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T cell responses to antigen: hasty proposals resolved through long engagements

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Abstract

T cells discriminate between peptide-MHC complexes on the surfaces of antigen presenting cells to enact appropriate downstream responses. Great progress has been made over the last 15 years in understanding varied aspects of T cell activation on short timescales (minutes), yet the mechanics and significance of long term T cell receptor signaling (hours or days) remain unclear. Furthermore, there remain some controversies regarding the correlation of the biophysical parameters of ligand-receptor interactions with the scaling of downstream effector functions.

Here we review recent studies that emphasize the importance of long-term engagement of antigens to fine-tuning the activation of T cells over the duration of the complete immune response. We discuss how T cells dynamically regulate T cell receptor signaling via antigen crosstalk, competition and consumption to accurately counter antigenic challenges.

Short-timescale controversies: what are the biophysical characteristics of antigenic ligands?

The exquisitely specific response of T cells to peptide-MHC (pMHC) antigens can be measured on very short timescales [1–4]. Indeed, signaling assays monitoring Ca^{2+} influx [5], T cell receptor (TCR) phosphorylation or ERK phosphorylation [3] in lymphocytes have demonstrated specificity and sensitivity within minutes of antigen engagement. On slightly longer timescales (30min to 3hrs), T cells reorganize their membranes to form immunological synapses with their antigenic targets [6], and are capable of effector functions such as cytotoxicity [1] and the upregulation of varied receptors (CD69, CD25). The observation that single point mutations in an antigenic peptide can trigger widely divergent activation patterns has been confirmed for all clones under consideration. Moreover, these altered associations have been quantified by surface plasmon resonance (3D-SPR) of soluble ligand/receptor pairs [7]. To summarize 15 years of biophysical characterization, stronger bonds correlate with greater signaling responses, and minute differences in parameters such as the lifetime of pMHC-TCR complexes map onto large changes in the functional potency of antigens [7,8].

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Recent measurements have challenged this “lifetime dogma”. Using a well-established cell-based adhesion assay to monitor the formation of complexes between T cell receptors and varied altered ligands, Zhu and colleagues [9,10] reported that single point mutations in the antigenic peptide impact large changes in the thermodynamics of pMHC-TCR interaction (up to 3000 fold changes in the equilibrium constant of pMHC-TCR binding, which would translate into differences of 8 k_BT in free energy released during pMHC-TCR bond formation). These surprisingly sizable differences could certainly account for the specificity and sensitivity of T cell activation. However, Zhu’s group paradoxically found that weaker bonds between pMHCs and TCRs, as measured in their assay, correlated with stronger functional T cell activation.

In contrast, results from [11] that use laminar flow chambers to monitor TCR-driven adhesion on MHC-coated surfaces are inconsistent with the cell adhesion results and more in line with the 3D-SPR conclusions. However, the different experimental settings (here, purified MHCs and TCRs loaded onto beads) could explain this discrepancy. More challenging are studies by the Davis group [12], which rely on a FRET system between fluorescently-labeled peptide and TCR to monitor the dynamics of pMHC-TCR bonds on the surfaces of live cells. Like the Zhu studies, these measurements characterized bond formation within whole membrane settings, and attributed faster association and dissociation rates for pMHC-TCR complex formation than 3D SPR measurements. Nevertheless, Davis and colleagues affirmed the canonical direct correlation between TCR ligand affinity and antigen potency in triggering effector functions.

Further work will be necessary to resolve this conundrum of pMHC-TCR interactions at the biophysical level. Yet no matter how agonist and self ligands initially engage T cell receptors on individual cells, many physiological factors and timescales are convolved in the mapping of immediate TCR signals to the regulation of the adaptive immune response at the systems level. We conjecture in this review that such physiological, long-timescale parameters might in fact reconcile the above stated discrepancies among biophysical measurements.

Antigens trigger a rapid, digital, and noisy signaling response

Immune responses spearheaded by T cells scale to the size of immune challenge, *i.e.* the quantity or quality of immunizing antigenic peptides or number of pathogens [13,14]. Paradoxically, many characteristics of the T cell signaling have been documented as essentially all-or-none [3,5,15]. This sharp initial response may be functionally essential, as T cells scanning the surface of inflamed antigen presenting cells (APCs) must rapidly commit to activation or move on. By deciding quickly, a T cell increases the probability of cognate antigen encounters—both between its own TCR and a high affinity antigen, and between the pMHC it has passed over and a different T cell clone. Digital signaling on short timescales might therefore be critical to ensure efficient engagement of a few antigen-specific cells from a large polyclonal population.

