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T-cell antigen recognition: catch-as-catch-can or catch-22?

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T-cell antigen recognition is invariably affected by tensile forces as they are exerted on T-cell antigen receptors (TCRs) transiently binding antigenic peptide/MHC complexes. In this issue of The EMBO Journal, Pettmann and colleagues promote the concept that forces reduce the lifetime of more stable stimulatory TCR-pMHC interactions to a larger extent than that of less stable non-stimulatory TCR-pMHC interactions. The authors argue that forces impede rather than boost T-cell antigen discrimination, which is promoted by force-shielding within the immunological synapse through cell adhesion via CD2/CD58 and LFA-1/ICAM-1.

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See also: **J Pettmann *et al* (2022)**

T-cells are central to adaptive immunity as they provide protection against pathogens and malignancies in an antigen-dependent manner while keeping healthy tissue unharmed. Integral for their function is their capacity to discriminate antigens from self-determinants by means of primary protein structure. For this T-cells express genetically recombined clonotypic T-cell antigen receptors (TCRs) which they employ to scan antigen presenting cells (APCs) for antigenic fragments. The latter are continually processed and then presented by APCs within the context of MHC molecules in an almost linear fashion (Fig 1A). Of note, TCRs bind nominal peptide-loaded MHC complexes (pMHCs) only with micromolar affinities (Matsui *et al*, 1991), which are three to four orders of magnitude lower than those featured by most antibody–antigen interactions. Remarkably, T-cells can nonetheless sense the presence of even a single antigenic pMHC among thousands of structurally similar endogenous bystander pMHCs that do not stimulate T-cells (Irvine *et al*, 2002). How they achieve this is far from being understood even four decades after the cloning of the TCR. What is known is that this degree of antigen sensitivity relies in most cases on the simultaneous interaction of the CD8- or CD4-coreceptor with a non-polymorphic region within the MHC class I of class II molecule, respectively. Yet evidence collected from diverse experimental avenues has rather given rise to divergent conclusions than a unified vision (Platzer & Huppa, 2023).

T-cell antigen recognition takes place within the confines of the immunological synapse, the bi-membranous T-cell-APC interface. Here TCRs and pMHCs are pre-oriented on opposing surfaces with consequences for the frequency of molecular interactions. Importantly and ignored for three of the last four decades, ligand-engaged TCRs are also subjected to mechanical tensile forces as a direct result of cellular motility and cytoskeletal rearrangements.

How are TCR-pMHC interactions affected by force? Most protein–protein interactions are best described as so-termed slip bonds, which become destabilized by imposed mechanical force (Thomas, 2008). When TCR-pMHC interactions were visualized via FRET-based live cell microscopy, they showed clear signs of slip bonds as they appeared cut short in duration in a fashion that involved actin polymerization (Huppa *et al*, 2010). This observation called into question the standard model of kinetic proofreading in T-cell activation, a widely held view that the lifetime of extracellular TCR-pMHC bonding is positively correlated with ensuing intracellular TCR-proximal signaling. Mechanical force could also act as a noise filter particularly if lower affinity interactions between TCRs and bystander pMHCs were more vulnerable to exerted force than nominal TCR-antigen interactions (Klotzsch & Schütz, 2013; Schütz & Huppa, 2019).

To approach this problem in an analytical fashion, George Bell provided a simple mathematical model which describes how bond lifetimes could be affected by tensile force within the confines of two opposing membranes (Bell, 1978). For this he resorted to Boltzmann statistics and introduced the force sensitivity parameter X_β to account for forces as they are imposed on receptor-ligand pairs:

$$k_{off}{}^{m(F)} = k_{off} \cdot exp\left(X_\beta F / k_B T\right)$$

with $k_{off}{}^{m(F)}$ and k_{off} describing the membrane off-rate under force and the off-rate measured *in vitro* under zero force, respectively, the applied force (F), the Boltzmann constant (k_B) and absolute temperature T *(in K)*.

