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Locked and loaded: Strong TCR signalling primes anti-PD-1 therapy

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In response to continuous T cell antigen receptor (TCR) signalling, T cells can attenuate subsequent antigen response by inhabiting a state of adaptive tolerance, which averts autoimmunity, but can also provide refuge to developing cancers. Various negative feedback mechanisms of adaptation, including TCR downregulation, ubiquitin ligase targeting of signalling, and checkpoint expression, have been previously described [1,2,3]. Elliot and coworkers add to our understanding of adaptation through checkpoints by exploiting an accelerated *in vivo* model of adaptive tolerance in the face of strong TCR signaling [4]. Their work offers insight into the role of TCR activation intensity and checkpoint molecule expression in tuning T cell restimulation capacity in the span of a day with large cohorts of synchronized cells enabling analysis of gene expression. Moreover, Elliot et al prove the value of a resulting genetic signature of strong TCR signalling by demonstrating the ability of this signature to stratify melanoma patients by response to checkpoint cancer immunotherapy.

Elliot et al establish an accelerated murine model for adaptive tolerance based on the Tg4 TCR transgenic model for experimental autoimmune encephalomyelitis (EAE) [5]. Tg4 mice express a TCR specific for a peptide from the central nervous system (CNS) autoantigen myelin basic protein (MBP) presented by MHC class II allele I-A^u. Paradoxically, mice in which 90% of T cells express encephalogenic TCRs are perfectly healthy. Lafaille and colleagues discovered that imperfect allelic exclusion leads to a small repertoire of CD4⁺ T cells with diverse TCRs, including CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg) that maintain peripheral tolerance [6]. On a Rag-/- background, this small population of Tregs is eliminated and mice spontaneously develop EAE [7]. Tolerance can also be broken by immunizing Rag expressing Tg4 mice with the MBP peptide in a strong adjuvant to generate disease causing T helper 1 (Th1) cells that enter the CNS. The model applied by Elliot et al is based on administration of a more potent altered peptide ligand or a high dose of the natural MBP peptide to differentiate the transgenic T cells into Foxp3⁻ type 1 regulatory (Tr1) T cells. These Tr1 cells express exhaustion-associated transcription factors c-Maf and NFIL3, lack proliferative capacity, display inhibitory molecules PD-1, LAG-3, TIGIT, and TIM-3, and switch to producing IL-10, all within 24 hours [4]. The very high frequency of responding T cells in the Tg4 model is un-physiological, but it may account for the accelerated development of large numbers of Tr1 cells that model some aspects of T cell exhaustion in cancer settings and is helpful experimentally to generate large numbers of cells for sorting and analysis.

Elliot et al use a *Nr4a3*-Tocky reporter to identify T cell states [8]. The Tocky reporter is based on a single fluorescent protein that initially displays blue fluorescence and then transitions to highly stable red fluorescence after 4 hours. The timer protein is under control of the *Nr4a3* regulatory sequence, a nuclear receptor expressed downstream of the calcium/NFAT pathway. Elliot et al define three dynamic activation states: initial activation (Blue⁺Red⁻), sustained activation (Blue⁺Red⁺), or arrest (Blue⁻Red⁺). Three antigen doses covering two orders of magnitude were administered to Tg4 *Nr4a3*-Tocky mice to investigate the impact of MBP peptide

Locked and loaded

dose on Tg4 TCR⁺ T cells at a population level. Initial and sustained signalling increased with antigen dose across several early timepoints. Controlling for activation state, the authors identified immunosuppressive gene expression, including that of coinhibitory receptors PD-1, LAG3, TIGIT, and CTLA-4 and immunosuppressive cytokine IL-10 (using a GFP reporter), that characterized strong T cell signalling. By comparing cells across synchronized activation states from different activation conditions, the authors aimed to control for potential kinetic changes to T cell activation resulting from changes to stimulation strength [9].

