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Computational Analysis of T Cell Receptor Signaling and Ligand Discrimination – Past, Present, and Future

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Abstract

Signaling through the T cell receptor for antigen (TCR) has been studied for years by conventional biochemical means. More recently, attempts have been made to develop computational models of signaling through this receptor, with a specific focus on understanding how this recognition system discriminates between closely related (self and non-self) ligands. Here we discuss recent advances centered on the role of feedback regulation, especially the key finding that a combination of digital and analog control circuits is fundamental to the discrimination properties of the TCR. We end by pointing to future, more biologically accurate models that incorporate spatial aspects of molecular organization in antigen-engaged T lymphocytes with this underlying biochemistry.

Introduction

For more than a quarter of a century, beginning with the initial studies of Stobo and colleagues showing that IP₃ generation and Ca²⁺ influx accompanied antibody crosslinking of the CD3 complex of human T cells [1,2], immunologists have probed the mechanistic underpinnings of immune recognition and signal transduction in T lymphocytes. Progress has included identification of peptides bound to the surface proteins encoded by the Major Histocompatibility Complex Class I and Class II loci (pMHC) as the proximate ligands for T cell immune recognition [3–5], cloning of the variable subunits of the T cell receptor (TCR) that recognizes these pMHC [6,7], description of the importance of the phosphorylation motifs termed ITAMs that are present in multiple copies in the cytoplasmic tails of the CD3 components of the receptor complex [8,9], uncovering of the role of the CD4 and CD8 coreceptors in antigen-dependent signaling [10], and identification and functional analysis of a large universe of kinases, adapters, enzymes, and target transcription factors that translate ligand engagement into gene regulatory events that control T cell differentiation and proliferation following antigen recognition [11–13].

Despite this impressive knowledge base, our insight into how pMHC binding to the TCR actually triggers the earliest steps in the signaling cascade is more limited than might be expected, with a number of competing hypotheses [14–19]. This same set of hypotheses

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inform ongoing debates about how differences in binding affinity or capacity for receptor allosteric change result in functional discrimination between structurally similar pMHC ligands by any individual TCR. The large body of imaging data showing marked surface molecule reorganization (microclusters and immunological synapse formation) during the signaling process [20–23] has yet to be placed in a robust conceptual framework that relates these changes to operation of the signaling apparatus and the functional effects of ligand recognition on the T cell. Finally, we know very little yet about how variations in the coupling of the same TCR structure to the intracellular signaling machinery during different stages of T cell development and differentiation allows for distinct outcomes upon ligand binding, such that self-ligands can promote initiation of developmental progression among thymocytes (positive selection) and yet be incapable of gene activation in mature cells expressing the same recognition structure and encountering the same self-pMHC [24,25].

Over the past few years, an increasing number of investigators have turned to modeling and simulation (computational biology) in an effort to better address these problems that have not been satisfactorily approached by conventional cell biological and biochemical methods. The majority of these efforts have focused attention on the key questions of ligand discrimination and signaling sensitivity that are characteristic of T cell antigen recognition. While some of these studies stop at the modeling stage, many involve a robust combination of computation and experiment. Here we discuss some of these efforts, especially what present models have contributed to our understanding and what are some of the limitations of the approaches taken to date. We then address how we hope to markedly improve on these existing efforts in the coming years using new computational tools, quantitative and/or global measurement technologies, and multiple rounds of prediction and experimental testing.

The Past

Kinetic proofreading as a core concept

The first widely appreciated set of models for pMHC ligand discrimination by the TCR developed out of the proposal that this receptor system relied on kinetic proofreading to regulate effective activation of the T cell [15]. The hypothesis was based on the notion that progressing fully through a linked cascade of enzymatic reactions required persistent occupancy of the TCR, given the underlying assumption of an overriding level of tonic negative signaling that returned the system to baseline as soon as ligand no longer occupied the TCR. Short-lived binding events would not provide enough time for the cascade of reactions to progress to completion. Because the elements of the cascade were enzymes, each step involved (potentially) a substantial amount of signal amplification. In turn, this would lead to very non-linear attenuation of functional signaling if the input driving flux through the cascade was aborted before the final steps occurred. The relationship between small changes in the length of occupancy (basically, a reflection of the dissociation rate between TCR and pMHC) was thus proposed to be a power function and a few fold decrease in binding strength would therefore be expected to result in a many fold larger loss of effective signal propagation. Kinetic measurements using surface plasmon resonance (SPR) and tetramer dissociation experiments emerging at this time that showed only a few seconds difference in the effective length of interaction between a given TCR and a strong vs. a weak agonist whose potencies differed by logs in terms of effective EC_{50} were consistent with this general thesis [26–30] (but not all studies agreed with this view – see [31]) and several simple mathematical models that seemed to explain the experimental findings were constructed based on this proofreading concept [32,33].