Theoretical analyses indicate that digital decisions are generally promoted through positive feedback regulation in signaling [3,15,16]. However, this elegant mechanism of digital cellular decision-making carries high functional risk, as positive feedback loops are notoriously “noisy” [17,18]. If T cell activation relied solely on these sharp, early signals, spurious activation by self antigens, reinforced by positive feedback loops, could trigger large-scale autoimmune disorders. Furthermore, purely digital decisions would constrain the dynamic range of T cell effector outputs for different TCR signaling inputs to mere variation in the proportion of activated cells (Figure 1A). However, empirical observations of large scalability in T cell responses show that this is not the case [14,19,20](Tkach et al.,

submitted). Although every proximal signaling event within the TCR cascade that has been measured with single-cell resolution has been found to be digital [3], analog outputs may be achieved further downstream [21]. Hence, additional timescales and layers of regulation are necessary to translate the rapid, digital and noisy signals of individual T cells into self-restricted, fine-tuned immune responses.

Building an analog T cell response: the importance of sustained antigen engagement

Studies probing TCR discrimination of pMHC complexes on APCs have correlated the success of early events such as the phosphorylation of ERK or initiation of cytokine secretion to T cells' ultimate magnitude of proliferation, differentiation and recall [14]. However, it is unclear how decisions made only minutes after antigen exposure are translated into differential outcomes throughout several days of immune response. Furthermore, in addition to titrating the percentage of activated naïve precursors, TCR signaling potency regulates the degree of activation within individual T cells [13,20,22] (Tkach et al., submitted). To examine how the digital processes following antigen encounter are converted into analog scaling of long-term T cell responses, we must consider an important tunable parameter of TCR signaling: signal duration.

Initial studies probing the role of TCR signal duration demonstrated that sustained TCR signaling was required to initiate effector function [23], and that earlier disruption of TCR-pMHC interactions yielded greater impairment of cytokine secretion [24]. A subsequent study indicated that T cells' downstream functions were activated in a hierarchical fashion, with lower antigen signaling thresholds for the initiation of IFN γ production than for the synthesis of IL-2 [25]. These results suggested that an initial burst of TCR signaling is insufficient to endow T cells with complete effector functions.

Investigators then sought to characterize the TCR signal duration requirements of CD4 and CD8 T cells by probing the consequences of signal withdrawal. Early studies in CD4 T cells reported that naïve cells need 20 hours of TCR signaling to commit to proliferation, with costimulatory signals shortening the necessary duration of TCR stimulation [26,27]. However, studies in CD8 T cells using an engineered antigen presentation system suggested that cytotoxic lymphocytes (CTLs) gained full effector function after only two hours of TCR signaling [28]. With advances in molecular visualization techniques, the effects of curtailed TCR signaling duration could be observed directly. Antibody blockade of TCR interaction with its pMHC ligand caused rapid extinction of PI3 kinase localization at the TCR synapse, and resulted in lesser cytokine production and proliferation on a 48 hour timescale [29].

Others have further dissected the role of sustained TCR signaling by controlling antigen persistence *in vivo*. A study that regulated antigen expression via a tetracycline-controlled promoter showed that persistent antigen is required to sustain the proliferation of CD4 T cells [30]. Another study titrated CD4 T cell signaling duration by synchronizing the start and end of antigen presentation via injection of peptide and an MHC-blocking antibody, respectively; the time between initiation and termination was varied to create different signaling duration periods. These experiments determined that CD4 T cells needed a minimum antigen exposure of 6 hours for functional activation, with longer periods of signaling yielding more robust proliferative and effector responses [31]. Similarly, diphtheria toxin-mediated depletion of APCs has demonstrated that titrating the duration of antigen availability scales the magnitude of CD8 T cell proliferation [32]. Therefore, while a short signaling period might be sufficient to generate some degree of functional response, the magnitude and the quality of T cell activation is not set on "autopilot" in the first hours of

signaling. In fact, CTLs also benefit from increased periods of antigen exposure through the delivery of effective CD4 help [33].

