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REVIEW

Lymphocyte mechanotransduction: The regulatory role of cytoskeletal dynamics in signaling cascades and effector functions

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Abstract

The process of mechanotransduction, that is, conversion of physical forces into biochemical signaling cascades, has attracted interest as a potential mechanism for regulating immune cell activation. The cytoskeleton serves a critical role in a variety of lymphocyte functions, from cellular activation, proliferation, adhesion, and migration, to creation of stable immune synapses, and execution of functions such as directed cytotoxicity. Though traditionally considered a scaffold that enables formation of signaling complexes that maintain stable immune synapses, the cytoskeleton was additionally shown to play a dynamic role in lymphocyte signaling cascades by sensing physical cues such as substrate rigidity, and transducing these mechanical features into chemical signals that ultimately influence lymphocyte effector functions. It is thus becoming clear that cytoskeletal dynamics are essential for the lymphocyte response, beyond the role of the cytoskeleton as a stationary framework. Here, we describe the transduction of extracellular forces to activate signaling pathways and effector functions mediated through the cytoskeleton in lymphocytes. We also highlight recent discoveries of cytoskeleton-mediated mechanotransduction on intracellular signaling pathways in NK cells.

KEYWORDS

actin, cytoskeleton, mechanosensing, NK cells, signaling

1 | INTRODUCTION

It has been recognized that physical cues from the environment transduce biochemical pathways that impact a wide range of fateful decisions in different cells.[1–3] The mechanisms of mechanotransduction, the conversion of physical forces into biochemical signals that might impact cell decision and cell fate, and mechanosensation, the effect of extracellular substrate rigidity on cellular functions, were documented in several cell types and tissues. Examples include studies in which force was exerted on kidney epithelial cells, inducing reentry into the cell cycle and DNA replication,[1] and studies that used substrates with different rigidities to induce muscle stem cell regeneration, or to induce dormancy in neoplastic transformed cells.[2,3]

The central regulator and mediator of both cellular mechanosensation and mechanotransduction is the cytoskeleton, which was shown to directly respond to mechanical stress.[4] The principal components of the cytoskeleton include actin filaments, myosin motor proteins, and microtubules. External signals and physical stress applied to cells

Abbreviations: ADCC, Antibody-dependent cell-mediated cytotoxicity; AFM, atomic force microscopy; APC, antigen presenting cell; ARF, actin retrograde flow; BCR, B cell receptor; cSMAC, central supramolecular activation cluster; DOCK8, Dedicator of cytokinesis 8; dSMAC, distal supramolecular activation cluster; ECM, extracellular matrix; FA, focal adhesions; FRET, Förster resonance energy transfer; HLA, human leukocyte antigen; ICAM-1, Intercellular Adhesion Molecule 1; IS, immunologic synapse; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; KIR, Inhibitory killer-cell immunoglobulin-like receptor; LFA-1, Lymphocyte function-associated antigen 1; MAC-1, Macrophage-1 antigen; MC, microcluster; MICA, MHC class I polypeptide-related sequence A; MLCK, myosin light chain kinase; MTOC, microtubule organizing center; NCR, natural cytotoxicity receptor; NK, natural killer; PA, polyacrylamide; PDMS, polydimethylsiloxane; PKC, protein kinase C; pSMAC, peripheral supramolecular activation cluster; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; ROCK, Rho-associated, coiled coil-containing kinase; SHIP-1, SH-2 containing inositol 5′ polyphosphatase 1; SHP-1/2, Src homology region 2 domain-containing phosphatases 1 and 2; SMAC, supramolecular activation cluster; TCR, T cell receptor; TFM, traction force microscopy; ULBP2, UL16 binding protein 2; VCA, verprolin, cofilin, acidic; VLA-4, very late antigen-4; WASp, Wiskott-Aldrich syndrome protein; WAVE, WASP-family verprolin homologue; WDR5, WD repeat domain 5; WIP, Wiskott-Aldrich syndrome protein-interacting protein

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can induce morphologic changes in the cytoskeleton, and in the cell, and produce specific structures and different cytoskeletal dynamics, depending on the environment.[5] As we will describe in this review, the cytoskeleton plays a pivotal role in mediating forces transduced to mechanosensitive receptors such as the T cell and B cell receptors (TCRs and BCRs), and on integrin orientation and affinity. Furthermore, we describe how reorganization of the cytoskeleton following cell seeding on substrates of different rigidities was shown in different cell systems to directly impact signaling events, emphasizing that the cytoskeleton acts as a master mediator between external physical cues and internal biochemical signaling cascades.

Studying processes of mechanotransduction/mechanosensation in nonadherent cells such as leukocytes and erythrocytes is more complex, given that they are subject to very different types of physical stress compared to adherent cells in solid tissues. Different forces are applied to and sensed by immune cells during circulation through blood and lymphatic vessels, and when they traverse into tissues.[6–9] Furthermore, force is applied to and sensed by lymphocytes during cell/cell contacts.[10] Advanced technologies such as atomic force microscopy (AFM), traction force microscopy (TFM),[11] tension gauge tethers,[12] and molecular force sensors[13] are enabling researchers to directly measure forces that these cells apply to different solid substrates.[14] Hence it is becoming increasingly evident that mechanosensation and subsequent mechanotransduction play a role in many of the tasks performed by immune cells.[14] Whereas most of the studies on mechanotransduction/mechanosensation in lymphocytes were conducted in T or B cells, the effects of mechanical force on the function and regulation of natural killer (NK) cells remains poorly understood. Additionally, it is unclear how intracellular signaling molecules that operate downstream to receptor engagement are influenced by mechanosensitive mechanisms. This review highlights force transduction in lymphocytes, with a focus on the role of the cytoskeleton in lymphocyte function, and specifically how it transduces physical cues from the environment into signaling cascades, which have important ramifications for the immune response. We will focus on recent discoveries demonstrating a novel role of the cytoskeleton in tuning the NK cell immune response through a force-dependent mechanism, which acts on intracellular signaling molecules.

