Book Chapter

Mechanical Forces in T cell Biology

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Abstract

The fate of the adult human body, in terms of tissue development and homeostasis, is governed by how well its cells interact with one another, and with their environment. While the biochemical aspect of such interactions has been extensively studied for decades, their mechanical features, have only more recently captured the attention of cell biologists. Such an over sight becomes particularly notable when studying immune cells that experience different mechanical milieus during their life cycles- from primary/secondary/tertiary lymphoid organs and peripheral tissues displaying variable substrate rigidities, to the blood and lymphatic circulatory systems presenting complex hydrodynamic forces- and that are capable of exerting a substantial amount of force against their interacting surfaces. Indeed, mechanical cues, both dynamic forces and spatial features, have been shown to regulate the development, activation, differentiation and expansion of immune cells. T cells in specific, however, depict a unique paradigm of mechano-immunomodulation as the T cell receptor (TCR) itself has been shown to both sense and convert forces into biochemical signals, as well as induce force exertion following triggering. Consequently, it is only reasonable to imagine that incorporating mechanical cues into our "classical" view of T cell biology will help us better understand and manipulate their behavior, and more importantly, address the still unresolved mystery of their activation. In this chapter, we will review the existing body of knowledge showcasing the influence

of mechanical forces on certain T cell surface and cytoplasmic proteins, the process of force generation during T cell interactions, how these forces come into play in T cell biology, and finally the ability of T cells to sense and respond to substrate stiffness and ligand mobility.

Keywords

Immune Cells; T Cells; Mechanical Cues; TCR; Forces; Substrate Stiffness; Ligand Mobility

Looking Back on History

In all forms of life, survival depends on the ability to adapt to environmental stresses, including mechanical stimuli such as external physical forces. It is a requirement so fundamental that it is at the core of all biological designs; virtually all organisms have evolved structures from the macro (organs, tissues) to the micro (cells) and even down to the nanoscale (molecular assemblies, single proteins) that are not only sensitive, but also responsive to forces.

The biological effects of these forces are perhaps most evident in the context of physical structure and activity- the skeleton provides structural support to sustain the force of gravity. The skin provides a protective barrier that is maintained upon the application of external stretch. Even the simplest of physiological functions, such as respiration and circulation, require the generation of forces. This could explain why the earliest understanding and quantifications of these forces were focused on the organism and organ levels. In 1917, biologist D'Arcy Thompson published his book 'On Growth and Form', in which he discussed how mechanical forces contribute to the shape and size of living organisms [1]. Near contemporaries of Thompson, Cecil Murray and Julius Wolff, proved respectively that shear stress controls the size of blood vessels [2] and that mechanical loading increases the thickness and density of bone [3].

This goes to show that the study of the interplay between physical forces and biological function dates back to well before the term ‘mechanobiology’ was even coined. Today, there is a general consensus that cells constantly sense the various mechanical cues (e.g. force, stress, strain, rigidity, topology and adhesiveness) of their micro-environment, *via* a process called ‘mechanosensing’. They then translate these cues into biochemical signals such as modified binding affinity, altered phosphorylation state, and/or a conformational change; a process called ‘mechanotransduction’.

These features are ubiquitous among different cell types and find themselves at the core of many physiological functions; in particular, it has been demonstrated that they are instrumental for key moments of immune cell life and function [4]. For decades, immunological research had focused on identifying the networks of secreted ligands, cell surface receptors, intracellular signaling pathways, and transcriptional factors mediating the immune response [5]. These networks have been predominantly regarded as chemical in nature, largely because the individual molecules that make them up have been characterized by their non-covalent molecular interactions and/or enzymatic activity. Though this chemical description may not be incorrect, it neglects the influence of physical cues, in particular mechanical forces, on signaling networks, as well as the influence of signaling networks on the mechanical environment within and outside of the cell. Such an oversight becomes particularly relevant when studying immune cells whose lives are intensely “physical”: regularly deforming, migrating through tight interstitial spaces, adhering under shear flow, and forming stable interfaces (known as immunological synapses; ISs) with other cells [6] (Figure1). Effectively, this means that the receptor-ligand interactions that govern immune cell function are likewise being subjected to and influenced by the same mechanically tempestuous microenvironment. And, given that several cell surface proteins (e.g. integrins) are known to be strongly connected to the actin cytoskeleton, which is in turn connected to other intracellular proteins, this makes the molecular machinery involved in signal transduction ideal for relaying physical information about the extracellular environment into the cell, as well as translating

biochemical signals inside the cell into physical forces exerted against that environment [7,8].

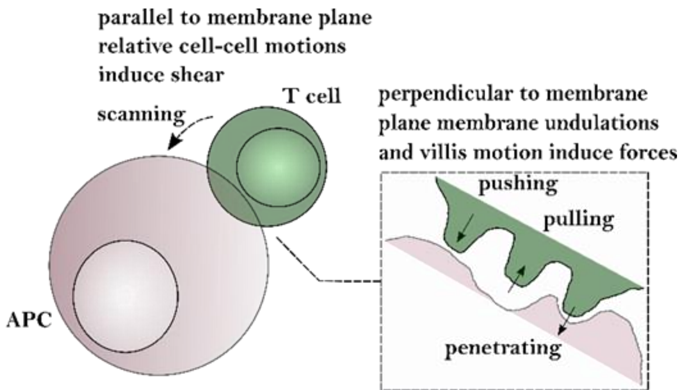

Figure 1: Origins and orientations of forces at the cellular and molecular scales in T cell recognition and function.