According to Bell's model, slip bonds feature a positive X_β. But how does X_β change over a spectrum of affinities between a TCR and variants of pMHCs? In case X_β increases with an increasing k_{off} – that is, bonds become increasingly slippier – forces would enhance discrimination. If – contrary to intuition – X_β decreases with increasing k_{off},

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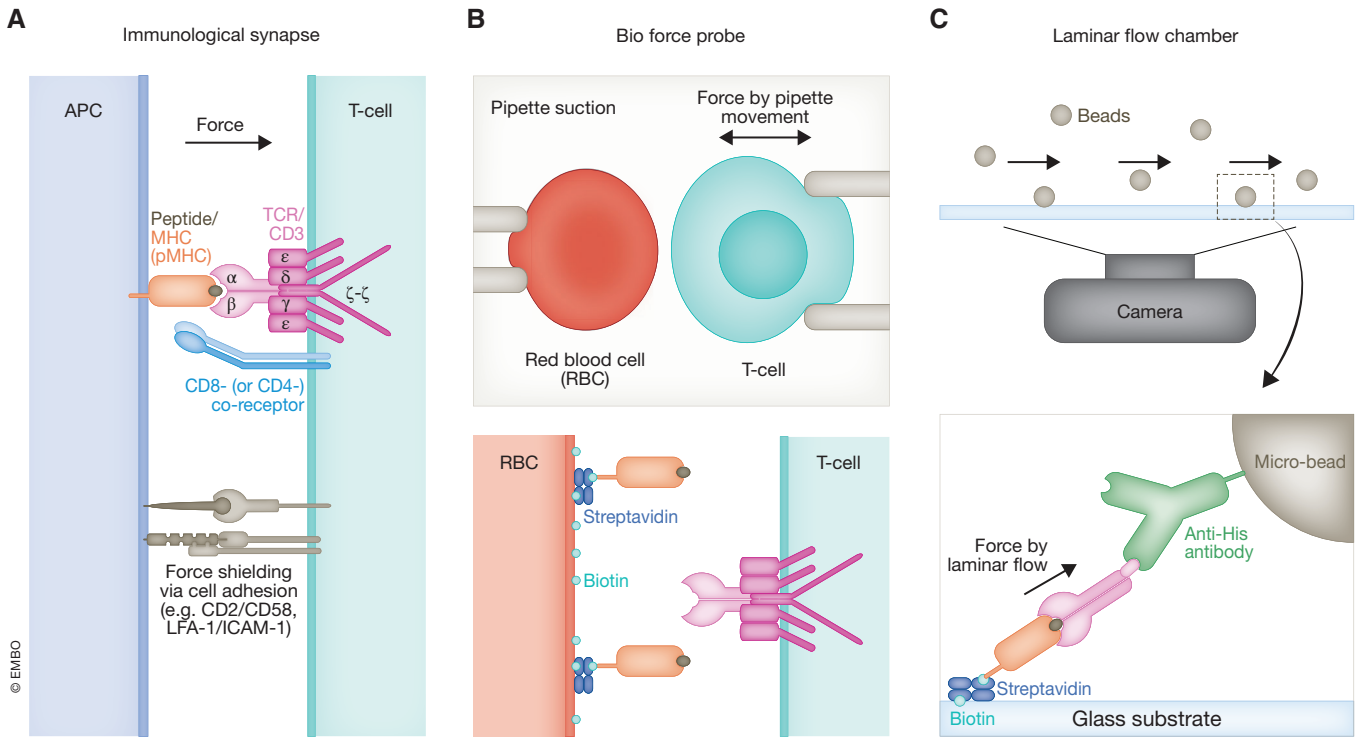

Figure 1. Mechanical forces acting on antigen-engaged TCRs within the immunological synapses and experimental approaches to study how they affect T-cell antigen recognition.