Antagonists raise questions about simple occupancy models

In the same time frame, concern developed over whether simple occupancy, even with the proofreading concept incorporated, was adequate to explain TCR-ligand relationships. The discovery of TCR antagonists was the key development in this area [34–37]. If all engagements of the TCR by pMHC ligand contributed to positive signaling events and it was the integrated sum of all such occupancy events that drove signal generation, then all pMHC ligands for a given receptor should fall into two categories. The first would be agonists of varying potency, based solely on the duration of occupancy. The second would be non-agonists, which in reality are extremely weak agonists whose capacity for stimulation could not be revealed experimentally because the maximally attainable density of pMHC on an antigen presenting cell (APC) was simply too low given a low affinity for the TCR to engender enough average occupancy to yield a measurable response [38]. What clearly should not be observed was antagonism – that is, the capacity of a pMHC to inhibit the response to more potent pMHC presented to the same T cell on the same APC membrane. This is because the only way such antagonists could mediate a negative effect would be to prevent occupancy of the TCR by the more potent ligand and they could only do so when occupying the TCR themselves. Thus, if all occupancy was equivalent, one would simply be replacing one binding event with another and no change in functional response should be seen. But in the early 1990's multiple laboratories using both mouse and human cell models show that slight variations in the amino acid sequence of potent agonist peptide could produce pMHC that inhibited responses to the parent agonist pMHC co-presented on the same APC – that is, they functioned as antagonists. Some early interpretations used the proofreading concept to develop a possible way out of this paradox and retain the core features of a simple occupancy model. It was suggested that in the context of a McKeithan-type proofreading scheme, the antagonists occupied the TCR for too short a time to produce an effective downstream signal, but at high concentrations, they engaged so many TCR briefly that they prevented the agonist pMHC ligand from accessing as many TCR as would otherwise be the case, thus limiting the overall effective stimulation of the T cell [34].

Data from this laboratory, however, suggested that antagonists might (also) operate through a more active mechanism [35] and this concept was given substantial support by studies using T cells from mice bred to express two distinct TCRs with different pMHC specificities [39,40]. Using this experimental tool, it was demonstrated that an antagonist to one TCR could antagonize responses to an agonist that engaged the other TCR. Such a result could not be explained by competitive occupancy, because the antagonist pMHC did not bind the same TCR that could engage the agonist pMHC offered on the same APC. These data provided evidence that antagonism could operate in trans, that is, between different receptors, and led to a search for molecular mechanisms that could account for this phenomenon.

Biochemical studies focused on active, TCR-induced, negative regulatory pathways, as these seemed most likely to account for such findings. The first clues came from studies in this laboratory and that of P. Allen, showing that antagonists were not null ligands but were able to induce a proximal TCR signaling pattern of incomplete ζ chain ITAM phosphorylation and recruitment without phosphorylation of ZAP-70 kinase [41,42]. This pattern of early signaling differed from the complete ζ phosphorylation and ZAP-70 phosphorylation/activation characteristic of agonist engagement of the TCR. The fact that the most proximal steps known in the TCR signaling cascade were already altered upon antagonist recognition, and the fact that some positive events, namely phosphorylation of some ITAM sequences in ζ , accompanied antagonist engagement of the TCR, could be explained either by arguing that the weak ligand could only produce a limited set of downstream events a la McKeithan, or that such stimulation induced unopposed negative feedback that prevented the full signaling response. If the latter view was correct, then an adequate amount of such negative

