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TCR SIGNALING

ZAP70 holds the key to kinetic proofreading for TCR ligand discrimination

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Full activation of the kinase ZAP70 downstream of T cell antigen receptor stimulation is necessary to enable T cells to discriminate between antigens of high and lower affinities and only respond to high affinity antigens.

T cells use their T cell antigen receptor (TCR) to recognize and bind to low affinity self-peptides derived from host proteins and to high affinity foreign peptides derived from pathogens, both of which are presented by major histocompatibility complex molecules (pMHC). However, only foreign peptides that bind with high affinity elicit a strong immune response. How the TCR is able to discriminate between antigens over a wide range of different affinities, yet respond with exquisite sensitivity to just a few cognate pMHC molecules has been a matter of considerable investigation[1]. In this issue of *Nature Immunology*, Voisinne et al.[2] explore how the TCR is able to discriminate between ligands of different affinities from a biochemical perspective. In an elegant study using in-depth time-resolved analyses of the total TCR-stimulated phosphoproteome and protein-protein interactions in $CD8^+$ T cells, the authors identify full activation of the TCR kinase ZAP70 as a key discriminating step in defining whether T cells will respond to a peptide ligand or not.

The main hypothesis used to explain the exquisite discriminatory power of the TCR is the kinetic proofreading model first described in 1995[3]. This model postulates that a certain number of rapidly reversible biochemical steps take place after TCR binding to pMHC before an essentially irreversible step is triggered that results in full commitment to T cell activation. The time taken to reach this irreversible decision is known as the proofreading time delay, and is a measure of how long the pMHC remains bound to the TCR (dwell time), which depends on the half-life of the pMHC-TCR interaction. Multiple experimental studies give credence to the idea that the dwell time of the ligand on the TCR is the main factor driving TCR decision making, in support of kinetic proofreading[4–7]. However, the exact signalling steps that allow small differences in binding duration to translate to a digital response are not currently known.

Voisinne et al.[2] used recently activated primary murine $CD8^+$ T cells expressing the well-characterized OT-I transgenic TCR to identify the earliest molecular events that evoke a digital response. Both cognate and altered peptide ligands with decreasing affinities for the OT-I TCR are known. These peptides elicit different magnitudes of T cell responses, even when similar numbers of TCRs are bound, making it an ideal system to study TCR ligand discrimination. Using peptides of different affinities to stimulate the TCR while controlling for equal receptor occupancy, the authors evaluated the differences in the phosphoproteome during the first few minutes of TCR activation. Phosphosites could be broadly grouped into those that were unaffected, those that showed a graded response and those that exhibited a digital response to the different peptide ligands. Interestingly, phosphorylation of CD3 chains of the TCR and recruitment of ZAP70 were unaffected by the differences in ligand affinities (Fig. 1). By defining those phosphosites that were largely

discriminatory for TCR ligand affinity, and investigating the kinases that might phosphorylate these sites, ZAP70 was identified as the main kinase driving discriminatory phosphorylation. Importantly, differences in signalling between strong antigen and weak ligands was not reflected by changes in very early signalling events, but rather by changes in later events focussed around ZAP70 activation and phosphorylation of the signaling adaptor LAT.

Full activation of ZAP70 is a multistep process requiring its phosphorylation on multiple sites[8]. Accordingly, recruitment of ZAP70 to the CD3 chains of the TCR is required, but not sufficient to activate the kinase. Phosphorylation of ZAP70 by the Src kinase Lck on interdomain tyrosines followed by phosphorylation of tyrosine (Tyr) 492 in the activation loop completely blocks the autoinhibition of ZAP70, allowing full activation of the kinase[8]. Voisinne et al.[2] found that phosphorylation of Tyr492 in the activation loop of ZAP70 only occurred when a high affinity peptide was used to stimulate the T cells (Fig. 1a). Phosphorylation of this site is thought to be preferentially induced by ZAP70 trans-autophosphorylation, thus requiring an additional step of recruitment of a second ZAP70 molecule to the TCR for full activation of ZAP70 (Fig. 1a). This is completely consistent with the predictions of the kinetic proofreading model whereby a certain number of molecular steps need to occur for the TCR to discriminate between ligands of different affinities. Of note, the additional step of full activation of ZAP70 through trans-phosphorylation fits well with the recent prediction of 2-3 steps needed for proofreading based on theoretical calculations[6] and experimental predictions[4,5]. In accordance with reports that the slow kinetics of phosphorylation of Tyr132 on LAT by ZAP70 also contributes to proofreading[7],

partially phosphorylated ZAP70 may not be sufficiently active to phosphorylate this key LAT tyrosine residue that recruits the phospholipase PLCγ1.

Assembly of multi-protein signalosomes on LAT and transmembrane receptor CD6 occur rapidly downstream of TCR activation[9]. To define the protein-protein interactions at the TCR induced by different affinity ligands, the authors go on to use a well-established pipeline for affinity purification mass spec (AP-MS) of one-strep-tag (OST) tagged signalling proteins that they had previously employed in $CD4^+$ T cells[10,11]. By measuring protein-protein interactions at the TCR, they find that binding of ZAP70 to the TCR occurs at similar levels regardless of the different peptide affinities. On the other hand, the association of ZAP70 to CD6 and the assembly of the CD6 signalosome is gradually reduced by reducing the ligand affinities used to stimulate the TCR. Moreover, the authors show that assembly of the CD6 signalosome has a much larger effect on dampening TCR signals downstream of the TCR when low affinity ligands are used (Fig. 1b), potentially through the effects of the phosphatases UBASH3A and UBASH3B, which can dephosphorylate ZAP70. Hence, an important take-home message from this work is that CD6 may contribute to the discriminatory power of the TCR by providing local negative feedback.

