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Review

The mechanobiology of NK cells- 'Forcing NK to Sense' target cells

Darren Chen Pei Wong [a,*], Jeak Ling Ding [a,b,*]

[a] Department of Biological Sciences, National University of Singapore, 117543, Singapore
[b] Integrative Sciences and Engineering Programme, National University of Singapore, 119077, Singapore

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ABSTRACT

Natural killer (NK) cells are innate immune lymphocytes that recognize and kill cancer and infected cells, which makes them unique 'off-the-shelf' candidates for a new generation of immunotherapies. Biomechanical forces in homeostasis and pathophysiology accrue additional immune regulation for NK immune responses. Indeed, cellular and tissue biomechanics impact NK receptor clustering, cytoskeleton remodeling, NK transmigration through endothelial cells, nuclear mechanics, and even NK-dendritic cell interaction, offering a plethora of unexplored yet important dynamic regulation for NK immunotherapy. Such events are made more complex by the heterogeneity of human NK cells. A significant question remains on whether and how biochemical and biomechanical cues collaborate for NK cell mechanotransduction, a process whereby mechanical force is sensed, transduced, and translated to downstream mechanical and biochemical signalling. Herein, we review recent advances in understanding how NK cells perceive and mechanotransduce biophysical cues. We focus on how the cellular cytoskeleton crosstalk regulates NK cell function while bearing in mind the heterogeneity of NK cells, the direct and indirect mechanical cues for NK anti-tumor activity, and finally, engineering advances that are of translational relevance to NK cell biology at the systems level.

1. Introduction

The study on how physical force may affect biological processes began in the late 1800s [1] and early embryologists and biologists embraced this idea by studying the physical nature of cells. With advancements in the understanding of cell and molecular biology, we now recognize that force exerted on or within the cell plays a central role in regulating protein functions, and thus, explanation by biochemical pathways alone is incomplete without the involvement of biophysical forces. For example, the focal adhesion protein, talin, is now recognized to undergo stochastic folding and unfolding and acts as a force buffer in the force transmission pathway [2]. Such examples of mechanotransduction underlie the process on how cells sense and translate physical force (e.g. matrix rigidity) into mechanical and biological responses.

The roles of mechanical force on adherent cell models have been relatively well defined. For example, cell surface mechanosensitive proteins and channels such as Piezo 1, directly respond to force and allows influx and efflux of ions (e.g. Ca^{2+}) [3–5]. It is evident that even homeostatic immune cells circulating the body are subjected to varying mechanical forces, such as fluid flow shear stress and extracellular matrix stiffness (Fig. 1). These forces can modulate various aspects of immune cell functions, such as cell trafficking, receptor activation and even cytokine production, all of which were previously discussed in general [6–9].

Chimeric antigen receptor (CAR) T-cell transfer has achieved substantial success in treating cancer [6]. The CAR T-cells form non-classical immune synapse with cancer cells to provoke mechanosignaling events which spatiotemporally remodel the T-cell cytoskeleton to facilitate CAR activation and kill the target cancer cells [10]. A number of reviews have documented the roles of mechanobiology in T-cell anti-cancer immunity [6,10,11]. Although CAR T-cells are effective against hematological malignancies, there are hurdles in treating solid tumours [12]. On the other hand, the innate immune NK cells that traditionally recognize cancer cells by the 'missing-self' signal, are gaining eminence due to their versatility in targeting tumour cells. Some advantages of NK cells include safety assurance, multiple mechanisms for activating and sustaining NK cytotoxicity and 'off-the-shelf' manufacturing possibilities [12]. However, hitherto, comprehensive reviews guiding readers on the roles of mechanobiology in shaping NK cell responses is lagging far behind that of T-cells. Furthermore, only limited attention has thus far been given to the roles of the intermediate filaments and septin

* Corresponding authors at: Department of Biological Sciences, National University of Singapore, 117543, Singapore.
E-mail addresses: dbsdwcp@nus.edu.sg (D.C.P. Wong), dbsdjl@nus.edu.sg (J.L. Ding).