regulatory function induced by large amounts of antagonist engagement could inhibit responses to low amounts of pMHC agonist provided to the T cell simultaneously. In a general sense, this concept added to the McKeithan proofreading the action of negative feedback and suggested that such feedback could be induced as a consequence of very early events in the cascade of positive enzymatic steps he proposed. If this was the case, then persistent engagement of the TCR by agonist pMHC in some manner allowed the cell to override these negative effects. The simplest way for this to happen would be for the amplification that accompanied progression through the cascade to quantitatively override the level of negative regulation induced by the smaller amount of signal characteristic of early states of the cascade. The very high ratio of antagonist to agonist necessary to reveal inhibitory activity could then be ascribed a requirement for the summation of many inputs feeding into the low level negative feedback loop to produce enough control to blunt the otherwise highly amplified signal generated by a few agonists. Consistent with this hypothesis of a key role for a negative feedback pathway, several groups then provided direct evidence that the hematopoietic phosphatase SHP-1 was crucial to antagonist function [39,43,44].

Negative feedback alone, though helping to explain TCR antagonism on a mechanistic basis, did not provide a full picture of how ligand discrimination operated. A striking observation made in our laboratory revealed that the ERK pathway directly modified Lck, the key proximal kinase in the TCR signaling pathway, and that inhibition of the ERK pathway resulted in conversion of the proximal signaling molecular signature from that of an agonist (fully phosphorylated ζ and ZAP-70) to that of an antagonist (partially phosphorylated ζ with docked by not active or phosphorylated ZAP-70) [43]. These findings could not be explained based on a simple amplification proofreading scheme, but instead indicated that a positive feedback event mediated by ERK was active in supporting agonist signaling. Our studies thus revealed two interconnected pathways that were sensitive to ligand quality (Fig. 1). The hematopoietic cytosolic phosphatase SHP-1 was tyrosine phosphorylated at position 564 [45] and then bound to the SH2 domain of Lck associated with the TCR complex (but not that Lck pool bound to the CD4 co-receptor) on CD4⁺ T cells within a minute of antagonist stimulation. In marked contrast, such binding of TCR-associated Lck by pSHP-1 was not seen until after 30–45 minutes of agonist signaling, in concert with an attenuation of ζ phosphorylation and ZAP-70 activity at this time. Treatment of T cells with a MEK inhibitor that prevented ERK activation allowed the rapid binding of SHP-1 to Lck even in the presence of agonist, consistent with the effect of this inhibitor on the pattern of ζ and ZAP-70 phosphorylation. These findings indicated that weak ligands predominantly evoked negative feedback regulation of TCR signaling consisting of tyrosine phosphorylation of SHP-1 by activated Lck, which led to binding of the phosphorylated phosphatase to Lck in the TCR complex. Further tyrosine phosphorylation engaged the SH2 domains of the SHP-1, activated the phosphatase and lead to loss of Lck activity, ζ phosphorylation, and ZAP-70 phosphorylation. These changes in turn shut down progressive signal transduction in the cascade leading to transcription factor activation, and hence, prevented the T cell from becoming functionally activated by ligands inducing this set of events alone. Agonists were able to induce ERK activation and ERK led to serine 59 phosphorylation of Lck, which prevented tyrosine phosphorylated SHP-1 from docking and hence, from mediating a negative effect on downstream signaling. This accounted for why agonists were effective even though pSHP-1 could still be formed by agonist signaling. These findings provided important insight into the biochemical control of TCR responses and suggested that the concatenation of two feedback loops opposing one another contributed to sharpening pMHC discrimination by the T cell (Fig. 1).

The Present – A New Feedback Regulation Model Built on a Proofreading Framework

The biochemical evidence supporting the SHP-1 negative: ERK positive feedback scheme was strong, but it did not lead to a rapid adoption of this scheme by most of the field as a critical aspect of TCR signaling and ligand discrimination. In part, this hesitancy arose because the ERK effect is not readily seen when using antibodies and transformed cells rather than non-transformed cells and pMHC stimuli. Given that the bulk of TCR biochemical studies have been and continue to be done using the former conditions [11,13], it was not clear whether the data we generated was generally applicable. However, a strong case can be made that results obtained with normal cells and physiological ligands should be given precedence over those arising from use of malignant cells and unphysiological stimuli. A second reason for limited acceptance was the claim that ERK, considered by the field to be a key player in nuclear gene activation events, was being proposed to regulate upstream biochemical signaling by the TCR, something of a heretical notion. Finally, on a more concrete basis, questions were raised about how what was essentially a bimolecular interaction involving Lck and SHP-1 could be overridden by the ERK pathways, with its many steps between TCR engagement and ppERK formation. In particular, why wouldn't the bimolecular pathway always dominate kinetically and hence, biochemically?