Exemplifying the importance of mechanosensing and mechanotransduction in their development and function are T cells, key players of the adaptive immune system [9]. Broadly speaking, T cells can be divided into three categories; Cytotoxic T cells that directly kill virally infected cells and cancer cells, and Helper and Regulatory T cells that activate and tune the effector functions of other cells in the immune system. In either case, T cells carry out the formidable task of identifying a particular cognate peptide bound to the major histocompatibility complex (pMHC) (Figure 2A), against a very noisy environmental background of endogenous self-peptides MHCs, many of which involve the same MHC molecule [10]. They do so even though the T cell receptors (TCRs) are cross-reactive and typically low in affinity when measured in isolation. One would expect that such high-fidelity decisions would be time consuming, however, T cells scan numerous antigen presenting cells (APCs) in a very short time (~ a few minutes) so that the immune system can react fast enough and avoid any potential significant damage to the body. The ability of T cells to perform their function properly while simultaneously abiding by all these constraints has baffled the scientific community for many years. Over the last decade, mechano-sensing/transduction has been

proposed to be the missing puzzle piece in our understanding of T cell function [11,12]. Different players may have different roles, as we will exemplify further on.

Integrins: The Prototypic Mechanoreceptors

As in any architectural structure, if mechanical load is to be transmitted across the cell surface into the cell, the simplest manner to do so would be through pliable structural elements that are physically interconnected [13]. Given that integrins link either the ECM or integrin ligands on other cells (through their extracellular domains) to the actin-cytoskeleton (through their cytoplasmic tails and adapter molecules) (Figure 2B), they represent excellent candidates for both mechanosensing and mechanotransduction. In fact, the demonstration that integrins are indeed mechanoreceptors was made almost three decades ago in a series of elegant experiments using magnetic twisting cytometry, where twisting ligand-bearing beads bound to β1 integrins caused endothelial cells to stiffen [14].

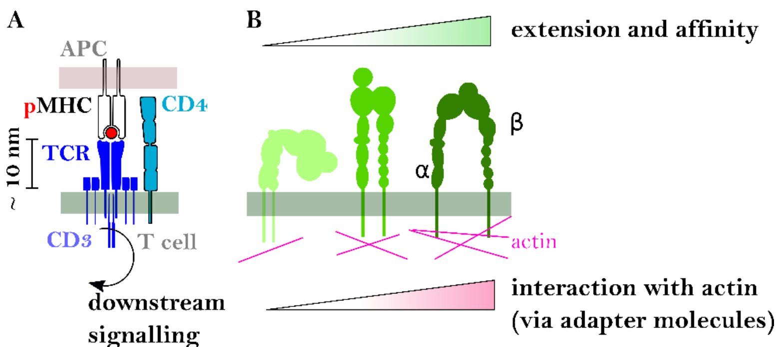

Figure 2: Key mechanosensory molecules for T cells. A: TCR interacts with peptide bearing MHC of an APC and directs the specificity of the adaptive immune response through signaling via the phosphorylation of CD3 cytoplasmic tails. B: Integrins can modulate their extension and interaction with the cytoskeleton depending on forces acting on them (outside-in signaling) or in response to e.g. T cell activation through the TCR (inside-out signaling) [15].

T cells specifically rely heavily on integrins, whether it is for adhesion during trafficking from the bloodstream, migration within tissues, immune synapse formation, or for signaling and

cell polarization [16]. Lymphocyte function-associated antigen-1 (LFA-1) is the predominant integrin on T cells, binding intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2) on partner cells (APCs or endothelial cells) [5]. Like other members of the integrin family, LFA-1 is a heterodimer comprising one α and one β chain, each containing a long, stalk-like extracellular domain, a transmembrane helix, and a short intracellular tail responsible for interacting with cytoplasmic signaling and cytoskeletal proteins [17].

The affinity of LFA-1 to ICAMs, however, is intimately coupled to its conformation, which is in turn set by the cell activation status [18]. In the cell resting state, LFA-1 exhibits a low affinity, bent conformation in which its ligand binding pockets are oriented towards the plasma membrane. In the presence of activating TCR signals during immune synapse formation, specific protein complexes (e.g. talin and kindlins) assemble on the cytoplasmic tails of the α and β chains and drive them apart [19]. The conformational change induces the extension of the extracellular domain, thus allowing ligand recognition. Although this extended conformation is capable of ligand binding, it can only do so at intermediate affinity. In fact, TCR signaling alone is insufficient to unlock the full binding potential of LFA-1 [20].

Only under applied tangential force, originating from the actin cytoskeleton (further elaborated later on), and transferred to integrins via interactions between cytoskeletal adaptors, such as talin, and the tail of the β subunit, does LFA-1 reach peak binding affinity (~ 100 fold increase) [21,22], a clear signature of catch-bond behavior. Catch bonds are an unusual kinetic behavior of ligand receptor interactions where the exertion of a physical force on a molecular complex counter-intuitively prolongs its bond lifetime, in contrast to the so called ‘ordinary’ slip bonds, where force intuitively shortens bond lifetime. Indeed, similar to other integrins [23], LFA-1 binding with ICAM-1 behaves as a catch bond [24].

Interestingly enough, the engagement of LFA-1 alone does not generate any measurable forces or intracellular signaling [25]. This observation suggests that the mechano-sensing/transduction

capacity of T cells could not be limited to conventional adhesion molecules such as integrins.

