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Authors
Zikherman, Julie
Au-Yeung, Byron

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"The role of T cell receptor signaling thresholds in guiding T cell fate decisions"

Julie Zikherman and **Byron Au-Yeung**

Rosalind Russell and Ephraim P. Engleman Arthritis Research Center, Division of Rheumatology, Department of Medicine, University of California, San Francisco, San Francisco, California, 94143, USA

Abstract

Canonical T cell receptor signal transduction has been extensively studied and dissected in cell lines and primary lymphocytes. However, a static depiction of this signaling cascade fails to capture the complex and dynamic process by which individual T cells discriminate TCR:peptide-MHC affinity, then integrate signals over time to drive discrete cellular behaviors such as thymic selection, proliferation, and cytokine production. Recent technological advances have made it possible to study complex lymphocyte behavior on a single cell level and are revealing how T cells interpret information about affinity and abundance of antigen in order to make life-and-death cell fate decisions individually and collectively.

Introduction

The earliest biochemical events detectable upon T cell receptor (TCR) triggering, such as tyrosine phosphorylation and calcium entry, occur on the order of seconds, yet sustained signaling lasting hours to days is required for critical responses such as thymic positive and negative selection, cytokine production and proliferation. Inherent in this dichotomy is a requirement that T cells not only interpret the quality and quantity of antigenic stimulation, but also its duration. Furthermore, an integrated signal must cross various thresholds to trigger biologically relevant events. How do T cells interpret such inputs at the level of an individual TCR, cell, or population?

Resolution of agonist and non-agonist peptide affinity

Kinetic proof-reading and related models, with varying degrees of experimental support, provide a conceptual framework for understanding how an individual TCR can distinguish between agonist and non-agonist peptides. This process must encompass strategies for distinguishing affinity, triggering the TCR, and incorporating both sensitivity and specificity

To whom correspondence should be addressed: julie.zikherman@ucsf.edu; byron.auyeung@ucsf.edu Permanent address: University of California, San Francisco 513 Parnassus Ave. Box 0795 San Francisco, CA 94143 Tel: (415) 476-1689.

of antigen detection. Mechanisms proposed to support this process include the most TCR-proximal signaling events such as recruitment of Lck via co-receptor[1], TCR-associated ITAM phosphorylation, Zap70 kinase recruitment and activation, and the complex interactions and feedback surrounding these components. This topic is extensively discussed in several recent reviews[2-5]. But how does a T cell distinguish between agonist peptides of similar affinity to produce widely divergent biological outcomes once signaling has been triggered?

Analog to digital signaling transformations

Classical studies of T cell responses *in vitro* and *in vivo* have demonstrated that on a population level, ‘stronger’, ‘more’, or ‘longer’ TCR stimulation generally gives a more robust output, such as secreted effector cytokine or proliferation[6-8]. Indeed, on a population level, such inputs and outputs appear on the whole to have a continuous relationship that conforms to the shape of a typical sigmoidal dose-response curve. However, careful studies of single T cell behavior reveals qualitatively different biology ‘under the surface’ that is characterized by much more switch-like digital responses that impose apparently sharp thresholds.

One of the most well appreciated examples of such digital all-or-none responses occurs at the border between positive and negative selection of double positive thymocytes. Palmer and colleagues elegantly demonstrated using fetal thymic organ culture (FTOC) of OT1 TCR transgenic thymi and a series of OVA-derived peptides, that the so-called ‘potency’ of positively and negative selecting peptides (as read-out by CD69 upregulation) correlated with selection[9]. However, it did so in an apparently digital rather than graded (analog) manner such that a relatively subtle change in ligand potency drove a dramatic and abrupt change in T cell fate from positive to negative selection. Interestingly, this work also illustrates the difference between peptide affinity and peptide dose such that most positively selecting and negatively selecting peptides drove only positive or negative selection irrespective of dose, while only a single ‘borderline’ peptide could straddle this abyss at the level of dose titration. This result suggests that individual T cells have evolved strategies to distinguish between peptides of very similar affinities, and moreover, that this imperative may trump the need to distinguish dose of stimulus at the level of a single cell. Certainly, the need to enforce central tolerance with great ‘fidelity’ may provide a clear impetus to evolve such a strategy.