2 | THE CYTOSKELETON AND MECHANOSENSITIVE MOLECULES

Mechanosensitive molecules change their conformation as a result of applied force, and subsequently transduce this information into biochemical cascades. Cells generate force through rearrangement of their cytoskeleton, a process mediated via actin polymerization and depolymerization, and myosin motor function. In addition, protein complexes which link the cytoskeleton to the extracellular matrix (ECM), known as focal adhesion (FA) proteins, serve as the principal mechanosensors and mechanotransducers of the cell. Actin polymerization is regulated by actin nucleating factors such as Arp2/3, which induces branching of new filaments from preexisting ones,[15] and formins, which promote linear actin polymerization from fast-growing filament ends.[16] Nucleation promoting factors, such as the Wiskott-Aldrich syndrome protein (WASP) family of proteins, facilitate actin nucleation by recruitment of Arp2/3 to their verprolin, cofilin, acidic (VCA) domains and facilitate its activation.[17] Nonmuscle myosin II also mediates cytoskeletal rearrangement via contraction of actin filaments. Myosins hydrolyze ATP in order to exert force on actin filaments, and this action is regulated through site-specific phosphorylation of their regulatory light and heavy chains by several kinases, the most notable of which are myosin light chain kinase (MLCK), Rho-associated, coiled coil-containing kinase (ROCK), and protein kinase C (PKC).[18]

Microtubules are an additional cytoskeletal component, which undergo dynamic rearrangement through polymerization of $\alpha\beta$-tubulin heterodimers, and depolymerization, primarily at the plus end where β-tubulin is exposed.[19] Microtubules play critical roles in NK and CD8 T-cell cytotoxicity, facilitating delivery of cytolytic granules to the contact site with target cells, the immunologic synapse (IS), via polarization of the microtubule organizing center (MTOC).[20,21]

In order to transmit force into the cell, the cytoskeleton must be docked to the cell's surroundings. FA proteins dock adherent cells and leukocytes onto the ECM by mediating binding of the cytoskeleton to integrins.[22,23] Super resolution microscopy showed that integrins and the actin cytoskeleton are separated by a roughly 40 nm FA complex which includes, among other proteins, talin, paxillin, vinculin, zyxin, and α-actinin.[24] A clear correlation exists between applied force and FA size and orientation. This is underscored by the fact that inhibition of acto-myosin activity reduces FA assembly.[25–27] In support of these findings, FA proteins have been shown to be intrinsically mechanosensitive. Examples of this can be observed in the member of the mechanosensitive Cas family, p130Cas, which was shown to become extended and phosphorylated under tension,[28] zyxin which is recruited to strained areas along actin fibers, thereby regulating their elasticity,[29] talin, which was demonstrated to alter conformation as a function of force exertion,[30,31] and vinculin, which requires force to localize to FA compelxes.[25,27] Quantitative measurements using cytoskeleton laser nanosurgery also provided evidence of direct actin stress fiber association to the substrate, recruiting FA proteins such as zyxin to areas with highest tensed anchorage.[32] FA molecules play important roles in various lymphocyte functions, specifically through regulation of integrins. In T cells, talin is important for inducing high-affinity conformation of lymphocyte function-associated antigen 1(LFA-1), facilitating migration.[33,34] Furthermore, talin-mediated regulation of LFA-1 plays a role in T follicular helper cell differentiation and T-cell activation.[35,36] In B cells, talin regulation of integrins LFA-1 and very late antigen-4 (VLA-4) is critical for migration to lymph nodes and bone marrow, as well as for response to T-cell antigen.[37] NK cells require talin for LFA-1-mediated adhesion and cytotoxicity,[38,39] and paxillin for lytic granule polarization.[40,41] Paxillin also strengthens binding of VLA-4 to the endothelial vasculature in T cells,[42] and was recently shown to play a role in the effector function of tumor infiltrating T cells by facilitating CD103 integrin-mediated adhesion and outside-in signaling.[43] In addition, vinculin facilitates high-affinity integrin binding in T cells and B cells.[44,45] Additional mechanosensitive

molecules include mechanosensitive ion channels, an example of which are Piezo ion channels, which were shown to respond to physical disturbances in lipid bilayers and to link these perturbations into ion flow.[46,47] The mechanosensitive Piezo1 ion channel was recently shown to be important in T-cell activation–stimulation of T cells generated calcium flux through the Piezo1 channel, and this induced calpain-mediated actin reorganization.[48]

In adherent cells, several lines of evidence demonstrate how the effects of substrate rigidity on cytoskeletal architecture (mechanosensation) impacts cell behavior. Cell differentiation, spreading, actin stress fiber formation, and FA formation can be regulated by ECM rigidity.[49–52] It is thus evident that the cytoskeleton can "feel" external rigidities, and alter cell morphology accordingly.[5] Importantly, this change in the actin cytoskeleton following mechanical stress can directly impact cell signaling (mechanotransduction).[53,54]

3 | MECHANICAL FORCE IN THE SERVICE OF LEUKOCYTE FUNCTION

In immune cells, it is becoming evident that physical force regulates immune responses at multiple levels. Leukocytes encounter different physical forces under distinct conditions. These can be categorized, on the one hand, into fluid shear forces encountered in blood and lymphatic vessels with cellular substrates such as endothelial cells on vessel walls. On the other hand, different mechanical forces regulate lymphocyte responses when they are activated in vitro with ligands for activating receptors, or during physiologic settings with antigen presenting cells (APC) or target tumor/virally infected cells.

3.1 | Force in the regulation of leukocyte rolling and diapedesis

In blood vessels, leukocytes encounter fluid shear forces that impact rolling and attachment to vessel walls. High shear flow reduces pseudopod formation and attachment of neutrophils and monocytes to vessel walls[7]; however, moderate shear is necessary for the formation of optimal bonds between the adhesion molecule L-selectin and its ligands to enable leukocyte rolling on blood vessels.[55] It was proposed that this process prevents aggregation of leukocytes under low shear conditions. In large veins and capillaries that contain low shear forces this mechanism may prevent abnormal aggregation of neutrophils, which were shown to roll on each other via L-selectin.[56] Furthermore, it is speculated that because shear flow is high at the vessel wall and low near the center of the vessel, that this mechanism also restricts attachment and rolling only at the vessel wall.[55] Shear forces additionally impact conformation of the integrin, LFA-1. Specifically, selectin bonds induce extended conformation of LFA-1 in neurophils,[57,58] and shear flow increases integrin-mediated lymphocyte adhesion.[59] Recently, it was shown that shear forces influence B-cell migration during antigen collection in the spleen, dictating marginal zone B cell movement into the spleen follicle for antigen delivery.[60] These elegant mechanisms act to both regulate leukocyte aggregation and enable attachment to vessel walls during inflammation, and to enable leukocyte migration to perform effector functions.