To address this latter issue in particular, we undertook the development of a new computational model of TCR signaling [46,47], based on the SHP-1: ERK hypothesis and also on a reconsideration of the models that had been developed using the McKeithan proofreading concept including one that included some of our early data on these feedback pathways [48]. A re-analysis of the results from simulations using these earlier models revealed that none adequately dealt at one time with three known characteristics of the TCR signaling response to pMHC:

1. The response is very rapid. We and others have shown that tyrosine phosphorylation of ζ and ZAP-70 occur within seconds of T cell-APC contact and even at these early times, one can detect the characteristic differences in phosphorylation described above for antagonist vs. agonist pMHC ligands [41–43]. Hence, any model that takes minutes of simulated time to develop a difference in signaling output between these two ligand classes fails to capture an essential feature of the system,
2. The response is sensitive. Our data using direct detection of the number of pMHC on the presenting cell [46], and data from several other laboratories [49–51], all indicate that as few as 1 and certainly as few as 10 good agonist pMHC can induce sustained calcium and other biochemical indicators of strong signaling by T cells and in longer term assays, induce functional responses. Thus, any model whose simulation requires hundreds or thousands of pMHC to trigger a T cell is not properly scaled to biological reality.
3. The response is highly discriminatory. A single amino acid side chain alteration in the peptide can change the dose response more than 20,000 fold, even though biophysical measurements indicate only a few fold change in the binding kinetics between the altered pMHC and the TCR that it engages, relative to the kinetic parameters involving the more potent agonist (reviewed in [47]). Any model that does not show such dramatic changes in efficacy for ligands with such a small difference in binding potential doesn't fit with a key known property of this recognition/response system.

Our review of models that were based on McKeithan proofreading without or with just negative feedback showed that all failed to combine these three features, and often 2 of the 3 were missing. We therefore sought to determine if adding in the ERK positive feedback pathway would fix this limitation and to see if such a model would help address the paradox described above concerning bimolecular vs. multimolecular pathways.

A great deal of effort building and testing such computational models with ERK feedback added initially failed to reproduce the 3 essential features of the TCR response, no matter how much we moved from abstracted and rather simple models with a few components to more complex models with dozens of steps or we varied the specific concentration and kinetic parameters. The breakthrough came not from the computational trials themselves, though they were important in indicating that some knowledge of the T cell signaling system was missing, but from addition biological experiments and a new method just introduced into the field. Up to this time, others and we analyzed signaling events using Western blots that averaged the responses of thousands or millions of single cells. The introduction of phosphoflow methods [52] using antibodies to the activated form of enzymes such as ERK allowed single cell resolution in such analyses. Unexpectedly, when we used phosphoflow to examine the ERK response of CD8⁺ T cells to pMHC, we found that increasing the quantity of pMHC per presenting cell did not lead to the expected gradual increase in average fluorescence intensity of ppERK staining in all the T cells in the responding population. Instead this dose titration caused an increase in the number of T cells that each showed the same high (~70–80% of maximum) conversion of ERK to ppERK. That is, the ERK response was digital on a per cell basis – a cell either remained at a low baseline level of activated ERK or rapidly reached a peak level of ppERK irrespective of the concentration of triggering pMHC; such changes only affected the fraction of fully responding cells. The triggered T cells were only found with an intermediate amount of ppERK for a few minutes during the onset of the response as they progressed from the resting state to the fully activated state. This digital behavior, see previously using transcriptional reporters in hybridoma cells [53] and verified since by others at the biochemical level [54,55], has major implications for understanding T cell responses. It implies that at low ligand densities, some cells are fully activated, where a proportional signaling model would imply that no cell is fully activated until much higher densities of pMHC are available (Fig. 2). This digital behavior of the MAPK pathway and its role in controlling switch-like behavior in cells has been emphasized in seminal work from Ferrell in non-immune biological systems [56,57].