Palmer and colleagues went on to explore the mechanism by which peptides with very ‘similar’ affinities could induce such distinct biological outcomes as positive and negative selection[9]. They found that extremely proximal signaling events such as CD3ζ-chain phosphorylation tracked with peptide affinity (Figure 1a), but further downstream signaling nodes (Lat phosphorylation, calcium entry, and most particularly membrane localization of the Ras signaling pathway) correlated more closely with cell fate, suggesting one or more ‘ultrasensitive’ signaling steps between these proximal and distal signal nodes, in which a small change in input leads to a dramatic change in output[10]. Since it has been shown that the TCR-associated ζ chain exhibits basal phosphorylation in a range of settings, Zap70 activation may serve to mark the difference between basal and active TCR signaling;

revisiting this proximal signaling node in the context of variant peptides will be illuminating[3,11-13].

Roose, Chakaraborty, and colleagues identified a clear example of such ultrasensitive signaling around allosteric activation of the Ras-guanine nucleotide exchange factor (GEF) Sos[14]. An 'analog' trickle of Ras-GTP (generated by the Ras-GEF Ras-Grp) is capable of binding and further activating Sos, thereby driving robust signal amplification, resulting in 'digital' Erk phosphorylation in lymphocytes (Figure 1b). Such a positive feedback loop could serve to generate an apparently 'sharp' boundary between positively and negatively selecting peptides and represents one of several strategies that cells employ for imposing signaling thresholds[10,14-16]. A potential pitfall of steep dose-response curves is the risk of oscillating around the threshold as soon as an input signal falls slightly, which is avoided by incorporating bistability and hysteresis into the signaling pathway[10,14,15]. This has also been postulated as a strategy to incorporate 'memory' into the signaling pathway, thereby permitting accumulation of signaling over time. More recent optogenetic studies of the Ras-Erk signaling module in the NIH-3T3 cell line have begun to uncover how duration and frequency of signal input are transmitted through this signaling node. This approach has revealed that signals of varying duration are faithfully transmitted from Sos to Erk, but short signals lasting < 4 minutes are suppressed or 'filtered' out, effectively preventing transmission of 'noise' rather than true signal[17].

Single cell digital behavior

In recent work, we used an inhibitor of Zap70 kinase activity to titrate TCR signal strength during thymic positive and negative selection in fetal thymic organ culture and in thymic slices[18]. In these studies, decreasing Zap70 activity (by increasing inhibitor concentration) resulted in a corresponding decrease in the number of thymocytes completing positive or negative selection in a graded, continuous manner. To visualize the 'amount' of TCR signaling integrated by positively selected thymocytes, we utilized a Nur77-eGFP reporter transgene[19]. Unexpectedly, the Nur77-eGFP reporter reveals that individual positively selected thymocytes express an invariant amount of eGFP irrespective of inhibitor dose. This result suggests the existence of a threshold for positive selection that selects for a minimum cumulative amount of TCR signaling experienced by thymocytes. Thus, titrating Zap70 activity alters the number of thymocytes that can cross this invariant threshold, but does not alter the threshold itself. The contrast between all-or-none responses on the single cell level that imposes invariant sharp thresholds, and continuous or graded dose-responses at the population level can be accounted for by postulating some degree of signaling heterogeneity at the level of a T cell population[15]. Optogenetic methods recently revealed that signal output generated by identical inputs was faithfully reproducible for individual cells, but varied from cell to cell for even a short and simple pathway (Ras-Erk) across stably transfected NIH-3T3 clones[17].

In a related report, we have demonstrated that an analogous threshold for TCR signaling is enforced at the level of T cell proliferation[20]. We show that on a population level *in vitro* T cell proliferation scales continuously with titration of anti-CD3ε stimulation or Zap70 inhibition (i.e. cumulative signal strength). However, individual Nur77-eGFP reporter T

cells that have divided express a uniformly high level of GFP independent of stimulus or inhibitor dose, suggesting that they have integrated an invariant amount of TCR signaling in order to commit to cell division. Moreover, this dose-independent 'threshold' appears to operate *in vivo* in the context of either immunization or infection. Furthermore, we demonstrated that T cells also exhibit a minimal required duration of TCR signaling to proliferate, beyond which they are insensitive to any further Zap70 inhibition and continue to divide. We interpret these results to mean that crossing an invariant TCR signaling threshold is not only necessary, but also sufficient to drive T cell proliferation. Furthermore, altering 'strength' of stimulation affects the number of cells recruited into the response, but does not change the threshold itself as indicated by Nur77-eGFP reporter expression. What is the purpose of such a signaling threshold? An obvious answer is to limit spurious responses. Conversely, a minimal duration of TCR signaling, beyond which further signaling is not required, may ensure that T cells are able to divide, differentiate and generate a complete memory response to infection even if antigen is rapidly cleared.

