Mechanoimmunology: molecular-scale forces govern immune cell functions

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ABSTRACT **Immune cell recognition of antigens is a pivotal process in initiating immune responses against injury, pathogens, and cancers. Breakthroughs over the past decade support a major role for mechanical forces in immune responses, laying the foundation for the emerging field of mechanoimmunology. In this *Perspective*, we discuss the mechanical forces acting at the level of ligand–receptor interactions and how they underpin receptor triggering, signal initiation, and immune cell activation. We also highlight the novel biophysical tools and advanced imaging techniques that have afforded us the recent progress in our understanding of the role of forces in immune cell functions.**

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AN IMMUNE CELL'S JOURNEY THROUGH A MECHANICAL LANDSCAPE

To efficiently defend an organism against injury, infection, and cancer, leukocytes must orchestrate a complex multiscale chain of events. For decades, immunological research focused on identifying cellular and molecular players that mediate the intricate interplay between cells of the innate and adaptive arms during a concerted immune response. Innate immune cells, including neutrophils, macrophages, and antigen-presenting cells (APCs) such as dendritic cells (DCs), are early responders recruited to sites of inflammation, where they can phagocytose foreign and pathogenic substances themselves or coordinate a wider immune response by recruiting lymphocytes that then clear the threat.

However, it is only within the past decade that evidence has emerged highlighting the critical role of mechanical forces in immune cell functions. To mount effective immune responses, immune cells must rapidly migrate to and contact APCs, or pathogen-laden or transformed target cells. As for all animal cells, actin-mediated force generation is the main driver of migration in leukocytes, which navigate an array of barriers and tissues of differing architectures either by responding to complex guidance cues or by employing highly evolved search mechanisms (Munoz *et al.*, 2014; Weninger *et al.*, 2014). Leukocytes are therefore endowed with considerable plasticity in shape and migratory regulation (Renkawitz and Sixt, 2010), as they continually probe and respond to the geometry and mechanical cues provided by their environment (Hallmann *et al.*, 2015). When an immune cell eventually encounters a target cell, it will physically "grasp" it and form a specialized synaptic interface, exerting forces on its conjugate in order to deliver its functions (Lim *et al.*, 2011; Basu *et al.*, 2016; Spillane and Tolar, 2017). In addition to these forces acting at the cellular level, recent progress in the field has demonstrated that immune receptors themselves respond to mechanical stimuli during antigen recognition, which is crucial for efficient discrimination of antigens. Indeed, mechanical forces acting directly on individual receptors influence receptor triggering and downstream intracellular signaling.

Given the multitude of functions carried out by immune cells within varying environments, it is not surprising that they experience mechanical forces ranging from piconewtons at the nanoscale to several orders of magnitude greater at the tissue level. Recent developments in imaging modalities and biophysical tools have finally allowed for the forces at the molecular scale to be probed. This

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Abbreviations used: AFM, atomic force microscopy; APC, antigen-presenting cell; BCR, B-cell receptor; BFP, biomembrane force probe; CAR, chimeric antigen receptor; CTL, cytotoxic T lymphocyte; DC, dendritic cell; F-actin, filamentous actin; FRET, Förster resonance energy transfer; ICAM-1, intercellular adhesion molecule 1; ITAM, immunoreceptor tyrosine-based activation motif; LFA-1, lymphocyte function–associated antigen 1; MPA, micropipette aspiration; MTFM, molecular tension fluorescence microscopy; NK, natural killer; OT, optical tweezers; pMHC, peptide–major histocompatibility complex; TCR, T-cell receptor; TFM, traction force microscopy; TGT, tension gauge tether.

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Perspective explores recent progress in the emerging field of mechanoimmunology, focusing on immune receptor–ligand interactions and highlighting the novel biophysical tools that have afforded us hitherto inaccessible insights into the role of molecular-scale mechanical forces in immune cell functions (see Box 1).

MECHANICAL CUES IN THE ENVIRONMENT REGULATE IMMUNE CELL BEHAVIOR

Within the past few years, it has become clear that immune cell activation is regulated not only by biochemical factors, but also by the stiffness of the environment the cells are interacting with. Cellular mechanosensing is the ability of cells to sense the physical characteristics of their environment (be it the extracellular matrix or adjoining cells). For this purpose, cells apply force on their environment and convert information about the resistance to this force into biochemical signaling through various mechanosensory proteins. Mechanosensing of substrate rigidity by macrophages has been shown to influence their phagocytic ability (Patel *et al.*, 2012), cell morphology and elasticity (Blakney *et al.*, 2012; Patel *et al.*, 2012), and production of both proinflammatory and anti-inflammatory cytokines (Blakney *et al.*, 2012; Previtera and Sengupta, 2015). Macrophages grown on stiff polyacrylamide gels (240 kPa) produced more proinflammatory mediators than those grown on soft substrates (~0.3 kPa), and Toll-like receptor 4 activity enhanced this effect in response to lipopolysaccharide, a bacterial proinflammatory agent (Previtera and Sengupta, 2015). This suggests that biological stimulants and tissue elasticity can work synergistically to regulate the pro- or anti-inflammatory characteristics of macrophages during an infection.