Once this property of the ERK pathway in T cells was recognized and encoded in the model, we were suddenly able to replicate many of the essential features of the T cell response to ligand, in particular the sharp transition between non-agonists and agonists within a narrow range of biophysical binding parameters between TCR and pMHC. This feature was something we were unable to achieve in any model in which the ERK behavior was analog and not digital [46]. With some parameter refinement, this model was able to predict a number of aspects of the T cell response to antigen that experimental tests validated. These included the key predictions that a modest drop in SHP-1 levels in a T cell would allow a previously non-stimulatory ligand to acquire agonist activity without a change in the nature of its binding to the TCR, and that ligands whose binding to the TCR more closely approximated that of agonists would be more potent antagonists than weaker binding ligands. This latter somewhat unexpected result is explained by the increasingly strong generation of pSHP-1 by the better binding ligands in the absence of a compensating ERK response, until the threshold for transition to early digital ERK production is reached and this addition of positive feedback overcomes the negative influence of pSHP-1 generation. Finally, the model explained the bimolecular – multimolecular paradox raised above. The SHP-1 negative feedback systems operates in an analog fashion, whereas the ERK feedback is digital. With an agonist, there would be a small amount of pSHP-1 generated and bound

to TCRs before the explosive ERK response ensues. This would begin to blunt the TCR response, but by modifying all Lck molecules not yet engaged by pSHP-1, an early burst of ERK protects the TCR signaling apparatus from further negative regulation until ERK phosphatases reverse the protection, as we see after 30–45 minutes of stimulation. Indeed, a careful re-analysis of blots probing the binding of pSHP-1 to Lck in the TCR complex showed just the predicted behavior. We observed small amounts of pSHP-1 binding in the first 1–2 minutes after initiation of TCR engagement by agonist and then a failure to accumulate any additional pSHP-1 until ERK activity decreased and Lck shifted from the ERK-induced serine 59 phosphorylated form [58] to the pSHP-1 receptive p56 form 30–45 minutes after signaling began.

This new model allowed all three of the canonical TCR response features to be reproduced in simulations and it also provided insight into several previously unrecognized aspects of TCR signaling. Biochemical support for this model, especially the role of ERK, can be found in an important study involving miR-181a [59]. More recently, the model was used to make several predictions about what physiological variation in the per cell levels of SHP-1, CD8, and ERK would have on the T cell response. These studies predicted that variations in CD8 would lead to a change in the EC_{50} , whereas a change in SHP-1 would affect the fraction of responding cells, without altering the EC_{50} for the remaining responsive fraction, and that variations in ERK would have no effect [60]. All three predictions were borne out by flow cytometry-based assays that permitted assessment of ppERK induction in concert with measurement of CD8, SHP-1, and ERK levels in the same cell. The cell to cell variation in function revealed by these studies even within a putatively homogenous TCR transgenic naïve T cell population is of substantial significance for our understanding of how distinct fates of T cells arise during an immune response and also raises intriguing questions about the issues of robustness and sensitivity that many models find difficult to reconcile. Our experiments suggest the possibility that the cell tempers the effects of the cell to cell variations and presumably, the temporal fluctuation in protein expression within a single cell, by counterbalancing the states of components within a pathway so that the net output of the pathway is maintained within acceptable levels; this pathway or network balancing also would then permit an acute change in the concentration or state of just a single component to unbalance the network and lead to a response to even a small input.

The Future

Moving from Ordinary Differential Equations (ODEs) to Spatially-resolved modeling

The model described above simulates all intra-cellular signaling processes as if they would take place in a single, well-stirred biochemical compartment. Hence, it does not take into account any of the data showing a key role for protein localization and molecular clustering on signaling by the TCR, or revealing a role for scaffolds in signal segregation and transmission. Furthermore, it does not allow one to assess the role of microcluster formation or synapse organization [20–23], issues some others have addressed in models that incorporate our feedback information together with spatial aspects of molecular behavior [61,62]. Finally, the lack of spatial aspects precludes studying the relationship between T cell signaling behavior and cell shape or dynamics, even though we know that T cells are highly motile in the lymph node and periphery [63–65] and data from studies of T-B collaboration clearly show that functional cell interactions are affected strongly by migratory dynamics and the duration of contact in vivo [66].