Davis and colleagues have gone further to show that the threshold for certain T cell responses is remarkably low[21]. They demonstrate that even a single agonist peptide-MHC II is sufficient to drive cytokine production by both naïve and effector CD4 T cells. Increasing the dose of peptide recruits more cells, but does not drive more cytokine release per cell. This type of behavior enables high sensitivity and dramatic digital behavior at the level of single cells (which do not distinguish dose at all), but apparently analog or graded responses to dose titration at the population level. Similarly, digital responses at the single cell level have been observed in a range of other cell systems [10,22,23].

Mechanisms of downstream signal integration and establishment of sharp thresholds

How are relatively proximal signaling events, such as Erk phosphorylation, which are detectable within minutes of TCR triggering, integrated over time to drive cellular decisions that occur on the order of hours or days later? Signal integration requires pathway intermediates with some degree of stability. One example is NFAT nuclear translocation, which has been postulated to provide cellular 'memory' of recent signaling that is cumulatively 'encoded' by NFAT-dependent gene transcription (Figure 1c,d) [24]. The transcriptional regulator Irf4 is another example; Irf4 is expressed within 8 hours after initiation of TCR signaling and its abundance scales continuously in accordance with peptide affinity[25]. Irf4 in turn drives expression of a critical set of genes involved in altered T cell metabolism, linking peptide affinity to clonal expansion[25-28]. A range of immediate early genes that accumulate in accordance with signal intensity and duration may serve analogous functions downstream of TCR signaling. Indeed, the Nur77-eGFP reporter is an example of such a signal integrator because of the relatively long half-life of eGFP protein relative to rapid reporter induction in response to TCR stimulation.

How then are signaling thresholds enforced in response to such integrated signals? We must posit another, more downstream, analog-to-digital transformation (Figure 1e). Irf4 protein expression varies continuously with peptide affinity, but Irf4-driven gene expression is discontinuous and requires high Irf4 expression, thereby imposing a *minimal* signaling threshold to drive transcription[25]. In contrast to relatively long half-life 'integrators' of

TCR signaling, an extremely unstable intermediate such as c-Myc with a short half-life could also enforce a minimal threshold such that signaling which is too short or too infrequent (i.e. noise rather than signal) would fail to drive complex downstream biological outcomes[29,30].

Modulators of affinity thresholds

Ultrasensitive signaling steps can transform graded inputs into switch-like digital output at the level of a single cell, but how are distinctions between non-agonist and agonist, or low and high affinity peptides established and modulated in the first place? Recently, Gascoigne and colleagues took advantage of the OT-1 peptide series to determine how Themis deficiency transforms positively selecting peptides into negatively selecting peptides (reviewed in detail elsewhere in this issue: Gascoigne et al.)[31]. They find that this occurs extremely proximally through release of SHP1 inhibition, and results in a cascade of signaling events that recapitulates the quantitative and kinetic characteristics of negatively selecting thymocytes as revealed by Palmer and colleagues[9]. Recent work from the Zamoyska lab also elegantly demonstrated that inhibitory phosphatases may help T cells set this proximal ‘threshold’ between peptides of subtly varying affinity [32]. The authors show that T cells deficient for the inhibitory phosphatase PTPN22, which were previously found to exhibit a fairly subtle shift in dose-response to potent anti-CD3ε ligation, are able to respond to very weak ‘subthreshold’ peptides. Collectively, these insightful studies suggest that inhibitory tone of TCR wiring (perhaps imposed as proximally as Lck kinase activity) may have evolved to permit T cells to resolve fine peptide affinity differences in order to enforce a threshold between benign and pathogenic peptides.

