Revie

Staggered starts in the race to T cell activation

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systems is inherently limited by the ability to find an altered peptide ligand (APL) that exhibits

Box 1. Optogenetics

Optogenetic approaches take advantage of naturally light-responsive proteins to create synthetic systems that can be controlled with light of specific wavelengths. Optogenetics have been used to interrogate the organization of signaling networks across many biological fields [98]. The past few years have seen a rapid uptake of this technology for manipulating T cell signaling. For example, optogenetic manipulation of T cell calcium signaling in a spatially controlled manner was recently achieved using a light-controlled STIM-1 construct that aggregates in response to two-photon stimulation [99]. Optogenetic approaches have been particularly informative for addressing questions of how the kinetics of receptor–ligand binding impact T cell activation responses. Studies using optogenetic receptors in the Jurkat T cell line and stimulating with cell-free ligands have been used to test the relationship between receptor–ligand binding kinetics and T cell activation [25,26] (see main text). Other studies have introduced cellular antigen-presenting systems opposite light-responsive CARs to examine the impact of signal frequency and duration [23,24]. By necessity, these systems use synthetic receptors, which may show differences from native TCR–pMHC interactions, and this needs to be considered when interpreting results. However, the development of these methods marks an important new era in the study of T cell stimulation strength, making it possible to precisely manipulate binding patterns under culture conditions that are otherwise identical. As such, the use of optogenetic systems has the potential to precisely define what we mean by stimulation strength.

naïve murine $CD8^+$ T cells [29,35]. By contrast, co-stimulatory receptor engagement rescued cytokine expression in primary human $CD8^+$ T cells under chronic in vitro stimulation [36]. Much remains to be understood about how co-stimulatory and cytokine signals integrate into T cell activation signaling to control the effective stimulation strength that a T cell experiences. One highly studied example is the cytokine IL-2, which is expressed in a stimulation strength-dependent manner by both $CD4^+$ and $CD8^+$ T cells and results in both autocrine and paracrine signaling through IL-2R [34,37,38]. Experiments in naïve murine $CD8^+$ T cells demonstrated that adding exogenous IL-2 could rescue translation and proliferation deficiencies seen in cells stimulated with low-dose or low-affinity ligands [31,32]. This is likely achieved by promoting the expression of the transcription factor MYC, which requires ongoing protein synthesis due to rapid turnover and controls division potential [31,32,39,40]. Future work examining the integration of other signals can shed light on the regulatory logic of intracellular T cell signaling.

A plethora of studies have demonstrated that reducing stimulation strength during activation of naïve or effector $CD4^+$ or $CD8^+$ T cells leads to a reduction in activation phenotypes, including signaling protein phosphorylation, calcium fluxes, transcription factor activation, mRNA expression, protein expression, proliferation, cytokine secretion, and cytolytic activity, as exemplified by [41–50]. The strength of T cell stimulation can also dramatically impact thymic selection, which falls outside the scope of this review [51].

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Historically, RNA and protein expression measurements were made on bulk cellular lysates, and functional tests used pools of T cells. These types of measurements describe the average behavior of a population but cannot discern how individual cells are affected. Thus, a reduction in average cellular activation in a given condition might be due to a change in the magnitude of activation within each cell or to a change in the proportion of cells that are activated. Single cell measurements are able to overcome this issue and provide more accurate insights into how individual cell responses combine to achieve a population response.

One of the original single cell methods, flow cytometry, enables quantitative read-outs of protein expression or modification in individual cells using fluorescently tagged antibodies, constructs, or dyes. This approach has revealed that some markers of activation exhibit simple ‘on/off’ behavior, such that the proportion of ‘on’ cells changes with stimulation strength. This type of response, termed ‘digital’, is exemplified in primary murine T cell activation by the phosphorylation of extracellular signal-related kinase (ERK) [52,53] and protein kinase D2 (PKD2) [54]. Other markers of

activation show a graded response, such that increasing stimulation strength shifts the marker intensity within each individual cell. IRF4 expression is the best characterized of these 'analog' responses, with extensive studies in murine $CD8^+$ T cells [55–58]. Recently, a hybrid digital/analog model has been used to describe certain activation markers that exhibit both "on/off" behavior and graded modulation of intensity within the 'on' population (e.g., expression of CD69 in $CD4^+$ T cells [59] and MYC in $CD8^+$ T cells [39,

strength on activation phenotypes, because they can lead to different interpretations of how the underlying intracellular machinery reads TCR signals.