To address these issues and related issues of cell signaling in the field of immunology and more broadly in cell biology, we have begun to develop a new suite of simulation technologies and software tools to support detailed, spatially-resolved modeling in the context of changing cell shape and migratory dynamics in complex multicellular

environments. These efforts are part of a computational systems biology effort in the Intramural Research Program of NIAID called the Program in Systems Immunology and Infectious Disease Modeling (PSIIM [<http://www.niaid.nih.gov/labsandresources/labs/aboutlabs/psiim/Pages/default.aspx>]). Composed of an integrated group of expert teams in the areas of immunology, cell and molecular biology, computational modeling, systems genomics, transcriptional networks, and proteomics, the PSIIM is developing a set of approaches that can support its own specific research agenda and also can provide biologists with a readily accessible set of methods and tools for undertaking complex modeling and simulation of their own systems of interest. The PSIIM is using new methods for global RNAi screening to discover more complete pathway and network connectivity for signaling systems such as TLR, TCR, and chemokine receptors, employing advanced mass spectrometry methods to permit absolute quantification of numbers of molecules in a cell or cell subcompartment, developing new ways to use multiplex SPR measurements to rapidly obtain the reaction rates needed for parameterizing molecular interaction network models, and creating novel software tools for modeling and simulation [67–69]. It is also developing methods for translating confocal and 2-photon imaging data into computable cell shapes and tissue environments [70,71], along with software tools that permit integrating signaling models with spatial features of the cell, and with the dynamic behavior of cells within a more complex tissue setting.

The Simmune Software Environment for Multiscale Modeling

There are 2 major classes of obstacles to moving to more precise and inclusive models of (immune) cell function. The first is the need for a comprehensive computational framework that allows the facile construction and simulation of spatially-resolved, multiscale models taking into account reaction-diffusion processes, intracellular compartmentalization, and higher order behavior of cells in complex tissue settings. The second is obtaining measurements of a sufficient number of parameters to reduce the degrees of freedom in such models to a manageable level that limits examination of biologically irrelevant conditions. The PSIIM is organized around efforts to address both of these key issues. M. Meier-Schellersheim and his group [<http://www.niaid.nih.gov/labsandresources/labs/aboutlabs/psiim/computationalbiology/Pages/simimmuneproject.aspx>] have developed a software suite called *Simmune* that deals with the computational issues. *Simmune* is designed to permit a biologist with detailed knowledge of a particular system to construct and simulate desired aspects of that biological system without concern for the complex mathematics and computer programming issues that normally limit such efforts [69]. Through a (reasonably intuitive) graphical users interface, the biologist can develop a model of, for example, trans-membrane signaling that is built from simple molecular binding event inputs and definitions of molecular complexes and transitions (Fig. 3). Based on such user definitions, the resulting signaling network, that is, the list of all reactions that can take place and lead to the formation of (multi-)molecular complexes, is automatically constructed by the software. This has several major benefits - first, assuming the binary molecular events are properly defined during the input process, even very complex network schemes will not contain user errors in connectivity; second, it is simple to add or remove elements and run a new simulation, without the risk of errors of mass balance or missing terms in equations of the type normally written manually for such modeling are now done automatically and without error by the software; third, the models are easily shared with others running the same software and these other users can readily simulate different conditions, change the model and test the effects of these changes, combine models of modules with shared molecular components and so on. Thus, *Simmune* eliminates many of the major impediments to the use of mathematical modeling and computer simulation in biology – the barrier to the involvement of most biologists lacking advanced mathematical or programming skills, the ‘one-off’ nature of most models built by

hand, the difficulty of changing such manual models over time, and the difficulty of sharing and integrating models that represent aspects of a larger biological system. *Simmune* runs on commonly used computing platforms (Windows, Mac, and Linux OS), and for models of modest size, does not require supercomputing facilities for simulation.

