triggering and signalling. However, the failure to detect activated ZAP70 (a marker for TCR signalling) in the cSMAC[74] led investigators to question whether signalling occurs in the cSMAC.

To further examine this question, a computational model[75] of mobile TCR and peptide–MHC ligands was developed. The molecules were placed on two membranes (to resemble T cell and APC surfaces). The receptors and ligands interacted with each other according to prescribed on- and off-rates. Receptor–ligand engagement resulted in signalling through a simplified model, and ZAP70 activation was monitored as a function of time. Fully phosphorylated internalized TCRs were degraded, and partially phosphorylated and unphosphorylated receptors were recycled to the plasma membrane. Directed movement of TCRs was initiated if the amount of activated RAC1 (a Rho GTPase) exceeded a threshold, thereby resulting in cSMAC formation. In the computational studies, unlike in the experiments, it is easy to turn off cSMAC formation by disabling this mechanism. This allowed the computations to directly analyse signalling with and without cSMAC formation without changing other conditions.

Results from Monte Carlo simulations (BOX 1) of this model, along with experimental tests, led to the following conclusions[75]: clustering of receptors and ligands in the cSMAC can enhance the rate of generation of fully phosphorylated receptors because higher concentrations can increase the rates of encounters between TCR, peptide–MHC and kinases (as described elsewhere[57,76]). But, the high concentration of receptors in the cSMAC can also enhance degradation, and this explains why active signalling molecules are not detected.

High-resolution images showed signalling molecules in T cells interacting with lipid bilayers loaded with appropriate ligands[48,49]. Active signalling was observed in microclusters of TCRs that formed continuously in the pSMAC. The microclusters were then transported to the cSMAC, where there was no evidence of active signalling molecules, as reported earlier[74]. These observations suggested that the cSMAC is exclusively a site for receptor degradation and cannot sustain signalling.

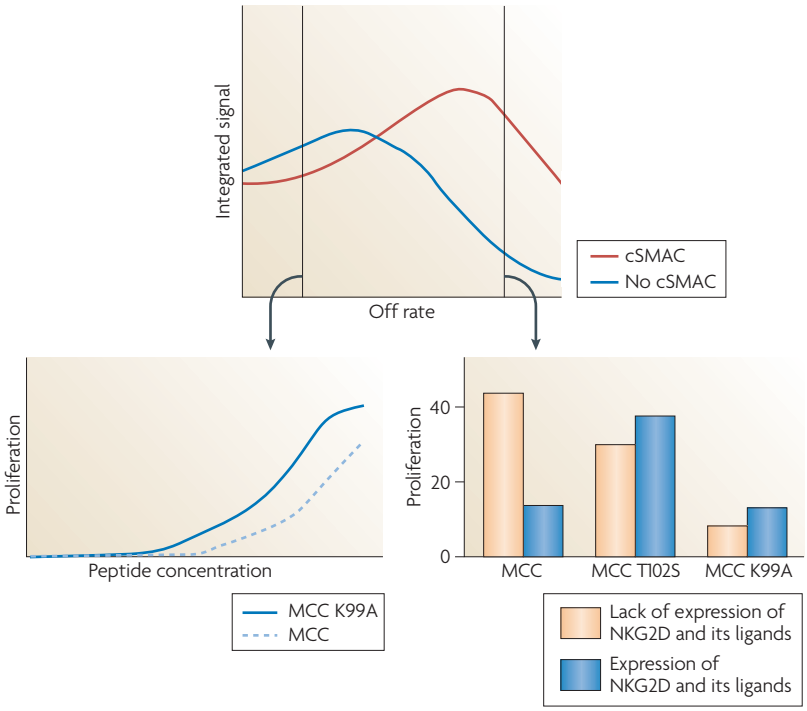


Figure 6 | **Signalling in the immunological synapse.** Two cases from a computer simulation are compared here, one in which a central supramolecular activation cluster (cSMAC) (red) is allowed to form regardless of the value of the off-rate and another in which no cSMAC (blue) is formed. For small off-rate values, cSMAC formation inhibited the total amount of integrated signal, whereas the opposite was true for ligands that bind the T cell receptor (TCR) weakly. These calculations were carried out with a peptide–MHC density of 1 molecule per μm^2 and an on-rate equal to 2200 $M^{-1}s^{-1}$. The proliferation of AND TCR T cells in response to wild-type moth cytochrome C (MCC), which efficiently forms a cSMAC, and variant MCC K99A peptide ligands, which do not form a cSMAC, is shown. The MCC K99A peptide is characterized by a faster off-rate for binding to the AND TCR, but induces a greater proliferative response than that induced by the wild-type MCC peptide (residues 88–103). Proliferation assays carried out with 5C.C7 TCR T cells support another prediction of the computational model. The variant peptides MCC K99A and MCC T102S, which bind weakly to the 5C.C7 TCR, resulted in enhanced stimulation when cSMAC formation is enforced by transducing the T cells and the antigen-presenting cells with natural killer group 2, member D (NKG2D) and its ligands, respectively.

Complementary computational and experimental studies have suggested that the situation may be more subtle[77,78]. Because proteins are concentrated in the cSMAC, stochastic effects are less important (see earlier). Therefore, a PDE-based model (BOX 1) was developed that accounted for spatial variations, but not stochastic effects. This model more faithfully represented receptor degradation than that used in previous studies[75].