It is not only cells of the innate immune system that respond to mechanical cues. T and B lymphocytes recognize a specific, so-called cognate antigen on the surface of an APC or an infected or cancerous cell. Both T- and B-cells have been shown to adapt their response to antigens based on the rigidity of the substrate they are presented on (Judokusumo *et al.*, 2012; O'Connor *et al.*, 2012; Wan *et al.*, 2013; Zeng *et al.*, 2015; Saitakis *et al.*, 2017; Shaheen *et al.*, 2017). Substrate stiffness modulates not only the level of activation, but also the nature of the cellular responses. In T-cells, this includes differentiation (O'Connor *et al.*, 2012), gene expression, cell migration, morphology, and cytokine secretion (Saitakis *et al.*, 2017), whereas in B-cells, proliferation, class switching, and antibody production are all influenced by substrate rigidity (Zeng *et al.*, 2015). Experiments using a biomembrane force probe (BFP; see Box 1) revealed mechanical feedback between the substrate stiffness sensed by a T-cell and the force it generated, with greater force applied on stiffer targets (Husson *et al.*, 2011). This was further confirmed with traction force microscopy (TFM; see Box 1), where Jurkat T-cells exerted stronger forces on stiffer hydrogels (Hui *et al.*, 2015). In a key study by Morgan Huse's group, stiffer target cells were shown to enhance the killing response of cytotoxic T lymphocytes (CTLs) (Basu *et al.*, 2016). Interestingly, there is a strong negative correlation between cancer cell stiffness and metastatic potential (Swaminathan *et al.*, 2011; Lekka, 2016). These studies revealed that metastatic cancer cells are softer than primary tumor cells, which raises the intriguing possibility of invasive cells evading CTL-mediated lysis by modulating their mechanical properties. Furthermore, Basu *et al.* demonstrated that mechanical force exerted on tumor cells by CTLs themselves facilitates perforin-mediated lysis. Using a micropillar array (see Box 1), they demonstrated that CTLs exert localized forces in areas into which they direct their lytic granules to deliver their cytolytic proteins, a process called degranulation (Basu *et al.*, 2016). The relationships between a cell and its environment or interaction partners are thus bidirectional and, in the context of mechanical forces, can be characterized as displaying "mechanoreciprocity."

IMMUNE RECEPTORS AS MECHANOSENSORS

The molecular mechanisms underlying the mechanosensitivity of immune cells are still being deciphered. Do immune receptors themselves have a mechanosensing capacity or do T- and B-cells perform mechanosensing through more conventional receptors such as integrins? Lymphocyte function–associated antigen 1 (LFA-1) is an integrin expressed on lymphocytes that binds to intercellular adhesion molecule 1 (ICAM-1) to promote adhesion during the formation of an immunological synapse at the interface between a lymphocyte and its conjugate. Previous studies suggested that lymphocytes are able to discern substrate stiffness independent of integrins (Judokusumo *et al.*, 2012; O'Connor *et al.*, 2012; Wan *et al.*, 2013; Zeng *et al.*, 2015), although the presence of adhesion molecules greatly enhances the ability of B-cells to discriminate between antigens (Shaheen *et al.*, 2017). In T-cells, engagement of LFA-1 alone did not generate any measurable forces or intracellular signaling (Husson *et al.*, 2011), suggesting that mechanosensitive receptors other than integrins are at play in lymphocytes.

The question of whether immune receptors are themselves inherently mechanosensitive has driven many new technological developments that experimentally uncouple force from antigen recognition. A T-cell will only recognize and respond to an APC or target cell if its T-cell receptor (TCR) binds to its cognate peptide bound to major histocompatibility complex (pMHC) on the surface of its interaction partner. The TCR itself is composed of an $\alpha\beta$ heterodimer that has no intrinsic signaling domain, but is noncovalently associated with CD3 chains ($\varepsilon\gamma$, $\varepsilon\delta$, and $\zeta\zeta$ dimers) that together contain ten immunoreceptor tyrosine-based activation motifs (ITAMs) that can be phosphorylated to initiate signaling (Figure 1). Signaling can also be amplified through association with coreceptors such as CD4 or CD8, the expression of which specifies the function of the T-cell. Novel biophysical echniques have exploited surrogate conjugates (such as pMHC- or antibody-coated beads, bilayers, or surfaces) and/or artificial ligands to activate T-cells. Early evidence that the TCR functions as a mechanosensor came from Ellis Reinherz's group (Kim *et al.*, 2009). They used beads coated with engineered anti-CD3ε antibodies that bind only one site per TCR and are thus unable to cross-link it to trigger signals. By trapping the cells and beads in optical tweezers (OT; see Box 1), they were able to apply tangential forces on the TCR, which induced cytoplasmic Ca^{2+} mobilization, a widely adopted marker of lymphocyte activation. Furthermore, Li *et al.* stimulated T-cells with artificial APCs presenting modified elongated anti-CD3ε antibodies that were unable to trigger calcium influx. Only when shear stress was applied to the T-cells through buffer flow from a micropipette tip, or when T-cells were physically pulled away from the APC via micropipette aspiration (MPA; see Box 1), was Ca^{2+} signaling initiated (Li *et al.*, 2010). In addition, optomechanical actuator nanoparticles that collapse upon near-infrared illumination, thus applying a mechanical load to the receptor–ligand complexes bound to the particles, were able to mechanically trigger calcium signaling in T-cells (Liu *et al.*, 2016b). Overall, these studies suggest that physical forces acting on the TCR complex can directly initiate signaling in T-cells.