In addition to the features already mentioned, *Simmune* in its next version will have a more intuitive and visually sophisticated user interface, so that biologists will find model construction even easier. The modeling software will also be linked to proteomic databases so that information can be imported rather than manually entered and links retained to allow others to understand the origins of elements of a model with respect to existing databases on molecular interactions or the literature.

The early version of *Simmune* instantiated cells as geometric objects of limited shape diversity. Using new algorithms introduced by M. Meier-Schellersheim and F. Klauschen, the software now is able to simulate the activities of cells of diverse and realistic shapes, which can be automatically imported in computable form from microscopic data, for example, a confocal microscope z-stack (Fig. 4;[71]). The same methods also allow *Simmune* to generate more complex cellular environments, including intracellular compartments like nuclei. They also permit placing cells in computational reconstructions of complex 3D extracellular environments that we extract from 2-photon live intravital imaging studies performed in the laboratory to examine cellular interactions [72], and soon, the spatially-resolved signaling dynamics of immune cells in the living animal.

To allow researchers to define and explore aspects of cellular behavior that involve combinations of processes on very diverse time scales *Simmune* also allows simulations using a hybrid agent approach in which higher order cell state changes, such as division, death, differentiation or motion, can be coupled to fine-grained biochemical simulations through rules formulated in terms of threshold molecular concentrations triggering those state changes. In this realm, *Simmune* does not operate in a Boolean manner with only two alternative states of the agent, but uses continuous functions so that although based on rules that use Boolean arguments such as ‘and’, ‘or’ and so on, the results better reflect the variation of functionality characteristic of a biological system, which is not typically binary in its behavior. In addition, the hybrid agent aspects of *Simmune* models can be directly linked to the fine-grained molecular simulations, with each feeding into the other as a simulation progresses.

Finally, *Simmune* also has a capacity to connect simulation of cellular biochemistry with simulations of cell shape changes and migratory dynamics, allowing models to be built that can address the behavior of immune cells within lymphoid or peripheral tissue environments, and permitting the models and simulations to predict outcomes that can be explored using intravital imaging tools. Thus, in single software suite, an investigator can go from a detailed analysis of biochemical events involved in signal transduction, to gene expression (using the agent based rules modeling), to control of cell polarity and shape, and on to the activity of many such cells in a complex extracellular or multicellular environment. Whereas other software tools for modeling and simulation implement some of the key elements of *Simmune* (these include BioNetGen [http://bionetgen.org/index.php/Main_Page] for automated generation of signaling networks and the Virtual Cell [<http://www.ibiblio.org/virtualcell/>] for spatially-resolved simulations), *Simmune's* strength lies in the combination of elements described here in terms of its integrated capacity to model across many spatial and temporal scales in a facile manner.

A Technological Infrastructure for Data Acquisition to Support Multiscale Modeling

While *Simmune* provides the computational infrastructure for advanced modeling and simulation, it would be difficult to employ its power effectively without the right kinds of data to constrain the parameters that go into constructing such models. For simulation of biochemical signaling pathways, it is important to be as complete as possible in defining the relevant members of the module to be modeled and the biochemical transformations these molecules undergo following a stimulation event. This means reducing to the greatest extent gaps in knowledge on molecular connectivity, obtaining information on parameters such as association and dissociation constants, measuring catalytic rates, and quantifying total protein amounts. I. Fraser and his group within the PSIIM [<http://www.niaid.nih.gov/LabsAndResources/labs/aboutlabs/psiim/Pages/MolecularCellBiologyGroup.aspx>] are focused on the first of these issues, using high content, highly multiplex (including full genome) RNAi screening in the context of relevant receptor stimulation events in the cell type of interest (for example, TCR in T cells, TLR in macrophages) to help better define the membership in the pathways/networks to be simulated. This team and also that of A. Nita-Lazar [<http://www.niaid.nih.gov/LabsAndResources/labs/aboutlabs/psiim/Pages/proteomics.aspx>] is using proteomic and protein biochemical tools such as mass spectrometry and surface plasmon resonance, also in high-throughput, multiplex mode, to rapidly quantify the absolute numbers of each molecule present in a particular cell type and to be included in a model, as well as to measure *K_{as}* and *K_{ds}* for the interactions of these molecules. This group will also assess post-translational modifications that arise as a result of receptor engagement. To enhance the rate of data acquisition for binding parameters, we are exploring the possibility of using linked transcription-translation with on-chip purification [73] in microfluidic-based analytic devices, to avoid laborious expression and purification schemes better suited to individual proteins and not the dozens of components included in models of the type we are seeking to build on an ongoing basis. Likewise, we will take advantage of the latest advances in mass spectrometry-based quantification that combine computational methods [74] with isotope-labeled peptide reconstructions and reaction monitoring schemes [75], to also improve the rate and the completeness with which such data can be gathered. The PSIIM will also use advanced microscopic methods to gain insight into the spatial distribution of molecules of interest and how this distribution changes over time post-stimulation of cellular receptors, using ultrahigh resolution in vitro analysis and also intravital dynamic imaging.