Computational results were obtained both allowing and preventing cSMAC formation (FIG. 6). For potent agonists present in high concentrations (as in most experiments), cSMAC formation was predicted to enhance receptor degradation and inhibit signalling. However, if a threshold off-rate was exceeded, cSMAC formation was predicted to enhance signalling. Results from experiments with ligands of varying potency seem to support these computational results. For example, the variant peptide ligand moth cytochrome C (MCC) K99A interacts with the AND TCR with a faster off-rate than the canonical wild-type agonist (MCC 88–103) and does not induce cSMAC formation but stimulates greater amounts of T cell proliferation (FIG. 6). If the MCC K99A ligand has an off-rate that is slower than the predicted point at which signalling by the cSMAC is neither inhibited nor enhanced (FIG. 6), this is consistent with the cSMAC inhibiting signalling for good agonists. Expression of natural killer group 2, member D (NKG2D) on T cells promotes cSMAC formation in a ligand-independent manner and does not affect T cell activation[77,78]. A weak agonist that does not induce cSMAC formation, or proliferation of AND TCR T cells, stimulates cSMAC formation and a proliferative response following NKG2D expression (FIG. 6). This is consistent with the prediction that cSMAC formation promotes signalling in cells stimulated by weak ligands. Also, consistent with predictions, active ZAP70 was observed in the cSMAC following stimulation of T cells with low doses of agonists[78].

The ability to ask 'what if' questions readily using computational models, and the experiments thereby motivated, leads to the suggestion that the cSMAC functions primarily as a location for degradation of receptors stimulated by potent ligands. But it can also function to enable less potent ligands to signal through the TCR. The physiologically advantageous or deleterious implications of its role in supporting signalling by less potent ligands need further study. Many other questions regarding the signalling function of the synapse remain unresolved. Why is it necessary to maintain the cSMAC over several hours for full commitment to activation[79] when activation of molecules immediately downstream of the TCR occurs in a few minutes? Even for potent ligands, signalling through co-stimulatory molecules (such as CD28) is detected in the periphery of the cSMAC[80]. Studying this process may shed light on how TCR signalling is integrated with co-stimulatory signals, with potential implications for factors that determine the induction of T cell anergy.

Concluding remarks

Extracellular inputs (such as cognate TCR ligands) are translated into specific functional outcomes, such as activation, anergy, differentiation and apoptosis, by the T cell signalling network. Signalling is mediated by cooperative dynamic processes that involve many components that must act in concert. Because it is often hard to intuit the consequences of such complex events, it has been difficult to establish the principles that govern T cell signalling and its aberrant regulation. Simpler cooperative dynamic processes have been successfully studied in the physical and engineering sciences using theoretical and computational approaches that complement experiments. Recently, such theoretical and computational approaches have been used together with genetic, biochemical and imaging experiments to uncover new aspects of the TCR signalling network. We have described how such a complementary approach works and illustrated it by summarizing some recent studies.

Anergy
A state of unresponsiveness that is sometimes observed in T and B cells that are chronically stimulated or are stimulated through the antigen receptor in the absence of co-stimulatory signals.

We focused on two broad areas of study. The first concerned how feedback regulation of signalling modules enabled digital activation of T cells and the ability to discriminate between ligands of varying potency. Although these studies suggest that certain feedback loops may have a dominant role in mediating these cellular responses, it is not known how the interplay between various feedback loops involved in T cell signalling[81–87] influences outcomes and this needs to be studied by bringing together complementary *in silico, in vitro* and *in vivo* methods.

We also described how combining computational and experimental studies has helped to clarify the early events in TCR triggering, with a specific focus on the role of the spatial organization of signalling components. Various models for TCR triggering (such as kinetic segregation, change in receptor conformation[88,89] and signal amplification by endogenous ligands[37,64]) are often thought to be mutually exclusive. We think that these hypotheses describe different effects, the relative importance of each being context dependent. Computational models should prove to be useful in defining such an integrated mechanistic description. As T cells are activated by minute numbers of stimulatory ligands[37,64], stochastic effects are important. Computational modelling can help in understanding the influence of intrinsic and extrinsic stochastic variations in experimental investigations[43,44]. Our discussion of the importance of spatial patterns of signalling components focused largely on membrane-proximal signalling (such as studies of the immunological synapse). But these effects need not be confined to events that occur at the membrane. For example, depending on the stimulus, RAS activation occurs predominantly at the plasma membrane or at Golgi membranes[90], and nanoclusters of active RAS proteins have recently been observed[91]. Spatially resolved computational studies coupled with biochemical and imaging experiments offer rich opportunities for elucidating the relevance of such phenomena and how they are connected to digital or analogue responses. Combining *in vitro* and *in vivo* studies with computational analysis should also help us to understand how co-stimulation and cytokine signalling pathways integrate with TCR signalling.

To facilitate such studies, we will need to develop more efficient ways to carry out parameter sensitivity analyses for stochastic computations[92] and more efficient algorithms for carrying out stochastic calculations that account for spatial inhomogeneities[93].

Most complementary theoretical and experimental studies today are the result of collaborations between experimental and computational scientists. This situation may change in the ensuing years as user-friendly computer codes that can carry out calculations for studying signalling in immune cells are becoming available[93–97]. Today, experimental scientists need to use these computer codes with caution. As we have pointed out, each type of computational method contains approximations, and it is important to establish whether a particular method is appropriate for answering a specific question of interest. Moreover, theoretical analysis that complements computation and experiment is key for understanding the underlying principles. Ultimately, however, we expect that a situation similar to that in organic and inorganic chemistry will prevail, in which synthetic chemists use standard quantum chemistry codes to inform and analyse experimental studies.

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Competing interests statement
The authors declare no competing financial interests.

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