Once immune cells arrest on vessel walls, they must leave blood vessels and migrate to inflamed tissue, in a process called diapedesis. Mechanical forces mediate this process in addition to 3D migration of immune cells through tissues. Improvements in 3D culture models are providing more physiologic tools that accurately depict the in vivo mechanics of cell migration through vessel walls and throughout tissues.[61] The force-dependent mechanisms governing 3D leukocyte migration are complex, because vascular endothelial cells are also mechanosensitive.[13,62–64] A recent study decoupled forces exerted by leukocytes and vascular endothelial cells in order to elucidate the function of each cell type during lymphocyte diapedesis. By utilizing 3D TFM, it was shown that leukocytes exert force to open junctions between vascular endothelial cells. Under inflammatory conditions, less traction stress is required for diapedesis, facilitating leukocyte extravasation.[9] Because less traction force is required for transendothelial migration during inflammation, this mechanism may also protect nuclear and DNA integrity, both of which were shown to undergo mechanical damage during invasive migration.[65]

Accordingly, another recent study demonstrated that T cells can sense 3D environments and density of the collagen matrix. 3D collagen matrices induced methylation of histone 3, lysine 4 residue (H3K4) by methyltransferases, and this methylation increased as a function of collagen density. H3K4 methylation in T cells in 3D environments was associated with less dense chromatin structure, deformable and less stiff nuclei, and lower nuclear viscosity.[66] This mechanism operated through the WD repeat-containing protein 5 (WDR5) subunit of methyltransferases. Importantly, the cytoskeleton was shown to couple the methylation of H3K4 through WDR5 in a 3D environment, shedding light on a novel mechanotransducing role for WDR5 in T cells. This mechanism may facilitate lymphocyte migration through tissues and protect nuclear content. Furthermore, this work sheds light onto how cytoskeletal alterations due to environmental changes act upon signaling molecules to shape immune cell output. This study compared the effects of collagen coated 2D wells to 3D collagen matrices in T-cell migration, emphasizing how 2D systems may not be optimal for delineation of mechanosensitive mechanisms, and implementation of 3D systems more accurately depicts these processes in migrating leukocytes.[61]

3.2 | Mechanical force at immune cell to target cell contacts

3.2.1 | NK cell signaling cascades IS architecture

NK cells are lymphocytes that play an important role in the innate immune response. Their central function is immune surveillance, eliminating virally infected cells and tumors.[67] NK effector functions include both a cytotoxic pathway and a cytokine secretory pathway. Cytotoxicity is mediated through release of cytolytic granules containing perforin and granzyme-B, inducing target cell apoptosis. Cytokine secretion influences various components of the immune system, and directly impacts transformed cells.[68,69] Unlike T cells that utilize a highly specific TCR to identify specific antigens, NK cells express

germline-encoded receptors to facilitate their function.[70] Ligation of activating or inhibitory receptors expressed on the surface of NK cells with their cognate ligands induces either activating or inhibitory signaling cascades, respectively.[70] The engagement of activating receptors with cognate ligands results in either immunoreceptor tyrosine-based activation motif (ITAM)-dependent or ITAM-independent pathways. The ITAM-dependent pathway includes ligation of activating receptors such as CD16 with the Fc portion of antibodies on target cells, which induces antibody-dependent cell-mediated cytotoxicity (ADCC),[71,72] or ligation of natural cytotoxicity receptors (NCRs) NKp46, NKp44, and NKp30, with their cognate ligands, such as viral hemagglutinins and heparin.[73] Ligation of NKG2D and 2B4 with cognate ligands transduces activation via an ITAM-independent pathway.[74,75] Ultimately both activating pathways induce a protein tyrosine kinase (PTK)-dependent cascade that promotes actin reorganization, MTOC polarization, cytokine secretion, and cytotoxicity.[76] Conversely, the ligation of inhibitory receptors containing immunoreceptor tyrosine-based inhibition motif (ITIM) with cognate ligands abrogates the phosphorylation of activating molecules via recruitment of protein tyrosine phosphatases (PTPs).[77,78] Inhibitory killer-cell immunoglobulin-like receptors (KIRs) and the inhibitory NKG2A/CD94 heterodimer recognize human leukocyte antigen (HLA) molecules, and can subsequently recruit phosphatases such as the SH-2 containing inositol 5′ polyphosphatase 1 (SHIP-1) or the Src homology region 2 domain-containing phosphatases 1 and 2 (SHP-1/2). Dephosphorylation of signaling molecules, such as VAV1, PLCγ-1, and LAT, by the SHP-1 phosphatase, increases the NK-cell activation threshold and induces inhibition.[79–82]

SHP-1 consists of a catalytic phosphatase domain near its C′ terminus, two phospho-tyrosine binding SH2 domains toward its N terminus, and sites of tyrosine and serine phosphorylation that might regulate its activity at the end of its C-terminal tail.[83] The crystal structure of SHP-1 revealed much about its regulation. In its inactive form, SHP-1 resides in a closed conformation with its N terminal SH2 domain bound to its catalytic domain. This prevents phosphatase activity by impeding access of substrates into the SHP-1 active site. Binding to phosphorylated tyrosines (such as phosphorylated ITIMS on inhibitory receptors, including KIR) through its SH2 domains causes a conformational alteration in SHP-1. This releases it from the inhibited state, and allows it to execute its catalytic function.[84,85] Thus, the molecular conformation of SHP-1 impacts its phosphatase activity,[85] and indeed, we recently demonstrated that SHP-1 is mechanosensitive, that is, regulated through cytoskeletal forces to tune NK-cell immune responses,[86] as will be discussed in the following sections.