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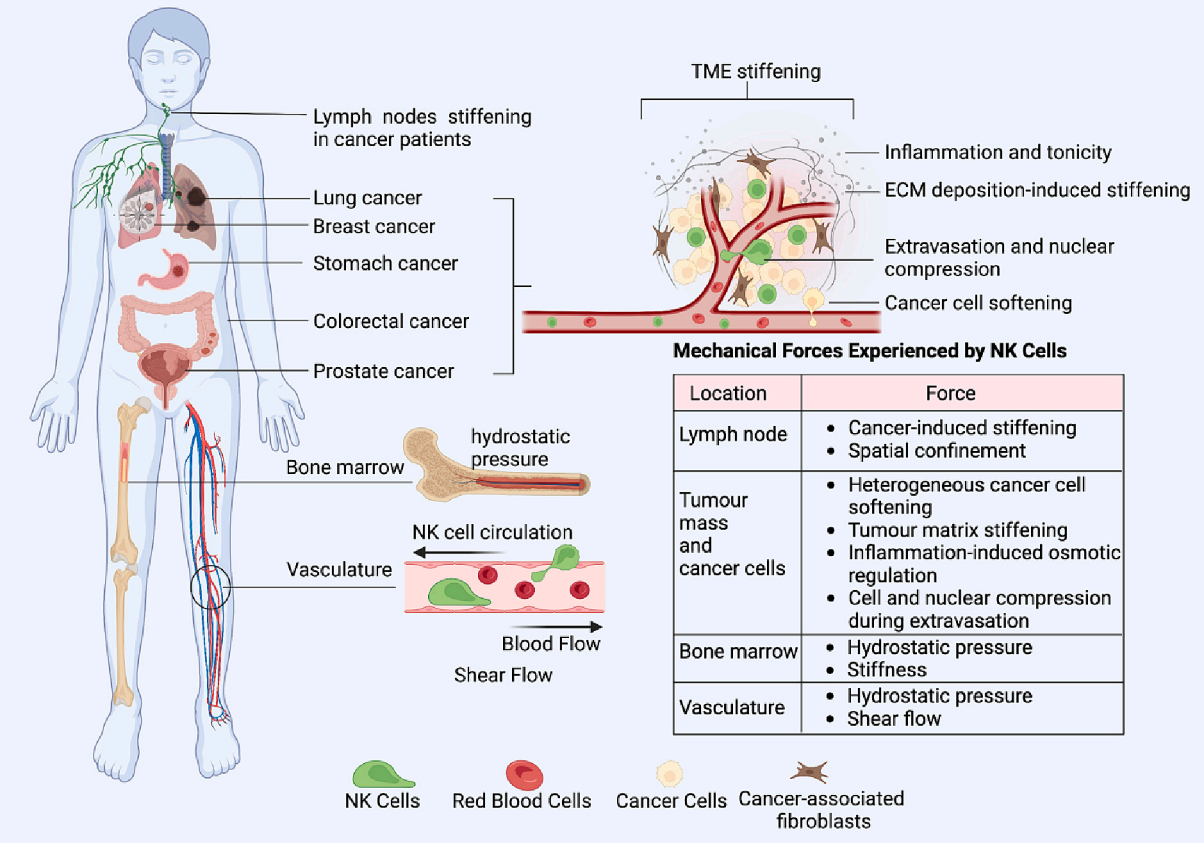