The TCR as a Mechanosensor

In the event that a cognate pMHC on an APC is encountered, TCR signaling will rapidly convert the ligand-binding event to the phosphorylation of up to 10 immunoreceptor tyrosine-based activation motif elements (ITAMS) in the cytoplasmic tails of the associated CD3 complexes. The ensuing signaling cascade ultimately results in developmental decisions, effects, or functions [26]. Unfortunately, our current knowledge of this signaling cascade far exceeds our limited understanding of how it is initiated upon TCR-pMHC binding.

The TCR-pMHC interaction is probably among the weakest protein-protein interactions that can initiate an effective biological response [27]. The affinity of a TCR binding to a pMHC is only around 10^{-4}-10^{-6}M [28], about 1000 times weaker than a typical antigen-antibody binding (10^{-6}-10^{-10}M [29]). Aside, shape-complementarity at the TCR-pMHC interface has been shown to be extremely poor [30]. Despite that, the TCR is still capable of discriminating as few as one to ten non-self antigens in a sea of endogenous self antigens that are presented by the same self-MHC molecule on the APC surface, and even a single pMHC is thought to be sufficient to trigger efficient TCR signaling and subsequent T cell activation [31]. All of this begs the question: How can a seemingly weak interaction simultaneously achieve such levels of specificity and sensitivity?

In an attempt to answer this question, in 2008, Ma and colleagues proposed a ‘receptor deformation model’ for TCR signaling. In this model, TCR signaling is initiated by significant conformational changes in the TCR/CD3 complex, induced by a pulling force originating from the cytoskeleton of the T cell and transmitted through pMHC-TCR binding interactions with enough strength to resist rupture [32]. Essentially, providing a mechanistic explanation to the specificity and sensitivity of the TCR.

A year later, Kim and colleagues provided the first concrete proof that the TCR behaves as a mechanosensor [19]. They used optical tweezers (OT) and nuclear magnetic resonance (NMR) techniques to characterize the distinct functional consequences of several anti-CD3 monoclonal antibodies (mAbs) binding to T cells. In parallel, they quantified Ca^{2+} levels as a measure of T cell activation. The NMR cross-correlation analysis showed that agonist Abs (i.e. those capable of triggering calcium fluxes) bind CD3 in a *diagonal* fashion, in comparison to CD3 Abs that do not trigger downstream signaling which bind CD3 in an upright mode (perpendicular to cell membrane plane). Interestingly enough, perpendicularly binding Abs were still capable of activating T cells but only when a significant *tangential* force, of ~50 piconewtons (pN), i.e. ~10-12 times the thermal agitation limit, was applied by OT. Based on these observations, the authors proposed a model in which external tangential forces generated following pMHC ligation during the scanning of the APC by the T cell, allow TCRs to mechanically sense and then transduce the first activation signals.

The TCR-pMHC Bond can Exhibit Complex Behaviors: The Catch Bond Proposition

In 2014, Liu and colleagues connected yet another piece of the puzzle [33]. Using biomembrane force probes (BFPs), they showed that the lifetime of the bond between a TCR and its specific pMHC was *prolonged* by the application of a ~ 10pN force, indicative of catch-bond behavior. Such a complex response was also associated with more robust and long-lived cellular calcium fluxes, suggesting that catch bond formation may be required for stronger T cell activation. By contrast, the affinity of non-specific TCR-pMHC bonds peaked at zero force, indicative of slip-bond behavior. OT experiments using DNA tethers further revealed that it is in fact the FG loop of the constant domain of the β chain that allosterically controls the V domain modules' catch bond lifetime and peptide discrimination, through a force-driven conformational transition [34]. Collectively, these findings demonstrated that by eliciting antigen-specific catch bonds, external forces may amplify the power of T cell antigen discrimination by separating agonist

pMHCs that induce catch bonds from non-specific pMHCs that exhibit only slip bonds.

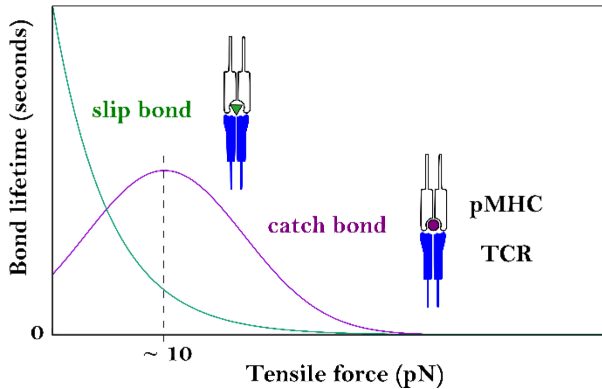

Figure 3: Different bond behaviors that have been proposed to play a role in antigen discrimination during the TCR-pMHC interaction. Slip bonds, whose lifetime only decreases when subjected to increasing forces, vs. catch bonds whose lifetime increases when subjected to increasing forces, up to a certain maximum limit (which has been estimated to be ~ 10pN for TCR/pMHC), beyond which the lifetime decreases as a function of force, similar to slip bond behavior [33].