3.2.2 | IS formation and organization

The IS refers to the interface between immune cells and target cells. Numerous molecules participate in the IS including cytoskeletal proteins, surface molecules, signaling molecules and cellular organelles. This process is suggested to enable stable contacts, induce signaling cascades, and to facilitate lymphocyte effector function[87–89]

The IS is characterized by three separate compartments known as supramolecular activation clusters (SMACs) based on actin morphology and dynamics, and by distinct signaling molecules present in each area. In T cells, during activation and IS maturation, TCR and major histocompatibility complex (MHC)-peptide interactions are formed in the distal SMAC (dSMAC) and are carried by centripetal actin flow (actin retrograde flow, ARF) to the peripheral SMAC (pSMAC) and finally to the central SMAC (cSMAC).[90,91] Adhesion molecules such as LFA-1 and its cognate ligand, intercellular adhesion molecule 1 (ICAM-1), are segregated from TCR-MHC clusters in the dSMAC, and migrate centripetally to form a ring in the pSMAC. The mature IS contains TCR and activating signaling molecules in the cSMAC where signaling terminates, whereas adhesion molecules are situated in the pSMAC.[88,90,92]

At the natural killer immunological synapse (NKIS), adhesion molecules such as LFA-1 and macrophage-1 antigen (Mac-1) in the pSMAC stabilize the interaction between the NK cell and the target cell.[89,93] Early studies showed that the most prominent difference between the activating and inhibitory NKIS is the ratio between accumulating activating signaling molecules (Syk, ZAP70, and Lck) and the inhibitory SHP-1 molecule at the cSMAC.[94,95] Activation of NK cells with co-ligation of LFA-1 and the activating NKG2D receptor induces cell spreading and a symmetric NKIS. On the other hand, co-ligation with the inhibitory NKG2A molecule abrogates the activating morphology, inducing a transient amorphic NK synapse that promotes cell migration.[96] During inhibitory interactions between NK cells and targets expressing cognate inhibitory HLA molecules, KIR receptors and their HLA ligands accumulate and are surrounded by LFA-1 and ICAM-1.[97,98] Importantly, it seems that actin rearrangement is not essential for KIR recruitment to the inhibitory NKIS.[99] This model predicts an actin-depleted inhibitory NKIS; however, it was shown that NK inhibition involves cytoskeletal recruitment and sequestration of activating receptors CD2 and 2B4 with KIRs to prevent their signaling function. Furthermore, super resolution microscopy showed that NK cell inhibitory synapses are indeed not completely actin-depleted.[98,100]

The nanoscale organization of receptor clusters in NK cells was further elucidated through super resolution microscopy experiments. Inhibitory KIR2DL1 receptors were shown to be organized in nanoscale domains under basal conditions at the NK cell surface. These clusters decreased in size and became denser upon ligation of the activating NKG2D receptor, demonstrating intricate crosstalk between both receptors upon NK cell stimulation.[101] These alterations in cluster size may act to increase activating signal intensity, because larger receptor ligand spacing was shown to decrease activation responses in NK cells.[102] KIR2DL1 localization was shown to be stable in the inhibitory IS, while retaining the ability to migrate to additional synapses during NK cell conjugate formation with multiple targets.[103] Activating receptors such as KIR2DS1, on the other hand, appear to organize into larger clusters than KIR2DL1, and to aggregate with the adaptor protein DAP12 upon NK cell activation. Larger KIR2DS1 clusters appear to favor NK activation through ZAP70 phosphorylation, whereas inhibition is favored in larger KIR2DL1 clusters as demonstrated by SHP-1 phosphorylation.[104] In addition, it was recently shown that different ligands for the NKG2D activating receptor, UL16 binding protein 2 and MHC class I polypeptide-related sequence A (ULBP2 and MICA) impact its surface organization. ULBP2 ligation causes NKG2D aggregation with the IL-15 receptor, whereas MICA induced shrinking of the NKG2D nanoclusters. Thus not all

ligands for the same receptor activate NK cells in the same manner, and induce different receptor organization accordingly.[105]

3.2.3 | Effector functions mediated by the IS

Effector functions, namely secretion of cytolytic granules, which induce target cell apoptosis, are facilitated by the IS architecture.[106] It was shown that cytolytic pore forming perforin proteins accumulate and are secreted predominantly in the cSMAC[107]; however, recent evidence demonstrates that perforin secretion is situated in areas of highest force exertion at the IS, and not necessarily limited to the center.[10] NK degranulation was defined in more detail through use of super resolution microscopy. These studies showed that degranulation occurs at low density regions of F-actin, which are precisely large enough to fit lytic granules. Upon primary NK cell stimulation with co-ligation of LFA-1 and NKG2D, granules dock to specific domains in which the membrane-bound actin network (cortical actin) becomes permeable.[100,108] It was shown that these hypodense actin regions at the NKIS are mediated through actin deconstruction by coronin 1A.[109] NK cells from a patient deficient in coronin 1A displayed deficient cytotoxicity,[109] and the importance of these mechanisms was also shown recently in Chediak-Higashi syndrome, a primary immunodeficiency caused by impairment of lysosomal trafficking. These separate studies verified that the cytoskeleton acts as a barrier to cytolytic granule secretion, impeding NK cell cytotoxicity.[110] Thus, it seems as though the actin cytoskeleton impedes NK cell degranulation, and forms a barrier at areas of the NKIS where granules are released. However, recent experiments using super resolution microscopy demonstrated that local nanoscale actin turnover, mediated through Arp2/3, is imperative for NK cell degranulation.[111] Thus a delicate actin architecture is assembled for degranulation at the NKIS, including nanoscale holes that enable granule exocytosis, and areas of high nanoscale actin turnover. Perforin-containing granules require microtubule function to polarize toward the NKIS.[107] Prior to microtubule polarization toward the NKIS, lytic granules converge onto the MTOC.[112] It was recently shown that this convergence is critical for directed secretion, sparing bystander cells from cytolysis.[113] Thus, the NKIS balances activating and inhibiting signals meticulously through cytoskeletal and signaling cluster reorganization. These processes emphasize the highly regulated mechanisms ensuring NK cell cytotoxicity in a precise and directed manner, orchestrated by simultaneous actin clearance and local nanoscale actin dynamism.