Fig. 1. Mechanical forces shape NK biology in the tumour microenvironment and circulation.
NK cells encounter diverse mechanical cues that warrant future studies on how NK mechano-immunomodulates cancer. The top five cancers, as listed by the World Health Organization (https://www.who.int/news-room/fact-sheets/detail/cancer), constitute ≈50% of all cancers worldwide. The tumour microenvironment (TME) contributes to biomechanical forces [17,18]. The stiffness of the extracellular matrix, a measurement of Young's Modulus (E) expressed in kilopascals (kPa), is one of the most studied biomechanical properties reported. The TME is generally stiffer than the surrounding tissues; cancer cells, on the other hand, are heterogenous in stiffness and usually softer than normal cells [19–21]. In addition, the secreted ECM deposits, cytokines and growth factors tend to alter the tonicity of the TME, creating a hypertonic tumor microenvironment that can affect the membrane tension [22] and intracellular contractility [23] of cells. Cancer progression and metastasis also affect lymph node stiffness [24] and NK cells may sense changes in stiffness in the lymph node, TME and even individual/ clusters of cancer cells. NK cells also constantly receive mechanical cues throughout development, maturation (in the bone marrow) and circulation in the vasculature [25]. These mechanical cues include hydrostatic pressure, spatial confinement, shear flow, and matrix stiffness (see table in Fig. 1). Many questions remain on how different mechanical cues under various environmental conditions shape NK responses. For example, whether cell and nuclear deformation during NK extravasation alter genomic responses and how shear flow affects NK uropod and surface receptor spatial organization remain to be understood.
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cytoskeletons in NK cell biology. The understanding of the regulation of NK cell cytoskeletal network is key to understanding the mechanobiology of NK cells. Various NK cell-related diseases [13,14] (discussed in detail below) are associated with defects in dynamic regulation of NK cell cytoskeletal components. NK cell sensing of substrate rigidity [15,16] has recently been reported to be an added level of control for NK cytotoxicity, and various advanced engineering technologies to leverage on NK cell mechanobiology has been undergoing development. Hence, the motivation for this review article.

Here, we first consolidate the available literature on NK cell cytoskeleton network and review how cytoskeletal components crosstalk to regulate NK mechanobiology. Importantly, we consider the heterogeneity of NK cells and discuss it in relation to the potential for next generation clinical trials. Next, we review several cancer therapeutic options that can affect NK cell mechanobiology and discuss advances in engineering materials and approaches used to study NK cell mechanobiology and their potential in contributing to advanced NK-based immunotherapies.

2. The cytoskeleton and NK cell function

We begin by examining the versatile and multifaceted roles of the cytoskeletal network constituting actin, microtubule, intermediate filaments and septin, all of which plays a vital role in sensing and transducing mechanical forces in cells. We consider the cytoskeletal network to be versatile and multifaceted because it serves as a molecular and structural scaffold to elicit cellular physiology and immune functions. For example, the microtubule network is well known to alter cell structure, maintain cell polarity [26], and even serves as a 'highway' for various microtubule-associated proteins (MAPs). Kinesins and dyneins, the two motor proteins that travel along microtubules, are examples of MAPs and have been implicated in regulating NK cell lytic granule release [27].

Given its versatility, it is unsurprising that all types of cytoskeleton play an essential role in regulating NK cell cytotoxicity. As the largest component of the cytoskeletal network, actin facilitates NK cell motility, infiltration [28,29] and organization of the immune synapse [30]. The microtubule-organizing center (MTOC) directs degranulation to target cells along the microtubule networks [31]. MTOC polarization towards the NK cell immune synapse (NKIS) is now known to be dependent on