Intriguingly, a very recent study demonstrated that the mechanosensitive ion channel Piezo1 is critical for TCR triggering (Liu *et al.*, 2018a). In their paper, Liu and colleagues propose a model in which

Box 1: Force measurement techniques.

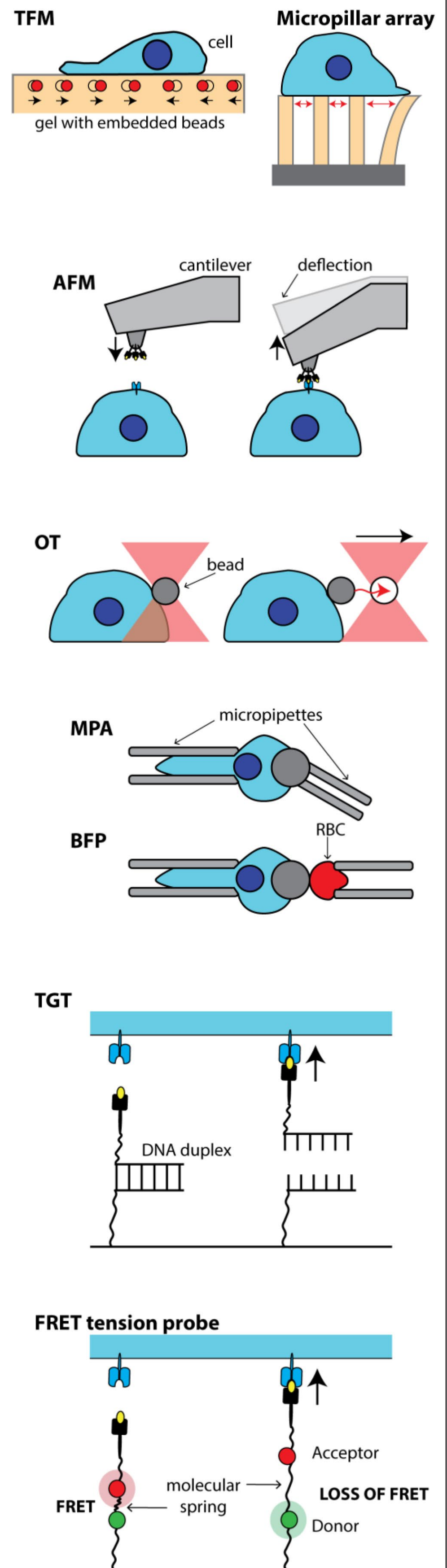
Traction force microscopy (TFM) relies on the principle that the elastic properties of a material relate the force per unit area (stress) it is subjected to and the ensuing fractional change in material length (strain) it experiences. Therefore, if the elastic properties of a substrate are known, and the strain it experiences can be measured, the forces exerted on it can be calculated. In TFM, induced deformations are usually determined by tracking the movement of tracer particles within a gel. Classically, cells are positioned on a layer of compliant material with an adhesion-functionalized surface (Style *et al.*, 2014). Alternatively, TFM can also be realized using arrays of deformable *micropillars*, such as those made of polydimethylsiloxane (PDMS). If the elasticity of the micropillars is known, their deflection can be used to calculate the applied force (Tan *et al.*, 2003).

Atomic force microscopy (AFM) employs a small scanning probe, consisting of a soft cantilever and a micrometer-scale tip, to determine minute tip–sample interaction forces (pN to nN). In close contact with the surface, attractive/repulsive forces induce deflection of the cantilever, which is tracked by a laser beam reflected from the back of the cantilever onto a photodiode detector. By functionalizing the cantilever tip with chemicals or biomolecules, or even replacing it with a live cell, interaction forces between biological systems can be measured (Müller and Dufrêne, 2011). Alternatively, AFM can be used to determine the mechanical properties (e.g., elasticity) of a sample by measuring the deflection of the cantilever, which reflects the loading force exerted on the sample, with increasing indentation depth (Kuznetsova *et al.*, 2007).

Optical tweezers (OT) are created by focusing a laser beam to a diffraction-limited spot by means of a high–numerical aperture objective. In the vicinity of the focal point, particles of a refractive index greater than that of the bathing medium (nm to μm size, e.g., polystyrene beads or even cells) experience a pN force directed toward the focus. For small displacements from the focal point, the restoring force depends linearly on the displacement. If a molecule attached to a dielectric bead or a cell interacts with a binding partner, then its displacement from the focal spot is directly proportional to the magnitude of the interaction force (Neuman and Nagy, 2008).