3.2.4 | Cytoskeletal dynamics at the IS

The movement of TCR signaling clusters is dependent on ARF, mediated by actin polymerization and de-polymerization, as well as myosin motor protein contraction.[114] Abrogating these cytoskeletal dynamics inhibits signaling microcluster (MC) formation and T cell activation.[90,115] Hence, centripetal flow of the actin cytoskeleton promotes the formation of new activating MCs and limits the lifetime of the T cell response. In the B cell IS, it was similarly demonstrated that the ability of B cells to spread and to subsequently aggregate antigen is dependent on actin dynamics.[116]

Cytoskeletal dynamics at the NKIS were also shown to be critical for NK cell activation and effector function. Principal signaling molecules such as VAV1 and WASp induce downstream actin reorganization in NK cells to promote the effector stage of the NK cell lytic synapse.[117,118] As in T cells, MC formation, mediated through the cytoskeleton, was shown to enhance NK signaling.[119] This is evident through studies demonstrating that MC formation of LFA-1, MAC-1, and CD2 depend on actin reorganization,[107] polarization of the MTOC is dependent on actin dynamics at the NKIS,[117] and myosin IIA function and actin reorganization (including actin polymerization and actin clearance at the IS) are essential for proper lytic granule release at the synaptic cleft.[108,109,111,120–122] Hence, it is evident that actin dynamics are important for lymphocyte MC formation, activation, effector function, and ultimately, for signal termination, and these dynamics are dependent on receptor ligation and specific signaling molecules[123,124]

Recently, a mechanical-dependent regulation of receptors and signaling molecules by force transduced through acto-myosin cytoskeleton dynamics was observed in NK cells by us.[86] This new concept will be discussed at length in the following section.

4 | INTERPLAY BETWEEN CYTOSKELETAL DYNAMICS, INTEGRIN AFFINITY MATURATION, AND SIGNALING

Integrin activity and affinity maturation are well established mechanosensitive mechanisms.[125] Applying tension on integrins was shown to recruit FA proteins and induce integrin conformational activation.[126] Coupling of integrins to the actin cytoskeleton through FA proteins such as talin and vinculin activates a molecular clutch, which responds to the physical characteristics of the substrate, altering actin dynamics.[127,128] The molecular clutch model is supported by evidence that the rigidity of the substrate has a direct effect on the clutch. For example, it was demonstrated that higher rigidity enforces conformational opening of talin, leading to transcriptional changes in mouse embryonic fibroblasts.[129]

Previous studies examining migrating epithelial cells showed that the rate of centripetal actin flow decreases in areas of FA formation, whereby areas of large FAs and slower actin rates are directly correlated with traction stesses.[130] These findings support a model in which engagement of the molecular clutch in high-tension areas decelerates ARF and contributes to force generation and cell migration. Vinculin was identified as one mediator of this process, linking the cytoskeleton to FAs, and slowing retrograde flow in order to generate high traction forces.[131] Evidence for molecular clutches has additionally been described recently in the context of lipid bilayer surfaces.[132] These surfaces do not display elastic properties, yet less viscous surfaces displayed fewer FAs, negatively impacting differentiation of myoblasts. Furthermore, this study showed that ARF velocity decreased on more viscous surfaces, providing evidence that cytoskeletal engagement with the clutch decelerates centripetal actin flow. These studies emphasize that the mechanosensitive integrin pathway that is