Recently, studies using intravital two-photon microscopy have characterized the kinetics of T cell-APC interactions *in vivo*. Several experiments visualized three distinct phases of T cell motility during activation [31,34]: a few hours of transient T cell contact with APCs are followed by a phase of stable T-DC interactions that can persist for up to 48 hours [22] and concludes with T cells remobilizing and proliferating. These studies found that increasing the strength of antigenic stimulation shortens the initial meandering phase [34,35] and that greater antigen availability extends the duration of the stable contacts [22,31]. Antibody blockade of the p-MHC ligand was sufficient to disrupt T-DC conjugates *in vivo* [31], suggesting that the termination of stable contacts is coupled to the loss of antigen.

Multiple studies have indicated that T cells integrate these discontinuous antigen contacts over time, and respond in proportion to the cumulative duration of TCR signaling [34,36,37]. Visualization of TCR dynamics at the cell surface has shown that despite receptor internalization following antigen engagement, TCRs are only depleted four-fold from the T cell surface, and therefore maintain continuous potential for antigen signaling [38]. Furthermore, the positive feedback loops that promote digital activation also enable memory of previous signals, a phenomenon known as hysteresis. Through hysteresis, T cells remain in a sensitive state for an extended period following antigen withdrawal [15], allowing the summation of sequential discontinuous signals [3,15,16]. Hysteresis can also be supported by the immediate upregulation of gene products that promote TCR signaling, such as c-Fos [39]. Thus, the integration of multiple TCR signals over time transforms serial digital events into an analog output that is capable of scaling with the quantity or quality of antigen (Figure 1B).

Long-term signaling can therefore resolve the strength of antigenic input better than all-or-none reactions on a short timescale. Indeed, experiments that tracked the fates of individual barcoded T cell *in vivo* revealed that the number of T cells that underwent digital activation was practically saturated across a one hundred-fold change in pathogen dose. However, a large dynamic range of response arose from the scaling of proliferation, which depended on the continued presence of antigen [13].

Several features of prolonged antigen engagement could underlie such an expanded resolution of antigen dose. Persistent TCR signals could allow for the enactment of slower cellular programs, such as epigenetic changes and the transcription of genes with latent kinetics [40]. In fact, certain gene products can accumulate non-linearly throughout the signaling period via amplifying feedback loops, creating wider dynamic ranges of response (Figure 1C) (Tkach et al., submitted). Furthermore, the persistence of antigen sustains TCR cross-talk with other pathways that can further modulate T cell fate. For example, several studies have demonstrated TCR-mediated inhibition of IL-2 signaling through pSTAT5 [41,42] (Tkach et al., submitted). Sustainance of this cross-talk has been implicated in the regulation of IL-2 scaling through a coherent feed-forward loop (Tkach et al., submitted), and of helper T cell subtype differentiation via modulation of transcription factor networks [42,43]. Time integration of such non-linear and cross-pathway signals extends each cell's response to antigenic potency beyond its initial phosphorylation events.

T cell population dynamics modulate TCR signal duration

The size of the antigen-specific T cell population is a significant variable in the progression of the long-term immune response. Many studies have established a negative correlation between a high clonal frequency of antigen-responsive T cells and the per cell degree of

CD4 and CD8 proliferation [20,22,44–46], effector function [45–48], and survival [49,50]. Clonal population size has also been implicated in shaping memory differentiation [51–53].

Some evidence of non-antigenic sources of interclonal competition [54] and cooperation [55] have been observed. However, many studies suggest that intraclonal competition for antigen drives the functional limitations of large clonal populations. This conclusion has been experimentally supported through the alleviation of competition by antigen replenishment [22,44], the exacerbation of scarcity effects through antigen blocking [50], and the lack of competition between clones of different antigen specificities [20,44]. Visualizing the physical dynamics of T cell populations on dendritic cells (DCs) *in vivo*, Garcia and colleagues have demonstrated that competition for available antigen, and not physical access to dendritic cells, limits the duration of stable contacts with DCs in the presence of large numbers of sister clones [22].

Given the significant variability of naïve T cell precursor frequencies [49], it has been proposed that intraclonal competition serves to normalize the magnitude of response for population size, allowing collective T cell function to instead scale with the strength of antigenic stimulation [44]. By shortening the TCR signaling period for individual T cells in a clonal population [22], competition for antigen curtails the integration of signal, and the resulting degree of activation per cell. However, these more limited individual responses can sum to generate similar overall quantities of proliferated effectors [44] and accumulated cytokine molecules (Tkach et al., submitted) as smaller populations that benefit from longer TCR signaling intervals. These studies provide important considerations for the design of adoptive immunotherapy protocols, as the number of antigen-specific T cells transplanted into a tumor-bearing host can affect the kinetics of activation and effector potency for individual cells, resulting in different disease outcomes [46].