While catch bonds have been observed in a broad range of molecules, TCR-pMHC catch bonds are still enigmatic, as their origin is still a matter of debate; numerous reports employing purely acellular systems have demonstrated that, outside the cellular context, the TCR does not exhibit catch bond behavior [35]. Aside, how can a tangential (to the membrane) force applied to the TCR-pMHC bond make it stronger? The same group attempted to answer this question using an integrated approach of steered molecular dynamics (SMD) simulation, MTs, and BFPs [36]. Their results showed that forces acting on the TCR-agonist pMHC complex induced a conformational change in the MHC that subsequently increased the length of the complex. Specifically, the increased force experienced by the TCR-agonist pMHC bond uncoupled the α-chain β2-microglobulin (β2m) interdomain interaction, resulting in a 5–10 nm extension of the MHC. They proposed that such pronounced extension would not only stabilize the TCR- agonist pMHC bond but also promote the formation of new interactions after forces

rupture the preexisting ones. Based on these results, the authors hypothesized that, in the case of agonist pMHCs, the forces acting on the TCR-agonist pMHC complex would induce a conformational change in the MHC, ultimately stabilizing the complex and creating a catch bond. The catch bond would then endow the TCR with the power to sensitively discriminate between peptides (self and non-self), plus, the increased chance of bond formation would make T cell activation easier. Nevertheless, this still does not explain the discrepancy observed by [35] and more work will be needed to clarify (i) if the catch bond behavior is indeed essential for T cell activation and (ii) if it is in fact a hallmark of TCR-cognate pMHC bonds, where is it originating from.

Mechanosensitivity Feature of the TCR Conserved at the Pre-TCR Level

Even before the TCR, force-based discrimination, is thought to be conserved in its developmental precursor, the pre-TCR, for the selection of efficient TCRs. Early thymic progenitors (ETPs; uncommitted thymic cells retaining some myeloid, NK and little if any B lineage potential) enter the corticomedullary junction of the thymus as double-negative cells (DN, stages DN1 to 4), lacking the expression of both CD4 and CD8, as well as the full T cell receptor (whether TCRαβ or TCRγδ). For the αβ T cell lineage, a surrogate preT-α chain (denoted pTα, which lacks Vα of final TCRαβ) is expressed on the surface of DN2 cells in place of the α chain seen in the final αβTCR. Shortly after, the cells enter the DN3 stage where they synthesize the TCRβ chain and express it on their surface in association with the pTα chain, forming what is known as the pre-TCR receptor [37]. Signaling through this pre-TCR marks the first major checkpoint in early thymic development, referred to as β-selection, whereby only DN cells with productive TCRβ are selected to continue their development. The question here is how does pre-TCR signaling occur?

Initially, pre-TCR signaling was thought to be ligand-autonomous [38,39] and purely dependent on pTα charge-based receptor oligomerization [40]. That theory was readily

discredited by Mallis and colleagues [41] who showed through NMR and BFP experiments that the pre-TCR, just like its mature form, and through the βchain alone, is capable of recognizing its respective pMHC (albeit with a broader specificity than its final TCRαβ form), as well as triggering calcium fluxes. Using OT, the same group later showed that this pre-TCR-pMHC recognition occurs specifically through the Vβ hydrophobic patch, in partnership with the Cβ FG loop of the TCRβ, and that the recognition is in-fact force-sensitive [42]. Indeed, the pre-TCR-pMHC interaction, similar to the TCR-pMHC one, was shown to exhibit features of catch bond behavior. Diminishing bond strength and/or bond lifetime (through mutating either the Vβ or the Cβ FG loop) negatively impacted pre-TCR ligand discrimination and ultimately reduced post-DN3 thymocyte proliferation and developmental progression (Li et al. 2021).

These observations show that only under force is pre-TCR signaling induced during β-selection. In this sense, the β repertoire is tuned prior to the αβ repertoire final tuning, with mechanotransduction through the β subunit serving as the first checkpoint towards ensuring a functional TCR. As for the diminished ligand specificity of the pre-TCR in comparison with that of the final αβTCR, it is possible that the broader ligand focus allows the β chain to interact with multiple self-pMHC ligands in the pMHC-rich stromal environment, affording DN3 growth/survival advantage to pMHC binding competent preTCRs and imprinting self-reactivity in the developing repertoire. Thus, DN progression selects for a self-reactive repertoire early in development. The Vβ patch may contribute to this behavior, relaxing peptide specificity requirements and functioning as a surrogate Vα domain whose replacement at the double positive (DP) stage (signaling through the pre-TCR marks the end of DN3 stage and the transition into the DP stage where the cells stop β chain rearrangement, undergo a period of proliferation, and begin to express both CD4 and CD8) where by an actual Vα domain then imposes more precise peptide recognition. Negative selection, that corresponds to the final selection before T cells leave the thymus where only DP T cells that bind self antigens at low affinity survive, therein purges high pMHC self-reactivity while maintaining a low self-pMHC bias.

Sensing and Exerting Forces on the Cellular Level: The Role of the Actomyosin Cytoskeleton

Moving up from the molecular to the cellular level, mechanical forces play a very important role in T cell function. However, before diving into that, one should first address how forces are generated and sensed on the cellular scale. Ultimately, mechanosensing/transduction, on any scale, and force exertion are tightly linked processes. Mechanically induced conformational changes, just as those described for activating the pMHC-TCR and LFA-1-ICAM bonds, only occur under the influence of force. Ergo, mechanotransduction necessitates that the cell exerts and receives forces from its environment. Conversely, force exertion is itself regulated by feedback from mechanosensing pathways, as we will see later on.