Micropipette aspiration (MPA) techniques exploit negative pressure to aspirate lipid vesicles or cells partly into a micropipette to hold them in place or change their mechanical properties. Given Laplace's law, if the pressure in the pipette and an aspirated spherical object are at equilibrium, the surface tension of the object can be calculated. In *biomembrane force probe (BFP)* measurements, a biotinylated red blood cell (RBC) is aspirated into a micropipette and a streptavidin ligand–coated glass bead is attached to the RBC. A cell is allowed to adhere to the ligand-coated bead and as cell and bead are separated, the deformation of the RBC is proportional to the interaction force (Gourier *et al.*, 2008).

DNA-based tension gauge tethers (TGTs) utilize DNA duplexes to attach cell–receptor ligands to a surface. Depending on their length and sequence, DNA duplexes are characterized by a specific rupture force. Using TGTs with increasing rupture force, the force required for cell attachment or cell activation can be determined (Wang and Ha, 2013). When the DNA duplex is flanked with a fluorophore and a quencher, fluorescence is low prior to rupture due to Förster resonance energy transfer (FRET), and the time point of detachment can be visualized in real time via an increase in fluorescence. Alternatively, in multiplex TGT constructs, TGTs that are sensitive to different force thresholds are labeled with different fluorescent dyes and are used to simultaneously map different tension levels in cells (Wang and Wang, 2016). Employing the same principle, *FRET-based tension probes* are short, mainly alpha-helical peptidic structures that extend under applied force. A fluorescent protein FRET pair is attached to the ends of the sensor, and loss of FRET reflects extension of the linker (Stabley *et al.*, 2011). In contrast to other force measurement methods, these fluorescent tension probes allow measurement of intracellular forces with molecular specificity at subcellular resolution. These techniques are sometimes grouped under the name *molecular tension fluorescence microscopy (MTFM;* Liu *et al.*, 2017). The mechanical spring element in MTFM probes can consist of DNA hairpins, protein domains, or polymer chains such as polyethylene glycol or elastic polypeptide. This probe design builds on earlier FRET-based tension sensors from Martin Schwartz's group, in which an elastic protein domain (e.g., TSMod, derived from the spider silk protein flagelliform) was incorporated within the protein of interest itself (Grashoff *et al.*, 2010).