Through these technologies, it will be possible to construct spatially-resolved models based much more on hard data than guesses for parameter values, markedly constraining the degrees of freedom in such simulations. This in turn should yield outputs closer to what will be observed in experimental measurements of the system, enhancing the ‘experiment - model building - simulation - prediction - experiment - model improvement - simulation - experiment’ virtuous cycle that lies at the heart of modern computational system biology efforts to advance biomedicine. The implementation of this paradigm using the new, integrated, multiplex experimental and computational tools described above should yield a refined model of TCR signaling and ligand discrimination in the near future that will markedly advance our understanding of this still intriguing aspect of immunobiology.

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Figure 1.

Colored-coded diagram of the key biochemical events in the feedback regulation of proximal TCR signaling. This cartoon illustrates the competing negative (SHP-1 mediated) and positive (ERK-mediated) feedback loops that sharpen TCR discrimination of pMHC ligands. p56Lck* = kinase active Lck. p59Lck* = kinase active Lck serine phosphorylated on position 59. ppERK is dually-phosphorylated, active ERK. pSHP-1 is tyrosine 564 phosphorylated SHP-1. The signaling starts with the interaction of peptide-MHC ligands with the TCR, which is coupled to the src-family kinase Lck in its p56 form. This leads to activation of Lck to p56 Lck* (blue arrow). The active Lck tyrosine phosphorylates SHP-1, producing pSHP-1, which in turn becomes activated within the phosphorylated environment of the active receptor, leading to dephosphorylation of key components of the signaling complex, including Lck itself, TCR ζ , and ZAP-70 (not shown). This negative feedback pathway is indicated by red arrows and dominates with poor ligands, explaining the lack of effective downstream signaling. TCR engagement can also lead to signal propagation via Ras-GRP and SOS to ERK, producing active ERK (ppERK), shown in green arrows. ppERK can phosphorylate TCR-associated p56 Lck to produce p59 Lck (green arrows). This form of Lck is resistant to recruitment of pSHP-1 (indicated by beige arrow with X). This active kinase (p59 Lck*) mediates prolonged signaling through the TCR complex that communicates with various pathways leading to gene activation (not shown). This positive feedback loop (inhibition of the negative SHP-1 feedback loop) dominates with highly effective ligands and explains their potency.

Figure 2.
Illustration of how classical Western blot analysis of signaling obscures analog vs. digital behaviors in T cell activation and of the markedly different biology that emerges in the two cases at the cell activation level.

Figure 3. Screen shot of the user interface of *Simmune2.0*. The image shows how the software keeps track of the investigator's entries of molecules and complexes (far left) and provides a dynamic graphical view of the pathway being input with precise definition of the sites of interaction for molecules with multiple interaction sites (center panel). Linked windows that are called up by highlighting specific entries in this window allow the user to input parameters for the molecules and complexes, such as numbers of molecules, kinetic constants, and so on.

Figure 4. Illustration of the conversion of confocal 3D imaging data into discretized, computable versions of the imaged cells, in this case a T cell bound in an antigen-specific manner to an antigen-presenting B cell tumor. The image on the left shows the wire-frame version of discretized space; the rendered version on the right shows the cells in a manner closer to the original confocal image. Red = T cell; green = B cell tumor.