A Left panel: T-cell antigen recognition is driven by T-cell antigen receptors binding with low to moderate affinity to antigenic peptide fragments as they are displayed by APCs in the context of the products of the MHC (peptide/MHC, pMHC). The clonotypic αβTCR is non-covalently associated with the invariant signaling chains of the CD3 complex, namely CD3γ, δ, ε, and the disulfide-linked ζ2 heterodimer to form the TCR/CD3 complex, a *sine qua non* for proper TCR-folding in the ER and surface expression. Depending on the T-cell lineage involved, the CD8- or CD4-coreceptor binds to a non-polymorphic region within the MHC class I or class II, respectively. T-cell recognition is tied to the formation of an immunological synapse between the T-cell and the APC, which is reinforced by cell adhesion. The latter is likely to shield both TCR and pMHC from mechanical forces as result from cell motility and cytoskeletal dynamics.

B Middle panel: Micrograph of the micropipette and bioforce probe (BFP) measurements. A T-cell (right) is aspirated by a pipette and aligned with a red blood cell (RBC) held stationary by another pipette (left). This experimental setup is employed to measure binding events as visualized by the deformation of the BFP in adhesion frequency assays. The sensitivity of these systems is reported to reach down to single TCR-pMHC bonds. Lower panel: Schematic diagram illustrating the BFP-based assay.

C Right panel: Schematic representation of the laminar flow chamber (LFC)-based methodology to measure the duration of TCR-pMHC interactions under force. Microbeads (diameter ~ 4.5 microns) which have been functionalized via antibody-coating with recombinant TCRs are floated in a laminar stream over a streptavidin-glass substrate featuring biotinylated pMHC as is indicated. Forces imposed on the TCR-pMHC bond can be precisely adjusted by the laminar flow and the size of the microbeads. Upon TCR-pMHC binding, the corresponding bead arrests and is released after bond dissociation. Such events are recorded by a camera to determine TCR-pMHC off-rates under force.

antigen discrimination would invariably suffer. Does X_β always stay positive? A bimolecular bond characterized by negative X_β would in fact increase in lifetime under force, a scenario which has been observed for cell adhesive bonds between P-selectin and its ligand PSGL-1 (Marshall *et al*, 2003). Such bonds have in turn been termed catch-bonds, and extensive research has revolved around the molecular mechanisms that underlie their existence (Thomas, 2008). And beginning in 2014, several studies put forward the concept that TCR interactions with nominal antigen are best described as catch-bonds, while weak TCR interactions with non-stimulatory pMHCs behave as slip bonds (Liu *et al*, 2014; Hong *et al*, 2018; Sibener *et al*, 2018). It was suggested that "catchiness" correlates with the stimulatory potency of TCR-pMHC interactions. "Catchiness" was furthermore described to be promoted by simultaneous CD8-coreceptor engagement (Hong *et al*, 2018) with a non-polymorphic region within the MHC class I heavy chain, which has been implicated in sensitizing T-cells for antigen.

Hence, understanding and quantitating TCR-pMHC slip- or catch bond behavior within the immunological synapse has been at the forefront of molecular immunology for the last decade (Göhring *et al*, 2022) yet carrying out and interpreting experiments are by no means trivial. To this date, the concept of TCR-pMHC catch bonds relies mainly on the interpretation of a bioforce probe (BFP)-dependent mechanical assay (Liu *et al*, 2014; Fig 1B). This experimental approach involves contacting pipette-attached T-cells with and retracting them from pMHC-functionalized probes. Reportedly even low-affinity TCR-pMHC interactions are registered at the single molecule level by the slight adhesion between the cell and the BFP, which can be monitored via standard life cell microscopy. When placed under experimentally defined strain, typically at a range of 10–15 piconewton, TCR-interactions with antigenic pMHCs appeared

to increase in lifetime. A limitation of these studies is that active signaling by the T-cells employed followed by or coinciding with nanoscopic changes in TCR-surface distribution and cytoskeletal rearrangements could increase the quality and number of TCR-pMHC bonds made prior to BFP-retraction thus complicating the data analysis.