Hypothesizing that a tolerant phenotype would impact the ability of individual cells to be restimulated, Elliot and colleagues then probed reactivation of CD4⁺ T cells at the single cell level using the Tg4 *Nr4a3*-Tocky model. T cells from mice initially given a high dose of antigen were arrested at high levels following a second *in vivo* administration of either low or high doses of antigen. *In vivo* administration of an initial high antigen dose followed by immune checkpoint inhibitors anti-Lag3 or anti-PD-1 and restimulation with a low antigen dose increased the level of T cells undergoing sustained activation. Genes differentially expressed in T cells undergoing sustained signalling in response to checkpoint inhibition overlapped with the strong TCR stimulation gene profile previously identified. By combining differentially expressed genes from high peptide antigen dosing and application of anti-PD-1 blockade to overcome adaptive tolerance, in addition to genes that were found to be differentially expressed in melanoma patients responding to anti-PD-1 (nivolumab) therapy, Elliot et al defined a 5-gene signature (*ICOS*, *TNIP3*, *TNFRSF4*, *IRF8*, *STAT4*) of strong TCR stimulation (TCR.strong), which they hypothesized would predict a T cell exhaustion phenotype underlying response to immune checkpoint blockade. This strong TCR stimulation signature was found to be enriched in an additional melanoma cohort responding to nivolumab, and application of this metric split patients by clinical response.

The *in vivo* system used by Elliot et al manipulated the potency and dose of TCR ligands, but didn't isolate the TCR signalling pathways. Along these lines, CD28 is required to induce tolerance in CD4⁺ and CD8⁺ T cells [10,11]. High resolution *in vitro* data also suggests that the TCR alone undergoes perfect adaptation and the nuanced regulation of adaptation relies on costimulation [1]. Steady state dendritic cells (DC) that are likely to be involved in the presentation of injected peptides express intermediate levels of MHC class II, CD86, CD58, and ICAM-1 that will amplify TCR dependent signals through engagement of TCR, CD28, CD2 and LFA-1 receptors, respectively, in the immunological synapse. The early production of IFN- γ and CD40L during activation of Tg4 T cells by strong or high dose MBP peptide-MHC complexes will also likely upregulate PD-L1 to provide an opportunity for counter-regulation by PD-1 that is upregulated on T cells to contribute to adaptation by 24 hours. TCR, CD28, CD2 and PD-1 engagement are co-organized in CD2 dependent immunological synapse compartments [12]. To illustrate how TCR and costimulatory signals are integrated, we examined Ca²⁺ flux, which is the major driver for the *Nr4a3* regulatory element. In human CD4⁺ T cells, we found that PD-1 engagement cancelled the CD28 contribution, but not the CD2 contribution, to TCR triggered Ca²⁺ flux (Fig. 1a,b). Elliot et al find that CD28 agonist antibodies do not enhance T cell restimulation as assessed by expression of *Nr4a3* in their model, which may suggest that the pathway is fully cancelled by PD-1 in this setting [13]. This analysis underlies the need for a comprehensive exploration of crosstalk between and amongst costimulatory and coinhibitory receptors to fine-tune T cell responses to antigens over a range of potency and doses to establish or break tolerance as required in different disease settings.

Locked and loaded

The Elliot et al TCR.strong signature is powerful in being able to track T cell responses to immunotherapy. This is remarkable given the seed data was collected in an acute *in vivo* experiment. A future goal will be to predict patients who are poised to respond – locked and loaded but with the checkpoint safety still on. Further refinement of this signature may depend on aspects of chronicity that cannot be fully recapitulated in a rapid *in vivo* assay. Elliot et al have taken an important first step in merging the power of modern molecular methodology with a classical model of adaptive tolerance.

Fig 1- Costimulatory contributions to TCR signals- In vitro analysis of T cell Ca^{2+} flux based on Demetriou et al [12]. A. CD28 contributions to Ca^{2+} flux is cancelled by PD1-PDL2. Inset- schematic of some receptor-ligand interactions that enable and amplify TCR-pMHC interactions and signals. B. CD2 contribution to Ca^{2+} flux is partly inhibited or partly intact in the presence of PD1-PDL2 interaction. Inset- CD2 corolla (Green, CD58, Red, anti-CD3, Blue, ICAM-1- all in supported lipid bilayers, see [12])

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Locked and loaded

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