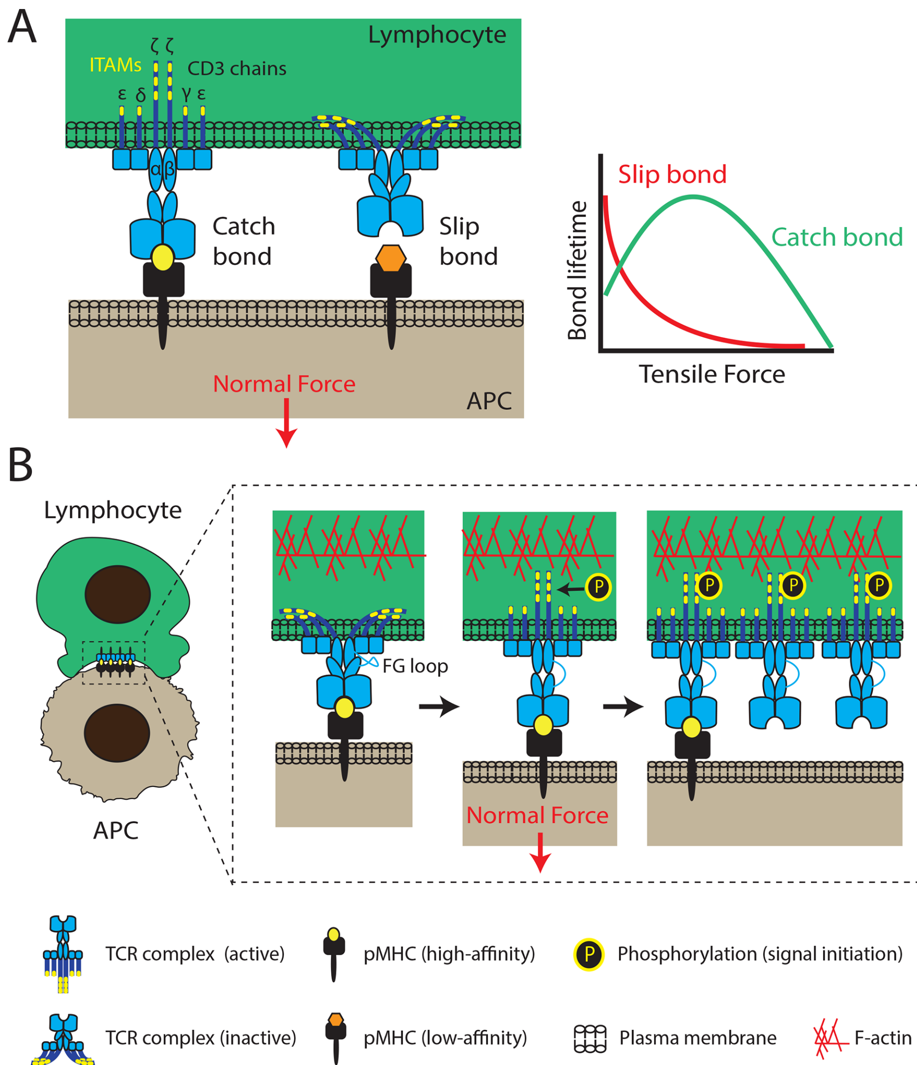


FIGURE 1: Mechanotransduction through the T-cell receptor. (A) The T-cell receptor complex consists of an αβ heterodimer that is noncovalently associated with ITAM-containing CD3 chains (εγ, εδ, and ζζ dimers). TCRs interact tightly with high-affinity pMHCs, forming catch bonds that are characterized by lifetimes that increase under load. Above a specific force threshold, lifetimes decrease. Longer TCR–pMHC interactions are more likely to lead to successful signal initiation and T-cell activation. Conversely, TCR interactions with low-affinity peptides exhibit slip-bond behavior, rupturing easily under low tensile forces. (B) Receptor deformation model of TCR activation. Force applied to the TCR upon pMHC binding triggers the unfolding of the FG loop region of the TCR and the exposure of its ITAMs (yellow bands) for phosphorylation and initiation of downstream signaling. FG loop unfolding facilitates extension of the TCR and its catch-bond behavior. Additionally, conformational changes of one TCR complex can propagate to its neighbors, producing clusters of active TCR complexes to amplify signaling. Both normal and tangential forces have been shown to initiate TCR signaling (normal forces shown here), with the contribution of each force component still under investigation. The F-actin cytoskeleton is thought to play a major role in both force generation and TCR clustering.

membrane stretch induced by immunological synapse formation triggers Piezo1 activation, thereby causing Ca^{2+} influx, and in turn driving cytoskeletal rearrangements that regulate TCR signaling (Liu *et al.*, 2018a). Although the mechanistic details underlying the role of Piezo1 in TCR activation require further study, these results bring into question the suitability of using calcium mobilization as an indicator of T-cell activation through TCR mechanosensing. Ca^{2+} fluxes are transient and heterogeneous between cells and involved in many different cellular processes, making it difficult to differentiate accurately between lymphocyte activation–associated calcium mobilizations and those resulting from other cellular phenomena.

There is long-standing evidence that reagents that disrupt the actin cytoskeleton abrogate TCR triggering (Campi *et al.*, 2005; Choudhuri *et al.*, 2005; Varma *et al.*, 2006). In an elegant study using atomic force microscopy (AFM; see Box 1), Hu and Butte demonstrated that an intact filamentous actin (F-actin) network is needed for T-cells to generate force at the TCR and thus to trigger T-cell activation (Hu and Butte, 2016). Both Ca^{2+} flux and force generation were abrogated when T-cells were treated with latrunculin A, a drug that sequesters monomeric actin and thus prevents its polymerization into F-actin. However, the most important finding of this study was that the application of an external oscillating force to the AFM tip, mimicking cytoskeletal forces, rescued calcium signaling in latrunculin A–treated cells (Hu and Butte, 2016), providing direct evidence that the necessary force-sensing machinery is intrinsic to the TCR itself. Intriguingly, it is not only the actin cytoskeleton of the T-cell that is vital during T-cell priming. Highlighting the importance of APC-T-cell cross-talk at the immunological synapse, the cortical actin networks of professional APCs such as DCs have been shown to regulate the lateral mobility of ICAM-1 molecules at their surface, with constrained ICAM-1 mobility promoting the formation of mature immunological synapses and T-cell proliferation (Comrie *et al.*, 2015). These data support a model in which ICAM-1 molecules with reduced mobility resist tensile forces exerted by the T-cell through ICAM-1/LFA-1 interactions more strongly, which in turn promotes firmer adhesion and maturation of the immunological synapse. Similarly, in natural killer (NK) cells, innate cytotoxic lymphocytes that recognize and destroy cancerous or virally infected cells, activation is regulated by the distribution and mobility of ICAM-1 molecules on the surfaces of target cells. Enhanced granule polarization and cytotoxicity were observed when ICAM-1 clusters were "tethered" to the actin cytoskeleton via ezrin and thus immobilized (Gross *et al.*, 2010).

NOVEL MOLECULAR TENSION PROBES ILLUMINATE MECHANOIMMUNOLOGY

To enable quantification of the minute forces acting at the single-receptor level, innovative biophysical methods with improved sensitivity have been developed. In 2011, Khalid Salaita's group pioneered the development of molecular tension fluorescence microscopy (MTFM; Stabley *et al.*, 2011; see Box 1). Here each probe, consisting of a donor–acceptor fluorophore pair separated by a DNA-based molecular "ruler" and immobilized onto a surface,