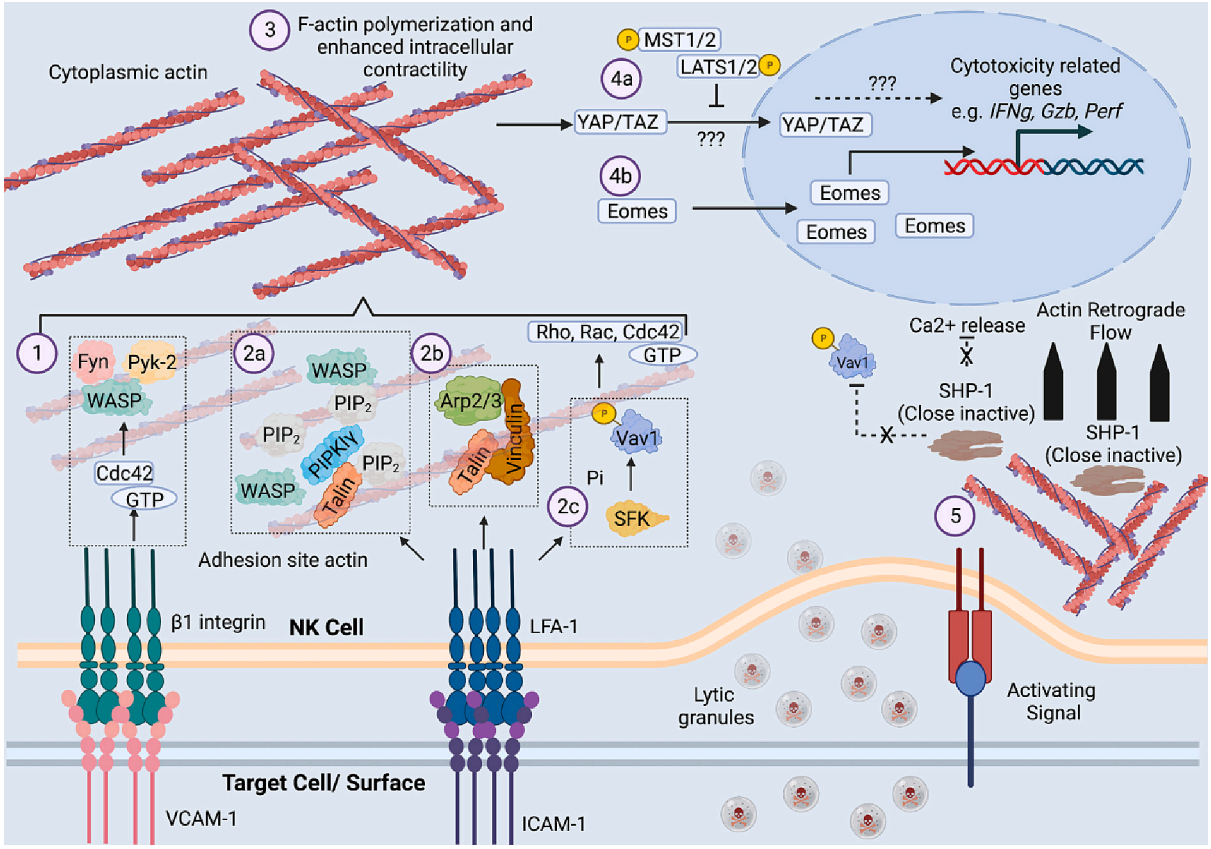

Fig. 2. Integrin signaling and activating signals remodel actin cytoskeleton dynamics for NK cell mechanotransduction. **(1)** Activation of β1 integrin by VCAM-1 expression on target cell/ surface results in the activation of the small GTPase, Cdc42, and WASP which associates with Fyn and Pyk-2 tyrosine kinases. Collectively, actin dynamics is altered. **(2)** Engagement of the β2 integrin LFA-1 to ICAM-1 on target cell/ surface induces actin reorganization in at least three known mechanisms. **(2a)** Talin binds to PIPKIγ, which synthesizes PIP_2. Wasp is recruited by the presence of PIP_2 and facilitate actin reorganization. **(2b)** ICAM-1 separates 2 chains of LFA-1 to expose the cytoplasmic tail of LFA-1 to talin, which is recruited to the plasma membrane with LFA-1-ICAM1 engagement. Talin association with vinculin recruits the actin nucleating protein Arp2/3 and promotes actin reorganization. **(2c)** Stimulation of LFA-1 recruits the Src family kinases to phosphorylate and relieve Vav1 from an inhibitory state. Vav1 acts as a Rho-GEF and promotes actin reorganization through small GTPases Rho, Rac and Cdc42. **(3)** Cytoplasmic actin relays mechanical signal from cell surface to alter intracellular contractility. **(4)** Transcription factors (Eomes) and cofactors (e.g. YAP/TAZ) translocate into the nucleus in response to enhance intracellular contractility. **(4a)** YAP/TAZ intracellular localization is subjected to kinase regulation. However, how intracellular contractility controls the regulatory kinase (MST1/2 and LATS1/2) activities and the roles of YAP/TAZ in NK cell biology remains elusive. **(4b)** The conserved Eomes transcription factor translocates to the NK cell nucleus to evoke early cytotoxic responses. **(5)** Activating signal initiated by activating receptors result in fast actin retrograde flow that prevents SHP-1 activation and binding to actin network. SHP-1 activation will result in dephosphorylation of Vav1, thereby affect actin dynamics and calcium release.
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the mechanical properties of the target surface [15]. The intermediate filaments consist of a large group of proteins which provide the cellular architectural framework [32], and their presence on target cells seem to affect NK cytotoxicity [33]. The GTP-binding septin protein family form hetero-hexamers or hetero-octamers and are required for NK cell degranulation [34]. Septins promote the fusion of lytic granules with the plasma membrane [34] and can bind to actin and microtubules. In the following sections, we will review how different cytoskeletal components sense and transduce mechanical force, and more importantly, how cytoskeletons collaborate during this process.