Cells exert forces against their environments via dynamic cytoskeletal remodeling; the cytoskeleton is a polymer network composed of three distinct biopolymers: actin, microtubules, and intermediate filaments. Typically, it is the filamentous actin (F-actin) cytoskeleton that bears the brunt of the mechanical load; It is a highly dynamic structure that undergoes continuous reorganization in response to external mechanical cues. This feature is what enables the cell to rapidly change its elastic properties and what consequently endows it with the capacity to apply forces against a substrate and move [44]. The classical model for F-actin dependent force exertion involves myosin motors consuming chemical energy in the form of ATP and walking on actin filaments in a general three-step process of binding, power stroke, and unbinding. This process is continuously repeated and leads to the generation of a contractile force (actomyosin contractile force) [44]. Although actomyosin contractility was initially characterized in muscle cells, it is now clear that it is a universal mechanism for force generation in most eukaryotic cells, fueling a wide range of processes including adhesion, division and motility. With that being said, it is important to note that actin polymerization alone, in the absence of myosin motors, does also generate force. However, such protrusive forces are far less characterized, most likely

because they are easily masked by the long-lasting, contractile ones [43].

Whether it is protrusive or contractile, in order for forces to propagate from the cytoskeleton onto the extracellular environment (substrate or cell), both parties have to be linked through *adhesive* contact points. The most characterized of such contact points are focal adhesions (FAs); FAs constitute large protein assemblies in which transmembrane adhesion receptors (e.g. integrins) and F-actin are bridged via a specialized layer of cytoplasmic scaffolding proteins (e.g. paxillin, vinculin, talin…) [45]. The size, composition, and structure of such adhesion sites are directly dependent on the mechanical forces that they are subjected to, whether it is from actomyosin contractility or from the extracellular environment. This explains why FAs are readily observed for fibroblasts cultured on stiff supports, while similar prominent contacts are harder to detect in-vivo, where the extracellular matrix (ECM) is much more compliant [46]. Interestingly, the process of building FAs from initial adhesion receptors is intricately coupled to the activity of intracellular signaling cascades, not through their possession of enzymatic activity, rather, their capacity to recruit specific, "classical" adhesome signaling components to the growing FAs [47]. For example, in the case of integrin mediated adhesions, the focal adhesion kinase (FAK) recruited to the FA site regulates diverse downstream signaling pathways, including those promoting cell growth and survival [48].

It has to be underlined that, unlike large adherent cells such as the fibroblasts mentioned above, many immune cells, among which the T cells, do *not* form distinct FAs-like structures in vitro or in vivo. Rather, they form *transient* adhesive contacts that contain cell surface receptors, F-actin, and cytoplasmic proteins such as the ones typically found in FAs. These contacts likely serve as sites for force exertion during migration and cell-cell interactions [4].

The most straightforward way in which cellular forces could contribute to T cell function is through enabling their migration and trafficking. Typically, as a cell moves on a substrate

(whether it is the ECM or simply a cover slide), it experiences external forces, mainly the viscous force/resistance from the surrounding medium and cell-substrate interaction forces, as well as internal forces that are generated by the cytoskeleton. In T cells, as in most animal cells, the cytoskeleton is the essential component in creating these motility-driving forces, and in coordinating the entire process of movement: First, a cell propels the membrane forward by growing the actin network at its leading edge, creating an F-actin rich lamellipodium. Second, it adheres to the substrate (for example through integrin adhesions in T cells) at the leading edge and deadheres (releases) at the cell body and rear of the cell (also known as uropod). Finally, the cell propels forward by the F-actin retrograde flow generated against the adhesive contacts present at the base of the leading edge of the cell; retrograde flow describes the variable movement of actin filaments rearward with respect to the substrate, generally in the direction opposite to cell movement [43] and it is caused by actin polymerization against the plasma membrane, which drives the growing fibers backwards, and myosin contractility, which collapses the leading edge F-actin network into linear bundles [4].

Aside from motility, cellular forces come into play at different time points in T cell activation. To begin with, the most basic requirement for T cell activation is for the TCR to interact with the pMHC. This may seem trivial to point out, however, there are physical barriers that make this interaction not as straightforward; The TCR-pMHC bonds (10-15 nm) are much smaller than individual TCR and APC glycocalyx proteins, such as the T cell receptor tyrosine phosphatases CD45 (28-50 nm) and CD148 (47-55 nm), and even LFA-ICAM bonds (45-50 nm for the couple). Though models such as the kinetic segregation one [49] were originally put forth to explain how the T cell overcomes these barriers, there still remains several key issues that the model does not account for [50]. Recently, Cai et al. combined time-resolved lattice light-sheet microscopy and quantum dot–enabled synaptic contact mapping microscopy to show how highly dynamic T cell F-actin-rich microvilli colocalized with TCR microclusters (MCs; upon ligand binding, TCRs coalesce into signaling microclusters containing >10

receptors each), and in the absence of external stimulus, scanned the entire area of opposing cells and surfaces (coated with antagonist/agonist pMHCs and ICAM-1) before and during antigen recognition, at a time frame (≃ 1 min) similar to that recorded for T cell–APC contacts in vivo [51]. These observations, coherent with earlier ones [52], suggest that T cell microvilli, with an average length of 380 nm, can promote TCR signaling by surpassing the size-related restrictions, penetrating the glycocalyx, and bringing the TCR into close proximity with the pMHCs. Additionally, one could imagine that the applied F-actin protrusive forces would further stabilize low affinity TCR-pMHC bonds, and with the microvilli containing pre-clustered TCRs, it would provide an easy access platform for signal amplification, explaining the high sensitivity of T-cells to low numbers of pMHC antigens.