A different study observed TCR-pMHC catch bond behavior with the use of an optical tweezer setup (Feng *et al*, 2017) while others, for example, the first FRET-based quantitation of synaptic TCR-pMHC interactions, failed to obtain evidence for catch bonds (Huppa *et al*, 2010). Moreover, the spectrum of forces acting on individual pMHC-engaged TCRs within the immunological synapse may require adjustment to values lower than 10 piconewton, that is, below the range of forces reported to promote "catchiness." The use of a FRET-based real-time analog single molecule force sensor provided evidence for TCR-imposed forces of maximally six piconewton at loading rates of approximately 1.5 piconewton per second. As a consequence, forces in the range of 15 piconewtons would need bond lifetimes of 10 s or longer. The majority of stimulatory TCR-pMHC interactions, however, falls short to survive 5 s. Of note, such force was only observable when TCRs were allowed to engage immobilized ligands. However, the large majority of pMHC are laterally mobile on the APC surface. When T-cells faced laterally mobile TCR-ligands, measured TCR-exerted forces gave only rise to two piconewton or less (Göhring *et al*, 2021).

Confronted with such incongruent findings, how can we arrive at a comprehensive perspective? A simple answer may entail breaking things further down into individual parts, for example, by measuring TCR-pMHC interactions under a defined force regiment with the use of an independent and more direct approach. To set it apart from previous experimentation and to avoid potential secondary effects as brought about by cellular behavior or cell-associated complexities, such approach would ideally be reductionist, conducted under cell-free conditions and allow for direct control over the choice and density of binding partners as well as the forces applied.

This is exactly what Omer Dushek, Philippe Robert, Anton van der Merwe and co-workers sought to achieve in their present study (Pettmann *et al*, 2022). The authors functionalized beads with TCRs of choice and had them migrate within a laminar flow chamber apparatus (LFC) over surface-conjugated pMHC which served as interaction partners (Fig 1C). By adjusting the densities of TCR and pMHC on beads and on surfaces, respectively, they managed to tweak the system towards the formation of single TCR-pMHC bonds between beads and surface, which they monitored by following the arrest of the TCR-functionalized beads. Force regiments acting on TCR-pMHC interactions could be precisely dialed in by means of applied bead sizes and flow rates. Recording the duration of bead arrests enabled the authors to reveal TCR-pMHC interaction lifetimes as a function of ligand quality and applied force.

The outcome of the study is indeed revealing and in some aspects startling: unlike previous studies using BFP-confronted living T-cells, the results of the LFC-based assays indicate that with increasing stability TCR-pMHC interactions show increasing slip bond behavior, at least when stripped down to their essential elements. Surprisingly, the exceptionally short-lived OT1 TCR- K^b/OVA interaction (solution half-life at 37°C ~ 0.2 s) showed longer lifetimes under force, demonstrating that the LFC-based system is well capable of revealing catch bond behavior and that catch bonds between the TCR and its nominal ligand do not rely on T-cell associated membrane dynamics. Most strikingly and counter-intuitively, lower affinity TCR-pMHC interactions with higher solution off-rates appeared to be less affected by external force than more stable TCR-pMHC interactions. If true, external force, at least when applied immediately without a force ramp as done in the LFC-system, would not promote but impede T-cell antigen discrimination.

To further extend their LFC-based observations, the authors conducted structure-based coarse-grained molecular dynamics simulations, in which they applied forces at constant pulling velocity to determine the force at which TCR and pMHC dissociate. They then varied in their simulations the pulling velocity and the degree to which the antigen was allowed to bind the TCR and, as they had done with their experimental wet-lab data, analyzed the simulations using Bell's model to finally arrive – again – at a negative rather than a positive correlation between solution k_{off} and X_β.

But how would such trend affect T-cell antigen discrimination and sensitivity? To assess the consequences, the authors applied the standard kinetic proofreading model of T-cell activation (McKeithan, 1995) in which they replaced solution off-rates with corresponding membrane off-rates under force. In good agreement with previously published T-cell activation data (Pettmann *et al*, 2021), both antigen sensitivity and discrimination appeared to suffer from applied force while benefitting from force-shielding via CD2/CD58- and LFA-1/ICAM-1-mediated cell adhesion. Of note, a previous study based on atomic force microscopy also arrived at a negative correlation between solution k_{off} and X_β for antibody–antigen interactions, suggesting molecular forces may also impact antigen discrimination by B-cells (Spillane & Tolar, 2016).