Digital to analog transformations

The cost of imposing sharp thresholds via switch-like digital signaling is lost input information (i.e. a dose-response curve with a very steep slope does not distinguish well among doses). Individual T cells can have difficulty distinguishing antigen dose and signal strength across a broad range precisely because they have specialized to generate digital all-or-none responses for the purpose of distinguishing antigens of similar affinity. Electrical circuits address this problem by introducing ‘dither’ or noise into digital to analog converters, thereby improving accuracy. Recent work nicely demonstrates that digital behavior at the single cell level can be transformed into analog behavior at the population level as ‘more’ or ‘longer’ stimulation recruits additional cells to cross an invariant ‘activation’ threshold into an immune response (either positive selection, cytokine production, or proliferation)[18,20,21]. The basis for population-level ‘dither’ or noise may be variation in receptor triggering as well as variation in expression of signaling proteins[10,15]. As a result, T cell responses to stimuli at the population level generally have a graded, analog appearance (Figure 1f) that conforms to a conventional dose response curve with a flatter slope (Hill coefficient closer to 1) and thus more accurately detect dose. Thus, certain biological responses to varying dose of antigen are detected by an entire population of T cells, rather than an individual T cell. This may prove particularly relevant in clonal T cell responses to infection.

Yet population behavior isn't always continuous, and needs to take into account a diverse T cell receptor repertoire. Chakraborty and colleagues suggest that digital behavior can be observed at the level of a population of T cells and may be imposed by IL-2 secretion such that a minimal 'quorum' of responding T cells is needed to promote a productive group response[33]. The authors postulate that such a strategy may have evolved to prevent rare autoreactive T cell clones from driving an inappropriate autoimmune response, while ensuring that once a 'threshold' number of responding pathogen-specific T cells are activated, a collective immune response can be triggered. Decision-making by T cells can thus occur at the level of the TCR (kinetic proof-reading), the individual T cell (invariant threshold sensing), and by a 'quorum' of T cells, providing an inter-locking series of safety nets to ensure sensitive and specific immune responses that are tunable at the level of proximal signaling apparatus.

B cell affinity discrimination

Like T cells, B cells also need to distinguish between varying affinity antigens. Perhaps the most unique function of B cells is their capacity to affinity mature their antigen receptors via somatic hypermutation in the germinal center (GC) in order to generate lasting and potent humoral immunity. To do so, B cell clones bearing very high affinity receptors must compete with one another for survival, and highest affinity clones must be selected. Some of the strategies evolved for solving this problem may be shared across lymphocytes. Shlomchik and colleagues reported recently that proximal B cell receptor (BCR) signal transduction in GC B cells is significantly dampened by inhibitory SHP1 and SHIP1 phosphatase activity[34]. Analogous to Themis in T cells, this may help resolve subtle affinity differences between very high-affinity somatically hypermutated BCRs. It will be important to determine whether single cell behavior, thresholds, and population dynamics in B cells resemble T cells or not, having evolved under different constraints (e.g. T cells must distinguish between similar low affinity peptides; GC reaction must identify the highest affinity clone).

Conclusion

Information processing capacities of single cells have been previously described. Recent work is beginning to relate these properties to the unique biological constraints imposed by antigen receptors that recognize and distinguish among a vast range of different ligands with varying affinities. Future studies will capitalize on technological advances to permit single cell as well as fine quantitative and temporal resolution of signaling in lymphocytes. We speculate that revisiting established signaling components and transcriptional regulators of T and B cells with these approaches in hand will unmask new biology.

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Highlights

- accumulated TCR signaling must reach thresholds for T cell responses
- positive feedback loops can transform graded inputs into switch-like digital output
- sharp signaling thresholds are enforced by switch-like digital responses
- resolution of peptide affinity is imposed at the expense of dose sensitivity
- population heterogeneity transforms digital single cell behavior into graded output