After TCR engagement, actin polymerization at the T cell-APC contact zone commences. The membrane deformation resulting from such polymerization forces allows the T cell to spread over the APC. This spreading process is critical as it not only allows the T cell to scan a larger area of the APC and thus increases the efficiency of antigen sampling [53], but it also exerts force on the receptor-ligand pairs engaged, such as the mechanosensitive TCR-pMHC and LFA-1-ICAM-1/2 bonds, further enhancing peptide discrimination and TCR activation.

As the T cell reaches its maximal spreading area, the same actin polymerization forces, combined with myosin contractility, create retrograde flow. Forces originating from this retrograde flow organize the various TCR MCs and signaling molecules present at the T cell-APC contact zone, and order them into the infamous spatially symmetric bullseye structure of the IS [54]. To be more specific, the TCR MCs are swept towards the center of the contact by retrograde F-actin centripetal flow at the periphery and then by myosin II dependent actin arcs closer to the center, leading to the formation of the cSMAC (central supramolecular activation cluster) surrounded by a ring of integrins (LFA-1/ICAM bonds) in the pSMAC (peripheral supramolecular activation cluster). The interruption of F-actin centripetal flow eradicates TCR MC signaling within seconds,

further confirming that force exertion is imperative for maintaining proper TCR activation. Interestingly enough though, the same actin machinery described above may also break TCR-pMHC bonds, allowing the serial engagement of the same pMHC with the other TCRs present in the TCR MC, consequently augmenting TCR signaling.

Once the IS is established, it has to be maintained for a long enough period of time (up to hours) to enable the proper activation of the T cell. This is a particularly difficult task as T cells are already highly motile cells and the T cell-APC interaction occurs in non-static conditions. By monitoring the T cell cytoskeletal organization during their interaction with both APCs and APC mimetic surfaces, Kumari et al. found that antigen recognition triggered the formation of actin foci (by the help of Wiskott–Aldrich syndrome protein) at the T cell-APC-substrate contact that, with the assistance of myosin II contractility, generated and sustained intracellular tension within the T cell that maintained the stability and symmetry of the IS for the activation time frame [55].

Finally, in an elegant series of experiments combining pMHC and ICAM-1 coated on beads bared by deformable micropipettes and on micropillar arrays, Basu et al. demonstrated that mechanical forces at the IS potentiate cytotoxic T cell (CTL) cytotoxicity: CTLs destroy target cells by secreting a mixture of the protein perforin and granzyme proteases, where perforin forms pores in the target cell membrane that enable granzymes to access the cytoplasm and induce apoptosis [56]. Specifically, their study revealed that altering the membrane tension of the CTLs using pharmacological drugs or osmotic shock strongly perturbed the pore-forming activity of perforin. Similarly, altering the membrane tension of the target cell by changing substrate stiffness modulated CTL killing, with cells on stiffer substrates exhibiting a higher sensitivity to perforin-induced pore formation. Taken together, these results point towards a model in which forces at the IS promote CTL killing by straining the target cell membrane, thus facilitating the formation of perforin pores. Considering that several reports have correlated transformation and malignancy with cellular softening, this work

puts forth a very compelling hypothesis in which tumor cells modulate their mechanical properties to relief forces at the IS and thus evade the immune system [4,57].

T Cells can Sense and React to Substrate Stiffness

Just as we do when we use our fingers to apply pressure on an object, T cells exert forces to test their mechanical environment, particularly stiffness. Pioneering work by Judokusumo et al. initially documented this property by stimulating naïve CD4+ mouse T cells with polyacrylamide gels of different rigidities, and functionalized with activating antibodies against CD3 and CD28 [58]. Their experiments revealed that T cells exhibited stronger activation, quantified as IL-2 secretion, with increasing substrate rigidities (over the range of 10-200 KPa), and that this mechano-sensing/transduction ability was largely affiliated with the TCR/CD3 complex rather than CD28. Intriguingly, this "stiffness sensitivity" property was observed only when the anti-CD3 antibody was immobilized onto the surface of the gel, rather than added as a soluble solution, and it was lost upon myosin inhibition. These observations are in accordance with the now commonly accepted idea that antigen receptors pull against their ligands for optimal signaling. Conversely, similar experiments done by O'Connor et al. on polydimethylsiloxane substrates with the same functionalization but using a different rigidity range (100-200 KPa), showed that naïve CD4+ human T cells were stimulated and proliferated more on softer substrates in comparison to stiffer ones [59]. Taken together, these studies suggest a possible biphasic response to stiffness sensitivity. Another crucial piece of information came from Tabdanov et al. who employed a combination of activating anti-CD3 antibody and ICAM-1 functionalized flat micropatterned PDMS substrates (5 KPa- 2000 KPa) and micropillar arrays to delineate the contributions of both the TCR/CD3 complex and LFA-1 in stimulated CD4+ human T cell activation [60]. In these experiments, early T cell activation, measured by the total phosphor-tyrosine levels, was weaker on soft substrates than on rigid ones. Though this stiffness sensitivity was observed in the absence of LFA-1 engagement, it was enhanced by its presence.

Even more interestingly, their results also highlighted a mechanical cooperation between the TCR/CD3 and LFA-1-ICAM-1 systems, whereby actin nucleation downstream of TCR signaling sustained the growth of the LFA-1 dependent actin network, which in turn provided the cytoskeletal tension to allow mechanical sensing, T-cell spreading and enhanced TCR activation.