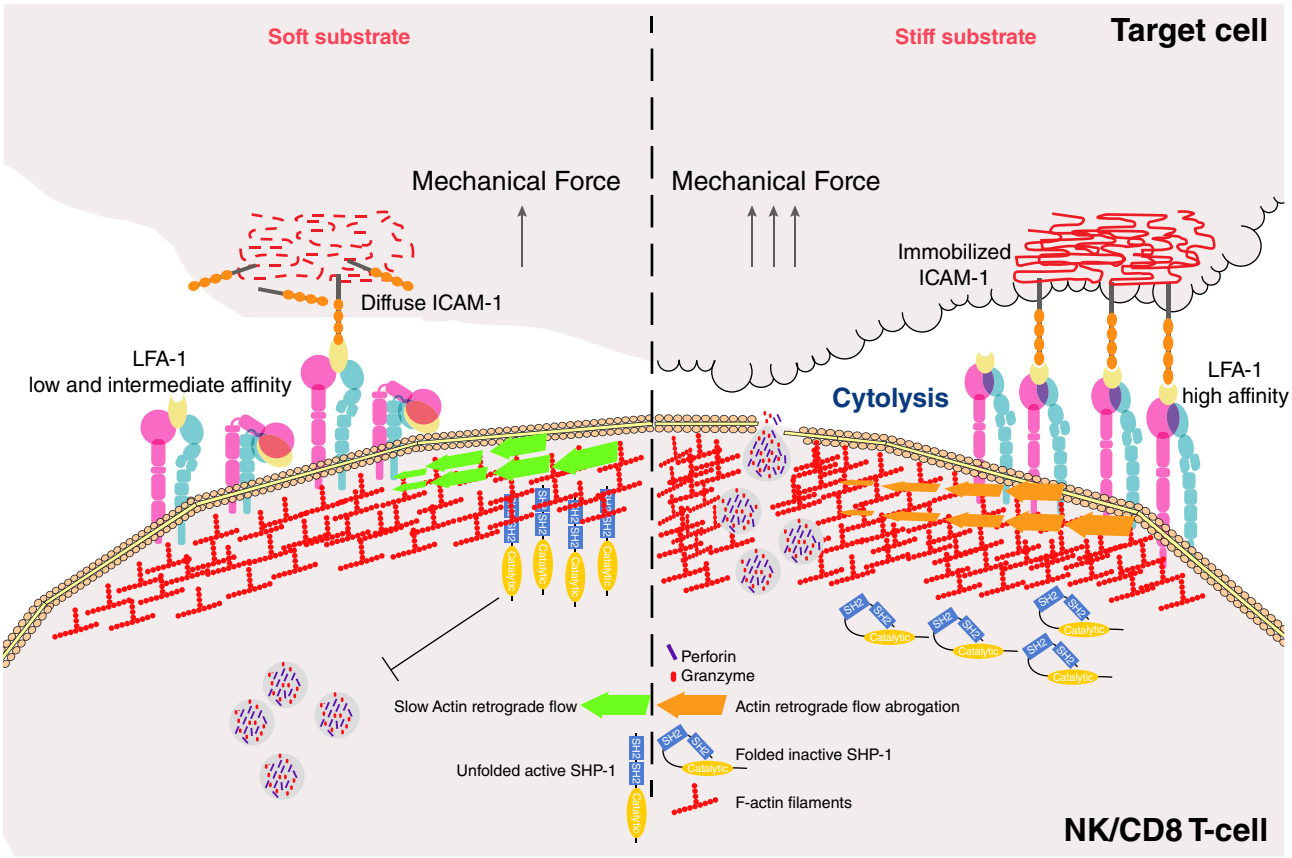

FIGURE 1 Variation in substrate stiffness and ligand mobility impact lymphocyte activation and effector functions: A potential role for mechanical force in SHP-1-mediated inhibition. Low substrate rigidity and high ICAM-1 diffusion are unfavorable for lymphocyte activation (left). Centripetal actin flow enables the SHP-1 open catalytic conformation, and increases the activation threshold (left). In contrast, high substrate rigidity and immobilized ICAM-1 favor lymphocyte activation and effector functions such as cytotoxic granule release.[10,135] Constrained LFA-1 greatly diminishes ARF, interfering with SHP-1 catalytic activity and reducing the activation threshold (right)

transduced to the cytoskeleton is influenced by elasticity of the substrate, as well as by the mobility of ligands on substrate surfaces.

In leukocytes, the effect of force on integrin conformation was shown in the context of migration, adhesion to vessel walls, and contact with APCs.[133] For example, shear forces induce integrin activation via selectin bonds to increase neutrophil adhesion with vessel walls,[58] and recently, it was shown that tension applied on the LFA-1 β_2 integrin subunit by the cytoskeleton induces a high-affinity LFA-1 conformation during T-cell migration.[34] Intriguingly, studies conducted in T cells showed the direct impact of ARF on affinity maturation of integrins. Specifically, ARF deceleration was shown to be critical for the high-affinity LFA-1 conformation at the IS.[134] Supporting the mechanism of the molecular clutch model, this study demonstrated that immobilized ICAM-1 is critical for LFA-1 conformational change, establishing that restrained cytoskeletal flow and tension are required for this process (Fig. 1). In accordance with these findings, an additional study of dendritic cells participating in the IS showed that constraining of ICAM-1 by the cytoskeleton is important for conjugation and T-cell priming.[135] Recently these observations were given additional support, as it was shown that in addition to affinity maturation, LFA-1 orients in the direction of ARF during T-cell migration.[136]

We recently demonstrated in NK cells, that the cytoskeleton not only facilitates effector functions and receptor alignment, but also regulates signaling directly through dynamic reorganization.[86] During inhibitory interactions, ARF velocity is significantly slower than during activating interactions. Association between SHP-1 and β-actin is favored under inhibitory conditions, and the slower moving actin filaments facilitate the catalytically active conformation of SHP-1, and subsequent NK inhibition, through dephosphorylation of key SHP-1 substrates such as VAV1 and PLCγ-1/2.[86] Critically, SHP-1 enzymatic activity is dependent on actin dynamics. In this study we implemented a YFP-SHP-1-CFP Förster resonance energy transfer (FRET) bio sensor to observe SHP-1 conformation dynamics in real time, during relevant physiologic interactions between NK cells and target cells. Abrogation of actin dynamics using small molecule inhibitors such as jasplakinolide, which stabilizes actin filaments, and cytochalasin D, which inhibits actin polymerization, retained SHP-1 in a closed inactive conformation, highlighting the cytoskeletal regulation on the enzymatic activity of SHP-1. Furthermore, this mechanism appears to be mechanosensitive. Following seeding of NK cells stably expressing F-tractin GFP, a small fluorescent actin probe, onto stiff (50 kPa) hydrogel substrates, we observed abrogated actin dynamics. Repeating this experiment with NK cells transfected with the YFP-SHP-1-CFP FRET bio sensor onto stiff (50 kPa) hydrogel substrates demonstrated that SHP-1 conformation was retained in a closed inactive state as measured by intracellular FRET efficiency. This process enhanced NK cell activity,

resulting in high tyrosine phosphorylation of SHP-1 substrates, and increased NK cell effector functions. The effect of actin dynamics on SHP-1 shows for the first time that dynamic actomyosin movement converts the conformation of a cytosolic enzyme in NK cells, and determines NK cell behavior. This mechanism also demonstrates how the environment impacts cytoskeletal dynamics in NK cells, influencing signaling cascades.

Our study complements the work conducted by Jankowska et al. who showed that the engagement of integrins with cognate ligands slows centripetal actin flow, subsequently causing a decrease in phosphotyrosine profiles of critical signaling intermediates.[137] However, it remained unclear why restraining actin flow at the IS would cause such a dramatic decrease in phosphorylation of downstream activating molecules. In a recent commentary, we compared both mechanisms, and proposed an integrated model suggesting a potential feedback response regulated through actomyosin dynamics. Accordingly, inhibition of actin dynamics through integrin engagement may activate phosphatase activity, thereby completing a negative feedback loop to quench the immune response.[138] Our findings may fill in the gap in our understanding of why slower-moving actin filaments decrease phosphorylation of PLCγ-1 and ZAP70 by providing an underlying molecular mechanism impacting SHP-1 catalytic activity. Both studies demonstrate a potential feedback loop mediated through cytoskeletal forces that initiate activation, and aid in termination of immune responses. This global mechanotransduction may also explain how cytotoxic and noncytotoxic synapses rapidly form and collapse, facilitating simultaneous rapid immune scanning of healthy and transformed cells. It remains to be determined what other events restrict actin dynamics at the NKIS, and it would be interesting to speculate whether KIR or other inhibitory receptor engagements physically restrict actin flow.