reports the force transmitted through receptors at single-molecule resolution via Förster resonance energy transfer (FRET). The Salaita group then further enhanced the sensitivity of these force sensors by immobilizing the FRET pair–decorated DNA hairpin onto a gold nanoparticle, with the probes now exhibiting dual quenching through both FRET and nanometal surface energy transfer (NSET) to the gold nanoparticle, providing a 100-fold increase in signal upon hairpin unfolding (Liu *et al*., 2016a). Using these tension probes, the authors were able to detect forces in the range of 12–19 pN experienced by individual TCR complexes during initial antigen recognition and preceding intracellular Ca^{2+} mobilization (Liu *et al*., 2016a). These results are in alignment with previous work in which single-molecule measurements showed that external forces in the range 10–20 pN are able to drive structural transitions in the TCR (Kim *et al*., 2009; Das *et al*., 2015) and increase the lifetime of TCR–pMHC interactions for strong agonists (Liu *et al*., 2014). When these tension sensors were incorporated into fluid lipid bilayers, the pN forces were shown to be sustained within TCR microclusters (Ma *et al*., 2016).

To measure forces applied on single receptor–ligand bonds accurately, Wang and Ha developed an alternative approach, known as the tension gauge tether (TGT; see Box 1), where a ligand is immobilized onto a solid surface through a DNA tether that ruptures at a critical force (Wang and Ha, 2013). By engineering a range of tethers with varying tension tolerances, this approach determines the forces required to activate cell signaling through single ligand–receptor bonds. Experiments combining TGTs with total internal reflection fluorescence (TIRF) microscopy were used to quantify B-cell receptor (BCR) accumulation and phosphorylation at the immune synapse as an indicator of B-cell signaling. Different classes of BCRs required different levels of mechanical force to induce activation, suggesting the existence of differing activation thresholds for different effector functions (Wan *et al*., 2015). The activation of the immunoglobulin (Ig) M (IgM)-BCR that is expressed on naïve B-cells before antigen encounter was dependent on the amount of mechanical force applied, with greater forces (>50 pN) resulting in more robust activation. In contrast, only limited mechanical force (<12 pN) was required for the activation of two other BCRs, IgG-BCR and IgE-BCR, which are expressed on differentiated memory B-cells that respond to a secondary challenge with the same antigen (Wan *et al*., 2015). This lower threshold could explain why memory B-cells exhibit faster and enhanced activation upon antigen reencounter.

Similarly, a recent study has shown that different B-cell subsets utilize different force thresholds to probe for antigen affinity (Nowosad *et al*., 2016). Using AFM, Pavel Tolar and colleagues demonstrated that B-cells use myosin-mediated contraction to pinch off part of the APC membrane to internalize antigen (Natkanski *et al*., 2013; Spillane and Tolar, 2017). In this process, the pushing and pulling of the membrane mediated by B-cell contractility contributed to antigen discrimination, with high-affinity antigens leading to stronger pulling forces and increased amounts of peptide being internalized (Natkanski *et al*., 2013; Spillane and Tolar, 2017). Using a degradation-sensitive DNA nanosensor, Spillane and Tolar then observed that in the situation where a B-cell could not mechanically remove the antigen, it secreted lysosomes that contained proteases capable of cleaving antigens from the APC surface (Spillane and Tolar, 2017). The mechanical threshold directing antigen internalization may be acting not solely at the level of individual molecules but rather at the level of antigen clusters. Naïve B-cells were found to gather antigen into large clusters, whereas germinal center B-cells undergoing affinity maturation formed smaller clusters of antigen that were trafficked to the cell periphery prior to endocytosis (Nowosad *et al*., 2016). A small cluster containing fewer antigens would require a higher affinity per molecule to surmount the mechanical threshold required for antigen extraction; thus only B-cells expressing BCRs with high affinity for an antigen will be selected for survival and antigen presentation to T-cells.

IMMUNE RECEPTORS EXHIBIT "CATCH BOND" BEHAVIOR

One of the most recent breakthroughs in the field of immune receptor triggering has been the discovery that the TCR forms ligand-induced "catch bonds" (Liu *et al*., 2014; Das *et al*., 2015). Catch bonds are characterized by lifetimes that lengthen with increasing force applied on the bond until a threshold force has been reached that results in increased frequency of bond rupture (Figure 1A). This is markedly different from "slip bonds," which are immediately destabilized when they experience force. Catch bond behavior was first described for selectins (Marshall *et al*., 2003) and integrins (Kong *et al*., 2009) and shown to promote cellular adhesion.

The group of Cheng Zhu used an adaptation of the BFP method to show that force affects the dissociation kinetics of TCR-pMHC interactions in a peptide-specific manner (Liu *et al*., 2014; Pryshchep *et al*., 2014; Hong *et al*., 2015). The application of forces in the range of tens of pN prolonged the lifetimes of single TCR-pMHC bonds for agonists (catch bonds) but shortened those for antagonists (slip bonds; Liu *et al*., 2014). This could potentially be achieved through extrinsic forces arising due to relative cell–cell motion, or through intrinsic forces generated by the actomyosin network transporting TCR clusters, both of which lead to a pulling force on the TCR that mechanically tests the strength of the TCR–pMHC interaction. Selectively prolonging bond lifetimes for rare agonists compared with abundant self peptides enhances antigen discrimination, a mechanism that allows the cell to distinguish between an appropriate immune response and injurious autoimmunity, and is therefore a critical aspect of immune recognition.