The study by Voisine et al.[2] answers a long-standing question of how kinetic proofreading is executed through biochemical processes downstream of the TCR. It will also serve, along with previous reports examining TCR-induced phosphorylation and interaction networks in primary murine and human $CD4^+$ T cells[10–12], as a useful resource to understand the signalling processes that cumulatively drive T cell activation. Indeed, several other novel interactions are identified here[2] as occurring at the TCR, such as WRNIP1 and ANKRD13A,

and the role of these proteins in TCR signalling will need to be characterized. The fundamental insights into how T cells discriminate between self and non-self ligands and what optimal TCR signalling looks like, will be useful for the rational design of next generation T cell chimeric antigen receptors for optimal activation of anti-tumour responses.

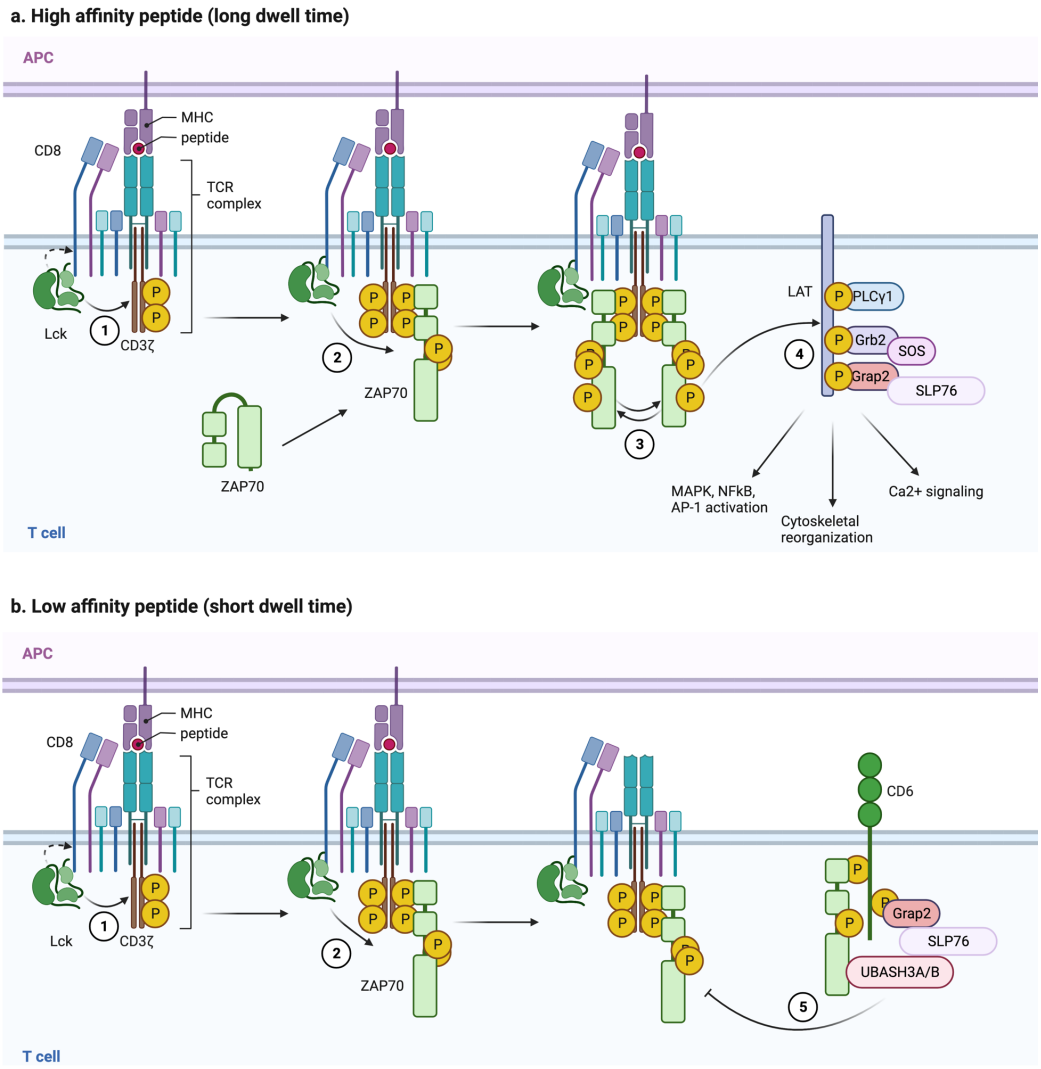

Figure 1. Kinetic proofreading for ligand discrimination by the TCR requires full activation of ZAP70. a) TCR signalling is triggered when the TCR binds to a high affinity peptide bound to MHC. Co-receptor bound or free Lck kinase phosphorylates the CD3 chains of the TCR (step 1). Autoinhibited ZAP70 kinase is recruited to phosphorylated CD3, where it is partially phosphorylated by Lck (step 2). Full activation of ZAP70 requires further

phosphorylation in trans by another ZAP70 molecule (step 3). Once activated, ZAP70 can phosphorylate the adaptor molecule LAT, permitting the assembly of the LAT-SLP76 signalosome (step 4). This signalosome then orchestrates multiple downstream signalling pathways that leads to full activation of the T cell. b) When the TCR binds to a lower affinity peptide presented by MHC, the half-life of the pMHC-TCR interaction is not long enough to permit step 3 of ZAP70 activation to take place. In addition, the CD6 signalosome, which assembles at all peptide affinities, is able to drive a negative feedback loop, potentially through dephosphorylation of ZAP70 by the UBASH3 phosphatases (step 5). The CD6 signalosome is also formed with high affinity peptide stimulation; however, once the LAT signalosome is formed, CD6 does not appear to have any effect in downregulating signalling (Created in Biorender.com).

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