Similar experiments were later repeated but on substrates with stiffnesses of more physiologic relevance in terms of T cell function, considering that APCs display a stiffness range between ≃ 200 Pa and 2 KPa [61]. Notable of which were those performed by Hui et al. [62,63] who used poly-l-lysine-antiCD3-coated soft polyacrylamide gels (1- 5 KPa) to demonstrate the contributions of actin polymerization and myosin contractility, as well as dynamic microtubules, to force generation and maintenance during enhanced green fluorescent protein (eGFP)–actin expressing Jurkat T cell activation (quantified as phosphotyrosine signaling). Their results, similar to what was originally documented by Judokusumo et al., showed that T cells exhibited higher levels of activation on stiffer gels in comparison to softer ones.

Though these studies are difficult to directly compare because they differ in substrate chemistry, antibody/protein immobilization, stiffness ranges, and more importantly the T cell types/subtypes used, they do overall reveal that T cells possess the inherent ability to sense stiffness. This, at least partly, explains their modified behaviors in mechanically distinct interactions, whether it is different APCs that have been activated by different stimuli and present a varying repertoire of agonist/non-agonist pMHCs, or endothelial cells in blood vessels, or infected/tumor cells inside tissues. Even if the change in stiffness between these surfaces may seem quite modest and inconsequential, it is nevertheless sensed and responded to by T cells. Wahl et al. recently proposed a model in which increased substrate stiffness heightens the TCR-pMHC resistance to cytoskeletal forces and thus increases T cell spreading and activation, that is to a certain limit, beyond which the tension on the bonds becomes too high and breaks them, which

consequently decreases spreading and weakens T cell activation [64].

T Cells can Sense and React to Ligand Mobility

Aside from stiffness sensitivity, T cells have also been shown to be sensitive to ligand mobility [65]. The interaction between a T cell and an APC necessitates extensive cytoskeletal and lipid membrane composition changes for both cells, as to allow for the spatial ligand/receptor re-ordering mentioned above. In an innovative approach, Mossman et al. investigated the impact of ligand mobility on T cell signaling by creating "artificial APCs" where nanofabricated 10–20 nm high chromium barriers were assembled on pMHC and ICAM-1 coated supported lipid bilayers [66]; set up as is, the bilayer would allow for free lipid diffusion, however, the barriers would block the movement of proteins with larger cytoplasmic domains, and more importantly, TCR MCs. Interestingly, trapping the TCR clusters in the in the IS periphery (as opposed to their natural position in the cSMAC of the IS) augmented early TCR-associated phosphotyrosine signaling and cytoplasmic Ca2+ levels in the spatially constrained IS in comparison to the native ones. In a similar approach, but playing on the lipid bilayer composition instead of using chromium barriers for limiting ligand mobility, Hsu et al. revealed that tyrosine phosphorylation and persistent elevation of cytoplasmic Ca2+ was in fact more pronounced for T cells (Jurkat and naïve or stimulated CD4+ murine) on mobile membranes than on less mobile ones [67]. Though these two studies seem contradictory, the immobilization of the TCRs differed between the two systems; in the former, the chromium barriers completely trapped the TCR clusters in the periphery, on the other hand, the latter still permitted the diffusion of TCRs but at a slower rate. This could underline a complex mechanism, potentially reliant on the spatial and temporal parameters of ligand constriction, by which T cell sensitivity to ligand mobility impacts T cell activation. However, this would require further experimentation to decipher.

More recently, pioneering work done by Bukhardt and colleagues [30,68] revealed that dendritic cell (DC) maturation-

a process characterized by an increase in DC cortical stiffness-induced a dramatic actin-dependent decrease in ICAM-1 mobility. The reported decrease in ICAM-1 mobility helped generate a counterforce that drove the centripetal flow of the actomyosin network in the T cells spreading over the APC. This flow, in turn, recruited LFA-1 to the IS, maintained it in a high affinity conformation, and consequently promoted efficient binding to ICAM-1. One could imagine that since LFA-1 connects the extra- and intra-cellular compartments, similar to other integrins, the tension on LFA-1 will also affect the dynamics of the underlying T cell actin network [69]; since the TCR is thought to be interacting with said network, this will indirectly influence tension on the TCR, potentially modulating TCR signaling [13]. This work is of particular importance as firstly, it explains how LFA-1 reaches peak binding affinity necessary for proper T cell activation, and secondly, it suggests that cells can regulate intercellular communication by altering the physical status of the signaling molecules in question, rather than just their expression level or spatial localization.

How to Relay the Message?

Although the influence of mechanical forces on the specificity and sensitivity of antigen recognition by the TCR is coming to light, how information regarding TCR-antigen binding is relayed into the cell still remains unclear [70].

As mentioned above, TCR signaling propagates across the membrane through the CD3 intracellular domains, specifically through ITAM phosphorylation. In their unphosphorylated state, ITAM chains have been shown to be buried in the hydrophobic interior of the membrane, hence inaccessible to Src kinases. Ligand binding by the TCR has been recently proposed to induce conformational change in the CD3 chains, extending them and exposing their ITAMs to phosphorylation [71]. Although there are currently no definitive studies directly linking mechanical forces applied onto the TCR protein to this CD3ζ conformational change (e.g. are the forces needed pushing/pulling on the complex to unlock it, similar to an umbrella?), a recent study using fluorescence resonance energy transfer (FRET) showed

that the TCR, under force, is able to decipher structural subtle differences between peptides by different bond conformations, independent of binding affinity and kinetics. Peptide potency then appears to directly regulate the amount of conformational change, which in turn dictates the degree of dissociation of the CD3 (ζ chain) from the inner membrane leaflet and consequently the exposure of its ITAMs to phosphorylation [72].