To engulf particles, macrophages and other innate leukocytes form a phagocytic cup—a highly organized synapse that forms in response to activation through phagocytic receptors (Goodridge *et al*., 2011; Niedergang *et al*., 2016). As with the TCR, the BFP method was used to uncover the catch bond behavior of the phagocytic integrin MAC-1 (Rosetti *et al*., 2015). Macrophage uptake of *Escherichia coli* was shown to be dependent on catch bonds formed between the bacterial adhesin FimH and the glycoprotein CD48 on macrophage filopodia (Möller *et al*., 2013). Force-activated catch bonds enable the long-lived interaction between a filopodium and a bacterium required to initiate phagocytosis, whilst a "shovel"-like lamellipodium protruding from the macrophage directly breaks interactions between the bacterium and the substrate (Möller *et al*., 2013). As the field develops, it will be interesting to discover what other surface receptors exhibit catch-bond behavior during ligand recognition.

TRANSLATING MECHANICAL STIMULI INTO INTRACELLULAR SIGNALING

One of the main outstanding questions in immune-cell signaling is how an extracellular stimulus can be translated into an intracellular signaling cascade. In particular, a variety of models have been proposed to explain how TCR triggering can account for the selectivity, specificity, and speed of the T-cell response (van der Merwe and Dushek, 2011). When the effect of mechanical forces on the TCR complex is considered, a model involving conformational changes seems the most favorable. Indeed, it has been suggested that the

TCR catch-bond behavior described above might be based on a force-induced allosteric change to generate additional intermolecular interactions that delay bond rupture. This idea is in agreement with the concept of the *kinetic proofreading model*, which postulates that completion of a series of reaction steps must occur during the TCR–pMHC bond lifetime in order to achieve T-cell activation (McKeithan, 1995), and an optimal dwell time of TCR–pMHC interactions has been identified for efficient T-cell activation (Kalergis *et al*., 2001). The implicit model is that a T-cell actively regulates forces transmitted to its TCR–pMHC complex to fine-tune bond lifetimes, thereby enhancing selective and differential levels of TCR activation. Emerging evidence supports a *receptor deformation* model (Figure 1B), in which mechanical force induces TCR-CD3 conformational changes to trigger signaling (Ma and Finkel, 2010). Forces generated through the actomyosin network and applied to the TCR would be the main driver, with any resistance to this force being converted into a conformational change in the TCR.

In a major tour de force, the combination of optical tweezers and DNA-based tether probes was used to show that the increased lifetime of TCR–pMHC bonds in response to tensile forces was dependent on a modification in the FG loop region of the TCR (Das *et al*., 2015). The FG loop is a well-structured element at the interface between the variable (Vβ) and constant (Cβ) domains of the TCR and has been shown to be stabilized through an interaction with the CD3γε dimer. Eliminating the FG loop abolished the catch bond behavior of the TCR, and conversely, stabilization of this domain resulted in enhanced bond lifetimes (Das *et al*., 2015, 2016). The FG loop is assumed to unfold upon experiencing force, leading to an extended conformation of the extracellular domain of the TCR complex (Figure 1B). This was also observed for pre-TCR–pMHC interactions, highlighting the novel role of mechanical forces during T-cell development (Das *et al*., 2016). The elongated conformation of the TCR favors catch-bond behavior and transmission of force across the TCR-CD3 domains, presumably leading to the release of CD3 chains from the membrane for phosphorylation. Indeed, the *safety catch* model postulates that the cytoplasmic portions of CD3ε and CD3ζ are embedded in the inner leaflet of the plasma membrane through a basic residue-rich sequence and that TCR engagement leads to dissociation of these chains from the membrane, exposing their ITAMs for phosphorylation by the kinase Lck (Xu *et al*., 2008; Zhang *et al*., 2011; Figure 1B). In support of this model, a recent study has shown that the cytoplasmic tails of CD3ε can exist in three conformational states with varying levels of association with the cell membrane (Guo *et al*., 2017). However, the mechanism by which force could be transduced from the extracellular region to the CD3 tails remains unclear due to a lack of structural information regarding the transmembrane organisation of the TCR complex. Despite the lack of direct experimental evidence, two models have been proposed: a first in which a pistonlike downward movement of the FG loop pushes on the cytoplasmic CD3 chains through the membrane (Kim *et al*., 2009; Wang and Reinherz, 2012), and a second in which the transmembrane domain of CD3ζ acts as a pivot point, resulting in ITAM exposure (Lee *et al*., 2015). The precise mechanical changes occurring in the TCR may depend on the direction of the force experienced by the TCR. Although both normal and tangential forces applied to T-cells were able to induce calcium signaling, tangential forces led to enhanced activation efficiency (Kim *et al*., 2009; Feng *et al*., 2017). The recent finding that T-cells laterally scan the surface of APCs or target cells for pMHC while extending dynamic microvilli (Cai *et al*., 2017) makes it even more difficult to determine the direction of physiological forces exerted on the TCR.