Advances in single cell-based studies of T cell activation

The advent of high-dimensional single cell technologies, including single cell RNA-sequencing (scRNA-seq) and mass cytometry (Box 2), as well as advances in live cell imaging (Box 3), have facilitated more comprehensive profiling of T cell activation to uncover the dynamics of individual cell responses (Figure 2).

In naïve T cells, one of the primary outcomes of TCR stimulation is the induction of gene expression. A recent scRNA-seq study examined the transcriptional changes downstream of in vitro naïve $CD8^+$ T cell activation [3] using the OTI TCR-transgenic mouse system [12], in which all T cells are specific for an ovalbumin peptide and for which APLs of varied affinities have been

which cells initiate a shared activation program. Due to the rapidity and transience of proximal signaling events, this latter study focused on TCR-distal signaling nodes, honing in on the coordination of ERK, S6, and STAT5 phosphorylation [61]. Thus, future work examining the simultaneous activation of TCR-proximal signaling mediators will be important to understand the initiation of this shared downstream signaling program.

Similar conservation of T cell activation processes was observed in flow cytometry experiments

Key figure

Model of a rate-based mechanism of T cell activation

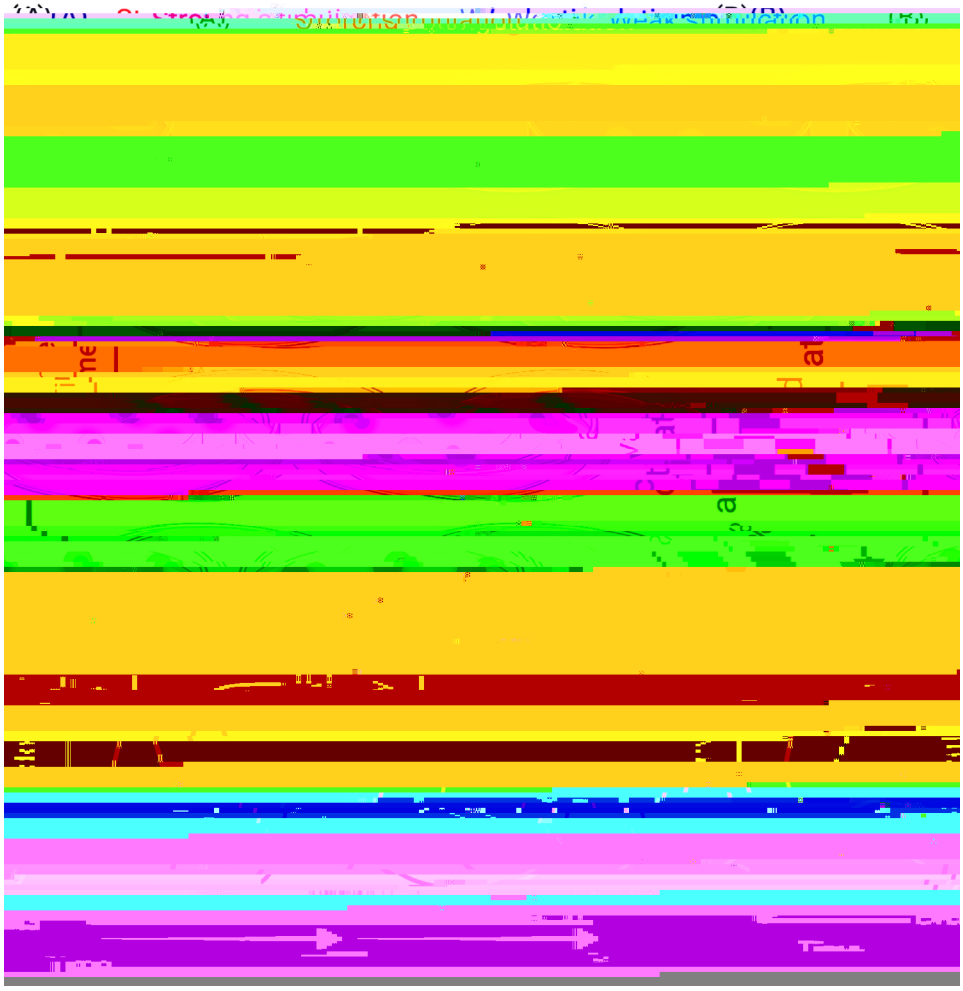

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Figure 3. (A) Schematic of a theoretical T cell activation series in which stimulation strength controls the rate of activation events, showing populations responding to Strong (red) or Weak (blue) stimulation. (B) Bar chart representation of how the percentage of activated cells at each timepoint might look comparing Strong (red, left) versus Weak (blue, right) stimulation. (C) Density plots of T cell activation events with Strong (red, left) versus Weak (blue, right) stimulation, simulated according to the rate-based model. (D) Cumulative distribution curves for simulated data from (C). Figure created using BioRender (BioRender.com).