Interestingly, other studies showed how different actin architectures impact NK cell peripheral tolerance and responsiveness. Guia et al. showed that in unresponsive NK cells, which develop in the absence of MHC-I ligands, activating and inhibitory NK cell receptors are confined together by the actin meshwork, reducing NK cell function.[139] Responsive NK cells, on the other hand, sequester inhibitory receptors separately from activating receptors, facilitating NK cell effector function. Interestingly, a recent study demonstrated that responsive NK cells exhibit more diffuse NKp46 movement that supported the NK cell response, whereas the inhibitory LY49A molecule was more confined in these cells. Importantly, this work showed that NKp46 interaction with the cytoskeleton is crucial for NK cell activation signaling.[140] It remains to be determined whether these mechanisms, which depend on cytoskeletal dynamism and architecture, are also mediated by external force.

5 | MECHANICAL REGULATION OF TCR AND BCR SIGNALING

In addition to force-mediated integrin activation, the cytoskeleton is emerging not only as a scaffold for MC formation[123,141,142] and for ensuring effector responses,[143,144] but has been further shown to directly influence signaling downstream to the TCR and BCR. The effect of mechanotransduction on the TCR is well established. The TCR deformation model proposes that force applied to the TCR induces a conformational change that initiates T-cell signaling.[145] Indeed, optical tweezer experiments which applied force on TCRs of individual cells induced pseudopodia formation,[146] whereas an additional study investigating TCR:pMHC interactions showed that specific antigens coupled with tangential force are required to induce calcium flux in T cells.[147] Application of optimal force (optimal at 10 pN) on TCR bound to agonist pMHC was shown to be required for a stable prolonged catch bond that is stable within the first minute of TCR-pMHC contacts. However, force implemented on TCRs stimulated with antagonistic peptides, induced slip bonds which reduce the lifetime of TCR:pMHC interactions.[148] Recent breakthrough experiments uncovered precise physical mechanisms by which the TCR converts force into signaling cascades.[149] By uncovering the structure of the TCR-α transmembrane subunit by NMR, it was shown that, under force, the TCR-α subunit transmembrane domain undergoes a conformational change which shifts two amino acid residues, Arg251 and Lys256, and causes TCR-$\alpha\beta$ and CD3 to dissociate.

Importantly, force-dependent activation of the TCR was shown to require cytoskeletal function.[150] Experiments that perturbed cytoskeletal dynamics abrogated TCR-mediated T cell activation,[90,114] and several experiments exposed the role of cytoskeletal dynamics in facilitating the forces required for TCR activation. TFM experiments showed that cytoskeletal-derived forces increased the mechanosensitivity of TCR stimulation, as small molecule inhibitors of actin dynamics led to reduction of traction forces produced by T cells.[151] Additionally, AFM cantilever experiments, in which the TCR was stimulated with antigen, showed higher activation as a function of higher force exertion. The effects observed depended on the cytoskeleton, as utilization of actin inhibitors abrogated T-cell responses.[152]

One of the molecular complexes that was shown to bridge cytoskeletal-mediated dynamics and TCR force exertion at the IS is a molecular complex consisting of Dedicator of cytokinesis 8 (DOCK-8, a guanine nucleotide exchange factor critical for NK and T-cell actin reorganization[153]) WIP, and WASp.[154] Specifically, DOCK8 deficiency was correlated with low actin foci formation, and lower phosphorylation levels of the mechanosensory molecule CasL (a member of the mechanosensitive CAS family). Accordingly, a separate study showed that actin polymerization is important for CasL phosphorylation in TCR MCs, highlighting the function of CasL in bridging between mechanical forces and the T-cell cytoskeleton.[155] Besides actin filaments and myosin motor proteins, microtubules were also recently observed to regulate T-cell traction forces.[156] In this case, inhibiting microtubule dynamics led to an increase in T-cell traction stress, and it was found that microtubules induce this effect by regulation of myosin II phosphorylation, and inhibition of ARF. Thus, there appears to be a regulatory role through other cytoskeletal compartments that down-regulate forces needed for T-cell activation. It is interesting to speculate whether this is a negative feedback mechanism that serves to down-regulate activation by inhibiting traction forces augmented by F-actin and myosin. However, additional biochemical experiments are

required testing inhibition of microtubule dynamics and its effects on signaling pathways.

Force transduction through the BCR was similarly demonstrated to be dependent on the cytoskeleton. B cells utilize myosin motor force to contract the F-actin cytoskeleton, and thus acquire antigen by centripetal pulling of BCR-antigen clusters and subsequently endocytosing them. Only high-affinity BCR-antigen bonds are internalized, demonstrating the ability of B cells to discriminate between high- and low-affinity antigen via physical force.[157] Furthermore, experiments utilizing DNA tension sensors demonstrated that naive IgM BCRs require higher tension (56 pN) to achieve maximum activation, whereas memory IgG or IgE class switched cells may be activated under minimal tension (12 pN).[158] This mechanism helps explain how IgG and IgE B cells are activated more rapidly than IgM counterparts, which have a higher mechanical threshold for activation. Similar to T cells, it was recently demonstrated through TFM experiments that F-actin dynamics and myosin contractility contribute to generation of traction force and B-cell activation.[159]

Physical force was recently shown to influence lymphocyte effector functions and development. Cytotoxic T cells that exert more force at the IS increase the potentiation of target cell killing by facilitating perforin pore formation.[10] This force exertion was dependent on myosin contractility, as abrogation of myosin dynamics reduced force exertion at the IS. Intriguingly, this study also proved that target cell membrane tension is also important for potentiation of perforin activity. Thus, physical properties of both sides of the lytic synapse regulate susceptibility to cytotoxic T-cell killing. It would be interesting to consider modifications of target cell rigidities as potential therapeutic targets. An additional recent study also showed how mechanotransduction through the TCR impacts T-cell differentiation in the thymus.[160] Through utilization of a bio-membrane force probe, it was demonstrated that ligands for negative selection of T cells in the thymus create long-lived catch bonds with TCR and CD8 receptors, whereas ligands for positive selection induced transient slip bonds. Catch bonds in this study were associated with more sustained force, and this ultimately distinguished between positive and negative selection. Importantly it was again established in this study that thymocytes generated forces on pMHCs through cytoskeleton dynamics.[160]