Antigen consumption through trogocytosis: a key regulatory mechanism to enforce ligand discrimination?

The T cell-mediated consumption of antigen, or antigen trogocytosis, has been characterized both molecularly and functionally. Visualization experiments have shown that T cells can acquire pMHCs from the surfaces of APCs, ripping them off through receptor internalization [56,57], then redisplaying them on their cell surfaces [58] or on their internal organelles [59].

Both positive and negative effects of antigen trogocytosis on long-term TCR signaling have been reported [60]. On one hand, internalization of antigens coupled to their receptors was shown to extend the duration of signaling responses through the trogocytosing TCR [59]. On the other hand, trogocytosis by high-affinity clones enforces competition for antigen, which prevents low-affinity T cell clones from maintaining long-term signaling and drives immunodominance in the T cell repertoire [61]. Similarly, other work has characterized antigen trogocytosis and subsequent presentation on the surface of the endocytosing T cell as a mechanism to deny other T cells access to these antigens on the surfaces of professional APCs; indeed, antigen activation through T-T contact was shown to be suboptimal and tolerance-inducing [58]. These studies demonstrate the role of antigen trogocytosis in shaping the clonal selection and differentiation fate of T cells by creating additional levels of regulation that influence antigen responses on a long timescale.

This persistent engagement between TCR and pMHC might be relevant to the discrimination of minute molecular differences in antigens, not only in the context of a cellular response, but also in biophysical experiments. As discussed above, there remains a discrepancy between the hierarchy of affinities obtained by adhesion assay [9] versus SPR in

vitro measurements [7] and in situ FRET measurements [12]. We propose that due to pMHC resampling and possible trogocytosis of antigen, adhesion assays may essentially reproduce the biophysics of TCR engagement over long timescales. The limiting step for re-adhesion might then be the depletion of pMHC ligands from the presentation surface by the probing T cells, particularly in the case of high affinity antigens [62], which could result in an inverted cell-adhesion hierarchy. Future biophysical experiments that prevent or quantify antigen trogocytosis by using covalently-linked pMHC, signaling blockage, or in situ imaging of pMHC-TCR interactions will be needed to test this hypothesis. Probing the lifetime of pMHCs on APCs may resolve these paradoxical measurements and highlight the relevance of antigen resampling and long-term engagement in establishing the discriminatory power of T cells.

Conclusion

The translation of short-term TCR engagement and T cell signaling into appropriately scaled, long-term immune responses opens many opportunities for systemic regulation. Response duration against, competition for, and consumption of antigens can normalize individual T cell signaling such that a population of T cells collectively scales its activation to the size of antigenic challenge. Such integration appears to be critical to overcome the noise and limited dynamic range of early TCR signaling responses. Future work will need to resolve how these integrative mechanisms contribute quantitatively to decision making in the immune system. The pay-offs will lie in the rational design of better antigen dosing and timing protocols to manipulate immune responses in clinical settings.

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Highlights

- Digital T cell activation exhibits a saturated dynamic range on short timescales.
- Persistent antigen engagement fine-tunes response through time integration of signals.
- Antigen-mediated cross-talk and feedbacks extend the dynamic range of T cell outputs.
- Competition for antigen limits TCR signal duration and normalizes for population size.
- Antigen consumption (trogocytosis) regulates T cell signaling over long timescales.

Figure 1.

Long-term engagement of antigens extends the dynamic range of the short-term digital activation of T cells. A. Short-term readouts of T cell activation (ERK phosphorylation, Ca^{2+} burst, upregulation of CD69) often display a bimodal distribution that is characteristic of all-or-none (digital) responses to antigens. Such distributions can be analyzed by measuring the fraction of cells that underwent activation. B. Time dynamics of T cell response encodes the antigen dose through varied activation frequencies and signal durations. C. Integration of regulatory loops downstream of antigen engagement over long timescales can extend the shallow dynamic range of short-term antigen responses. Here we present the example of IL-2 accumulation, which is amplified through positive feedback loops: this long-term regulation results in power law scaling with the dose of stimulating antigen.

Figure 2.
Examples of regulatory loops invoked during the long-term engagement of antigens.