Collectively, the new study by Pettman and colleagues places forces exerted on pMHC-ligated TCRs in a new light: more likely than helping the process of antigen recognition they need to be dealt with and contained to allow for precise and sensitive antigen scanning. Cell-adhesion, as brought about by the constitutively active CD2/CD58 as well as the activation-enforced LFA-1/ICAM-1 adhesion axis, fulfills the criteria to absorb many of the cellular forces and helps pushing both sensitivity and discriminatory capacity of T-cell antigen recognition to their limits, which are ultimately set by the transient nature of TCR-pMHC interactions and the sophisticated membrane-proximal signaling machinery.

As is most often the case with new studies, the work by Pettmann *et al* leaves open a number of both technical and conceptual questions for further analysis. The first that comes to mind concerns the precision in measuring low affinity interactions down to the single molecule level in a noisy setting shaped by non-specific interactions. Clearly, conducting experiments in a cell-free system is highly advantageous as it allows for control without losing sight of effects that may affect the readout. In this sense, the LFC-based assay offers a complementary alternative to BFP-based assays which – especially after repetitive testing – ignores the state of the T-cell and the nanoscopic changes as they may occur on the T-cell surface and the underlying cytoskeleton over time while conducting the experiment. The flow-based assay is free of such cell-related concerns, and hence the most direct path to higher precision is repetition, that is, to collect as

much data as possible to reduce the effect of noise in the final analysis. It will be interesting to see how future work may improve noise levels and deal with some inconsistencies in the data landscape, which cannot be fully ignored as they clearly impact the fits made when applying Bell's model.

Another aspect concerns the fact that TCR-pMHC interactions are stripped down to the absolute necessary, i.e. the extracellular portions of the interaction partners, which is perceived as both a strength and a weakness of the study. When interpreting the findings, we need to consider that both the αβTCR and pMHC have been taken out of the cellular context in which such interactions typically occur. After all, the extent to which (i) the association of the clonotypic αβTCR heterodimer with the invariant signaling chains of the CD3 complex and (ii) the TCR's embedding within the plasma membrane alter binding behavior remains to be determined. Of note, the cytoplasmic CD3 tails harbor, unlike the αβTCR heterodimer, the signaling domains which interact with the membrane proximal signaling machinery and the cytoskeleton. Co-expression of all CD3 subunits is in fact a *sine qua non* for TCR-surface expression. Conceivably, the quaternary of the TCR/CD3 complex may affect the tertiary structure and orientation of the αβTCR (and vice versa), and as a consequence the TCR-pMHC binding behavior, especially when placed under force. Binding may also change as a result of previous interactions, which, if productive, initiate major rearrangements of surface molecules on the T-cell and may alter binding conditions with outcomes for binding lifetimes and on-rates. At least some of the differences observed between this work and previous studies using BFPs could be explained by the lack of CD3-association and the dynamic membrane context provided by living T-cells. As suggested by the authors, conducting BFP-based measurements using non-T-cells (e.g., fibroblasts or epithelial cells) which ectopically express the TCR-CD3 complex and optionally also the CD8- or CD4-co-receptor, may be helpful for unraveling the contribution of active T-cell signaling and co-receptor-binding to what has so far been interpreted as TCR-pMHC catch bonding.

Catch-as-catch-can, catch-22 or something in between? The journey towards a comprehensive understanding of T-cell antigen recognition is far from over. More often than not we learn especially from differences in observations, which places the emphasis on the strengths and limitations of the methodologies applied. We are well-advised to remain humbly open for more surprises, as more work will continue to shape our understanding of T-cell antigen recognition in ways we cannot foresee.

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