Experiments that utilized surfaces with tunable properties provided further proof that activation of T cells and B cells is force dependent. Some of these studies reported contradictory results,[161] yet lymphocytes tend to exert more force on rigid substrates due to stabilizing counter forces, and therefore such substrates are associated with increased activation (Fig. 1). A recent study showed that T cells seeded on stiff substrates (100 kPa vs.6.4 and 0.5 kPa polyacrylamide [PA] hydrogels) were more activated and proliferative.[162] Remarkably, this study also utilized polydimethylsiloxane (PDMS) substrates of varying rigidities (1.5 and 28 kPa) to tune the mechanical properties of APCs. When $CD4^+$ T cells were seeded onto stiffer APCs, they secreted more IFN-γ and TNF-α.[162] Though the variation in APC rigidity was modest, the differences evoked significant changes in T-cell responses. This highlights the physiologic importance of mechanical properties in evoking immune responses. Additionally, it was recently shown that B cells seeded on stiff (2.6 vs. 22.1 kPa when utilizing PA substrates and 20 vs. 1100 kPa when using PDMS substrates) substrates demonstrated stronger activation facilitated by PKCβ and FA kinase signaling,[163] in accordance with previous studies demonstrating the magnitude of B-cell activation over a variety of surfaces with tunable rigidities.[158,164] It remains to be determined if similar force-driven events that regulate TCR and BCR activation operate on NK cell receptor engagement, and how this translates to tuning the NK cell activation threshold. Due to the germline encoded receptors expressed by NK cells, and the dynamic equilibrium and co-ligation required between receptors, this task is more challenging, yet it could shed important light on how force is also integrated into the delicate balance of signaling downstream to NK cell receptors.

6 | CONCLUDING REMARKS

Observing mechanotransduction in the immune system is more complex than in adherent cells; however, recent technological advances have enabled scientists to directly measure forces exerted by leukocytes during an immune repsonse.[165,166] It is becoming evident that external forces are sensed by lymphocytes during activation and inhibition at the IS, and these are converted into biochemical signals that impact the immune response. Often, it is the cytoskeleton that mediates between external physical stimuli and signaling pathways.[4] In lymphocyte responses, it is becoming clear that the cytoskeleton is not simply a platform for signaling molecule organization and facilitation of effector functions, but rather, dynamic cytoskeletal remodeling in response to external cues directly impacts immune cell signaling. Centripetal actin flow can impact receptor and enzyme conformation, affecting phosphorylation profiles, and this is often dependent on ligand mobility.[134,135,137,150,167] It would be interesting to further investigate how actin polymerizing and depolymerizing factors are differentially regulated in relation to different levels of mechanical tension, and how these subsequently influence actin morphology. In NK cells, force-dependent mechanisms have been less fully explored, yet it is becoming clear that dynamic actin processes affect signaling events, and activation threshold.[86,139]

Nevertheless, several questions still remain. One unresolved question is how downstream signaling molecules, and not only mechanosensitive receptors and FA proteins, are impacted by force-driven events during lymphocyte functions. It is not yet known how different forms of receptor engagement impact actin dynamics, beyond simple actin recruitment at the NKIS, and how different combinations of receptor ligation are impacted by physical properties of the substrate. Direct measurements of force applied between NK cells and susceptible activating or inhibiting target cells with different combinatorial receptor engagements, could provide a great deal of information about the force-dependent mechanisms that regulate the NK-cell response. Furthermore, it is necessary to explore whether other signaling molecules, and specifically inhibitory phosphatases, such as SHP-2 or SHIP-1, are also recruited to the cytoskeletal machinery and are impacted by actin dynamics during different NK

cell responses. A recent study shed novel light on some of these issues by utilizing a nanowire based system coated with the NKG2D ligand MICA to measure force induced by NK cells, and found that NK cells displace a single nanowire with minimal force of 10 pN.[168] This study showed that similarly to T-cell mechanotransduction, the actin cytoskeleton was actively concentrated in areas of force exertion on the nanowires. Interestingly this study revealed that nanowires coated with activating NK ligands induced higher activation than flat surfaces coated with the ligands. This provides novel evidence regarding how mechanical inputs, including both rigidity and topography act together with biochemical inputs to tune NK cell activation. Recent super resolution microscopy experiments elucidated the highly regulated and intricate cytoskeletal architecture at the NKIS, in addition to organization of different KIR clusters, their sizes, and the effects of cluster size on signal transduction.[104,108,109,111] Whether and how force impacts these delicate aggregates is not known. It would be interesting to observe how this architecture, and how actin dynamics at the NKIS behave when exposed to substrates of differing stiffness and topographies. It can be speculated that stiffer surfaces, up to a certain threshold, would induce more NK cell degranulation; utilizing super resolution microscopic techniques may elucidate if this is merely a function of an increase in activation signaling,[86] or also due to a more permissive NK synapse. Furthermore, elucidating how inhibitory molecules, that is, SHP-1, are localized at the NKIS as a function of substrate rigidity, and if they are recruited to the dynamic actin puncta as a function of NK cell activation or inhibition can provide information regarding the regulation of these intricate mechanisms. Additional aspects of NK-cell biology, such as maturation and proliferation, should be tested when cells are situated in environments of differing rigidities. Though it is clear that cytoskeleton dynamics affect NK cell responses and signaling during activation and inhibition, more work clearly needs to be conducted in these cells to elucidate the regulatory outcome of force transduction.

Further work is also required to delineate the force-dependent process regulating lymphocyte responses in more physiologic settings. Our knowledge regarding how intracellular signaling molecules, and not only well defined receptors (such as the TCR, BCR, and integrins) are affected by mechanical force, are poorly understood. Implementing 3D systems and methods to quantify force transduction in vivo could shed important light on these unexplored issues.

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AUTHORSHIP

A.B.-S., B.S., and N.J. performed research through the literature, collected relevant information, and prepared drafts of the manuscript. M.B.-S. is the senior author; she corrected the drafts, and provided feedback and suggestions regarding presentation of the information.

DISCLOSURES

The authors declare no conflicts of interest.

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