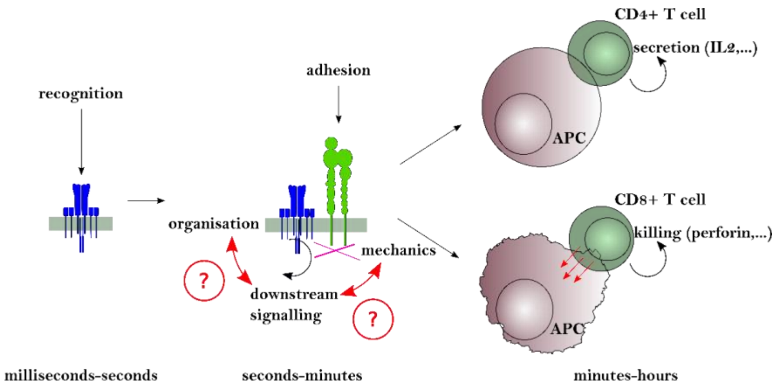

Figure 4: Typical times scales for signal propagation and consequences. The question marks indicate the interactions between different cell biology "modules" [73] that still need clarification in order to fully understand the entire process of T cell mechanotransduction and activation.

Another important question to address is the link between the different scales (Figure 4), particularly the TCR and the actin cytoskeleton. As mentioned previously, in adherent cells, the maintenance, growth and signaling through FAs are completely dependent on cytoskeletal forces. As such, FAs act as mechano-sensors/transducers bridging transmembrane adhesion receptor binding and actin flow with cell signaling. It is intriguing to imagine that the TCR MCs serve similar purposes. Using novel ratiometric tension probes, Ma et al. have demonstrated that TCRs undergoing clustering within the first few minutes of stimulation experience tension in the pN range [74,75]. It is thus highly likely that TCR clustering is stabilized by the underlying F-actin network or even through direct tethering of the TCR complex to cortical actin. Interestingly, the force-sensing protein lymphocyte-specific Crk-associated substrate (Cas-L) has recently been proposed by Santos et al. to mechanically link

TCR MCs to the underlying actin network [76]. Their experiments showed that Cas-L participates in a positive feedback loop whereby, upon TCR triggering, Cas-L localized to the TCR MCs undergoes actin-polymerization dependent activation (through phosphorylation), leading to Ca2+ signal amplification, regulation of TCR MC transport, inside-out integrin signaling, as well as actomyosin contraction [76].

Concluding Remarks

Besides the mechano-sensitive/transducive abilities of the TCR and integrins, there are several other membrane receptors, ion channels, cytoskeletal proteins, and transcriptional factors that are thought to be also affected by mechanical forces. For example, pulling forces on bound Notch receptors during endocytosis of Notch ligands induce a conformational change in Notch that ultimately drives early thymic progenitors to commit to the T cell lineage [77,78] and mechanical stretch of the membrane during IS formation activates Piezo channels, thereby triggering Ca2+ flux and regulating TCR signaling [79].

What is truly interesting is that these different elements do not function in isolation, but rather as parts of a complex mechanical signaling network with cross-talks and feedback loops, that ultimately regulates T cell mechanics, gene expression, and behavior. The challenge, now that some of the key elements have been described separately, is to understand how these mechano-signaling components and pathways are intertwined and integrated across time and length scales, and in different intra-cellular compartments, to shape the T cell response [7,73]. To take the TCR and LFA-1 as an example, Bernard and colleagues attempted to decipher the mechanical link between these two molecules by imaging T cells on anti-TCR Ab micropatterned soluble lipid bilayers (SLBs) [80]. Their results showed that the TCRs do in fact aggregate into MCs that colocalize with the anti-TCR Ab patterns, however, the clusters do not move (by the means of retrograde actin flow) to the center of the contact area, as seen during the formation of the central supramolecular activating complex of the IS. Only upon the addition of ICAM-1 to the SLBs, do the TCR MCs centralize with the actin and form

a peripheral ring around them. This study, in addition to many others [60,81,82], supports a model in which the actin cytoskeleton couples the TCR and LFA-1 in a positive feedback loop that coordinates IS formation and growth. It also puts forward a very exciting concept of the actin cytoskeleton acting as a mechanical intermediate that integrates force-dependent signals coming from different receptor-ligand interactions, and then coordinates outgoing responses over large distances [50].

Over the last decade, a sturdy foundation has emerged for measuring and interpreting mechanical forces in T cells. Nevertheless, the field remains in its infancy, we still don't know much- for example, how are mechanical forces transferred and integrated across different molecules, different scales, different time intervals, and different partner cells- however, what is becoming more and more apparent is that forces represent a fundamental component of the T cell response that can no longer be ignored. It is our hope that the literature and arguments presented in this review raise awareness to this emerging area of research in T-cell biology. It is also worth noting that the concepts presented here for T cells apply to all immune cell types, with basic similar phenomena and subtle differences for other lymphocytes such as B cells and NK cells, but also APCs such as dendritic cells, macrophages and neutrophils [4,6].

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