Additional evidence of stimulus-dependent tuning beyond a shared activation program comes from studies of TCR-induced gene expression changes. In the scRNA-seq study described above [3], after accounting for the activation status of each cell, a small number of genes remained differentially expressed at the mRNA level between cells stimulated by strong and weaker ligands. Likewise, observations of hybrid digital/analog expression of induced proteins support the idea of tuning beyond a shared response, such that a common program initiates expression in a digital manner and subsequent stimulus-dependent effects tune this expression in

an analog manner within each cell (e.g., [40,59]). Moreover, extensive work using APLs to stimulate naïve $CD8^+$ T cells in multiple murine TCR transgenic systems showed that, starting from approximately 1 day after activation, T cells express IRF4 in a graded manner, reflecting stimulation strength [55–58]. As IRF4 can enhance effector differentiation [57,58], this suggests that subtle tuning of gene expression during the early days of naïve T cell activation might alter differentiation outcomes, as has been observed in in vivo models for both $CD4^+$ and $CD8^+$ T cells [47,67–72]. Similarly, experiments stimulating murine $CD4^+$ T cells with varying antigen doses found that, after 24 h, stimulation strength correlated with the expression of IL-12Rβ2, which facilitates Th1 polarization in response to IL-12 signaling [72]. These results again suggest a means by which stimulation strength can impact differentiation fate. Together, these data indicate that, although shared activation programs may exist, the strength of T cell stimulation can further tune resulting activated T cell phenotypes.

This raises an important question: if the rate-based model for activation is accurate, how are responses tuned beyond a core activation program according to stimulus? One potential explanation is that cells continue to receive stimulation beyond an initial activation event. An elegant optogenetic study tested the impact of sustained signaling on T cell activation responses [23]. Using an optogenetic chimeric antigen receptor (optoCAR), in which light induced the dissociation of the intracellular signaling moiety from the receptor–ligand complex and its subsequent inactivation, the authors quantified the persistence of TCR-induced signals including calcium flux, ERK and FOS phosphorylation, and gene transcription, in the human Jurkat T cell line. Results showed that, upon proximal signaling disruption, downstream activation events rapidly dissi-

which corresponded to TCR–pMHC affinity. Moreover, measurements of nuclear translocation of the transcription factor NFAT (as an activation marker) revealed that successful activation was associated with either a single long dwell time, or sequential, short, spatially correlated binding events. In this way, all activated cells received the same total input, regardless of ligand affinity. The model put forth by this study is that activation events occur in a probabilistic manner, taking place when sufficiently long dwell times (real or effective) are stochastically achieved. This interpretation provides an intriguing mechanism that might explain how ligand affinity as well as concentration can alter the rate of cellular activation.

Theoretically, converting TCR–pMHC binding dwell times into a highly discriminatory activation switch requires a thresholding mechanism. One of the most popular models for this is kinetic proofreading, which posits that signaling steps introduce a delay between ligand binding and subsequent activation cascades, such that weak interactions often dissociate before responses are triggered [75–77]. Two recent optogenetic studies explicitly tested the concept of kinetic proofreading in T cells, using light to alter the binding half-lives of synthetic ligand–receptor pairs in an otherwise uniform environment [25,26]. One study used a LOVTRAP system in which a CAR expressed on Jurkat cells was bound in a light-controlled manner to LOV2, presented on a supported lipid bilayer [25

Downstream of ZAP70, the LAT signalosome assembles, recruiting and activating multiple signaling intermediates, including phospholipase C gamma 1 (PLCγ1), which cleaves phos-

of T cell responses. Such a mechanism is intellectually appealing because it enables a range of T cell responses at the population level without requiring infinitely diverse responses from each individual cell. A probabilistic model for initiating a molecular program has been proposed in the context of in vivo T cell differentiation, in which individual cells exhibit extensive heterogeneity in their progress along a shared differee

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