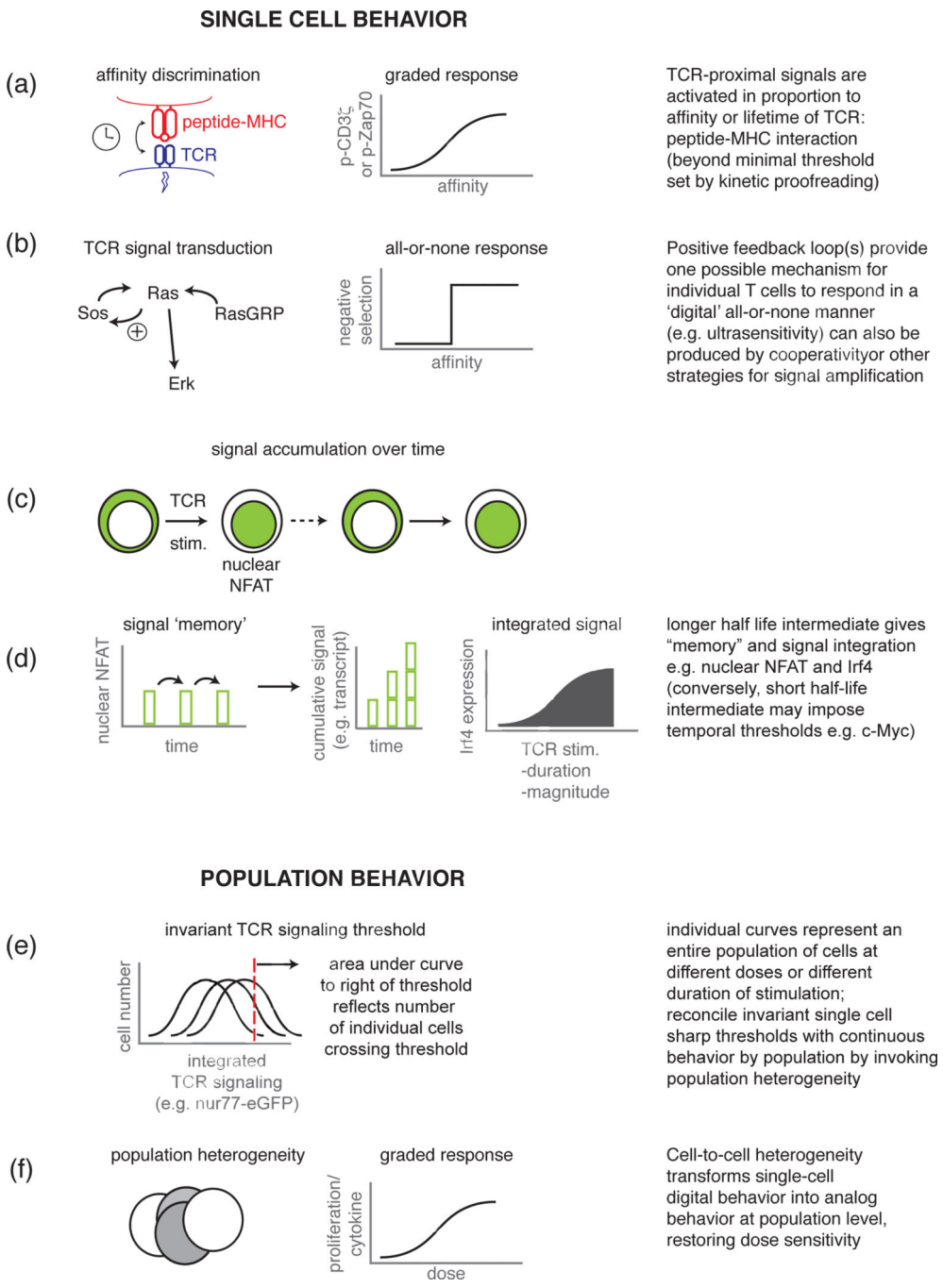

Figure 1.

A 'grand unifying theory' of T cell activation. (**a**) T cells are able to discriminate peptide-MHC affinity with high sensitivity. Dwell times and affinity of TCR:peptide-MHC interactions correlate with the magnitude of TCR proximal signal transduction events, such as phosphorylation of the CD3ζ chain or phosphorylation of the TCR proximal kinase Zap70 (left). The relationship between affinity and proximal signal intensity is graded and resembles a sigmoidal dose response curve (right). (**b**) Some signal transduction nodes downstream of the TCR, such as the Ras-Erk Map kinase pathway, incorporate positive

feedback loops through the activation of Sos (left). This is one way of transforming the continuous behavior of TCR proximal signaling in response to affinity titration, into switch-like or all-or-none downstream signal transduction (right). (**c,d**) Signaling through the TCR must be integrated over time to induce transcriptional and cellular responses. Translocation of NFAT transcription factor in the nucleus and subsequent activity on gene transcription is one example of how TCR signals can transmit area under the curve, or integrated, signaling information. A second example is Irf4 transcription factor expression, which scales in proportion to TCR:peptide-MHC affinity. (**e**) There is an invariant threshold of integrated signaling that must be crossed to induce cellular responses, such as proliferation. This invariant threshold was revealed using a reporter of integrated signaling, Nur77-eGFP. (**f**) T cells exhibit heterogeneity with respect to their integrated TCR signaling, resulting in a broad distribution at the population level. This heterogeneity, in combination with enforcement of an invariant threshold, transforms switch-like behavior at the single cell level and enables graded dose responses at the population level.