Ligand-induced conformational changes in CD3ε are thought to propagate to adjoining TCR complexes within the same cluster, and this cooperation between TCR complexes could explain the high sensitivity of T-cells to low numbers of pMHC antigens (Martinez-Martin *et al*., 2009). In addition, earlier work demonstrated that both receptor clustering and conformational changes in the CD3 chains were required for full TCR triggering (Minguet *et al*., 2007). Furthermore, Kuhns *et al*. have proposed that conformational changes that occur in the AB loop of the TCR Cα domain may regulate TCR oligomerization (Kuhns *et al*., 2010). Thus, it is not improbable that force-induced conformational changes lead to enhanced signaling by inducing TCR clustering (Blanco and Alarcón, 2012; Figure 1B). The positive relationship between TCR clustering and signaling efficiency at the nanometer scale was recently revealed using single-molecule localization microscopy. Only TCR complexes in dense clusters were phosphorylated and associated with downstream signaling molecules and the density of TCR clusters was dependent on the quantity and affinity of pMHC (Pageon *et al*., 2016). To bring these two concepts together, the Salaita group developed novel ratiometric tension probes that can simultaneously map receptor forces and clustering at the immunological synapse (Ma *et al*., 2016). The authors report colocalization between the ratiometric signal representing tension density and TCR clustering within the first minute of stimulation, showing that TCRs undergoing clustering are experiencing tension in the pN range (Ma *et al*., 2016). It is highly likely that TCR clustering is stabilized by the underlying F-actin network, potentially through membrane compartmentalization or direct tethering of the TCR complex to cortical actin. Additionally, receptor clustering may also be driven by the retrograde flow of actin (Yi *et al*., 2012) that is observed during cytotoxic synapse formation and target-cell engulfment (Ritter *et al*., 2015).

When these findings are considered as a whole, a unifying model emerges in which a force-induced mechanical switch occurs in the TCR upon receptor engagement, driving conformational changes and receptor clustering, and thus leading to robust intracellular signaling and effective cell activation. Receptor clustering has also been linked with activation in B-cells (Mattila *et al*., 2013) and NK cells (Pageon *et al*., 2013; Oszmiana *et al*., 2016). In macrophages and neutrophils, it is well established that phagocytosis is initiated by the lateral clustering of Fcγ receptors upon ligand binding (Sobota *et al*., 2005). It is likely that mechanical forces influence receptor clustering in these cell types too, although this has not yet been investigated and may involve different mechanisms. The cellular force-sensing machinery may also be involved in downstream signal-transduction events. For example, the force-sensing protein lymphocyte-specific Crk-associated substrate (Cas-L) has recently been implicated in physically linking TCR microclusters to the underlying actin network (Santos *et al*., 2016). Following initiation of TCR signaling, evidence suggests that Cas-L undergoes a conformational change in response to actin-induced stretch, leading to amplification of signaling, regulation of TCR microcluster transport, and inside-out integrin signaling, as well as actomyosin contraction (Santos *et al*., 2016). Further investigations will reveal the full extent of the involvement of mechanical forces in immune-cell signaling and effector functions acting at varying length scales and in different cellular compartments.

OUTLOOK

Within the past decade, pioneering biophysical approaches have contributed to our understanding of the mechanobiology at play during immune responses. The overall emerging picture is one where immune receptor signaling is governed by a complex

regulatory network involving cross-talk and feedback loops between chemical and physical signals. We have discussed the key role of molecular-scale mechanical forces in effecting immune responses, but this probably also holds true for most receptor–ligand interactions (Chen *et al*., 2017). With the field of mechanoimmunology still in its infancy, further studies are required to elucidate the exact mechanisms that allow immune receptors to sense and regulate mechanical stimuli.

In recent years, immunotherapy has emerged as the biggest breakthrough in modern cancer treatment. With the development of chimeric antigen receptors (CARs), we are getting closer to achieving high specificity with reduced risks of off-tumor cytotoxicity, with clinical trials employing CAR T-cells achieving unprecedented remission rates (Frey and Porter, 2016). The role of mechanosensing in antigen discrimination is key to engineering improved CARs that will amplify minute differences in antigen structure to exclusively target tumor antigens. To this end, a deeper understanding of TCR-mediated mechanosensing is required. Key functional insights will no doubt continue to emerge with the design of ever-improving tension probes (Liu *et al*., 2017) and the development and refinement of novel biophysical tools. Improved in vivo imaging capabilities will likely be crucial, since immune cells move through and operate in such a variety of mechanically distinct 3D microenvironments within organisms. The ability to visualize cells in intact tissues directly will deepen our understanding of the unique mechanobiological mechanisms regulating immune cells and the influence of the mechanical landscape on their migration and functions. This is already becoming a reality, with Eric Betzig's new adaptive optical-lattice light sheet microscope (AO-LLSM) allowing high-speed, high-resolution in vivo imaging of dynamic subcellular processes in 3D (Liu *et al*., 2018b). By combining this technology with genetically expressed force sensors, we may soon be able to map molecular-scale mechanical forces in and on cells deep within the complex tissues of living organisms.

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