2.1. Actin- a signal transducer and the regulator of intracellular contractility

The actin cytoskeleton consists of individual G-actin polymerized into actin filaments (F-actin). F-actin, along with actin-associated proteins, is the major force-generating module in a cell. An important aspect of mechanobiology is the capability of actin to generate 'force'. This process is mainly driven by the interaction between actin and myosin. Together, the force-generating subunit is known as the 'actomyosin' complex. The actomyosin complex in non-muscle cell types consists of F-actin and motor protein non-muscle myosin II. The hydrolysis of ATP in non-muscle myosin II translates to power strokes and mechanical cues for intracellular contractility. Various excellent reviews have illustrated the formation of F-actin from G-actin, F-actin dynamics, and generation of force from the actomyosin complex [35,36]. Here, we highlight and review how the subcellular localization of actomyosin complex determines the biological outcome of force generation, particularly in natural killer cells, NK (Fig. 2).

2.1.1. Actomyosin complex- its subcellular localization and immune cell activities

In most *in vitro* 2D studies, cells are perceived to form focal adhesions that sense and mechanically couple the extracellular matrix (ECM) to the nucleus. For instance, the association of talin with vinculin at the focal adhesion complex depends on stretching force [37] and various transcription factors/cofactors shuttle in and out of the nucleus, depending on sensing of the force exerted on substrates [38,39]. Although the presence of focal adhesion complex *in vivo* is controversial, multiple studies implicated the importance of actomyosin in regulating

cell biology, including NK cells. Mechanical cues alter actomyosin contractility and are known to induce YAP (a transcription cofactor) nuclear localization in fibroblasts [40,41]. However, the contraction of the circumferential actin belt in epithelial cell monolayers suppresses YAP nuclear localization [42]. Furthermore RhoA activation, which leads to increased contractility, needs to be coupled with the loss of the *Cdh1* tumor suppressor gene for YAP activity [43]. These findings suggest that cellular contractility and biochemical pathways require coupling to elicit a meaningful physiological response. Therefore, more studies are required to delineate the crosstalk between biophysical and biochemical interactions, and such understanding in NK cell anticancer activity is lagging far behind.

The actomyosin cytoskeleton governs several aspects of NK cell function. These include: (i) NK cell immune synapse (NKIS) dynamics and organization, (ii) NK transcription factor localization and (iii) NK cell motility and infiltration to target sites requiring immune defense. Since the establishment of cell-cell contact between NK cells and the target cells is important for NK cells to elicit their cytotoxicity against the target cells [44], we will first discuss how actomyosin regulates the NK cell immune synapse.

2.1.1.1. Actomyosin – regulation of NKIS (NK cell immune synapse) in effector and target cells. It is well known that the innate immune NK cell activity is governed by activating and/or inhibitory signals at contact sites (NK cell immune synapse, NKIS) formed between the NK cell and target cell [45]. The NKIS has been commonly described to be organized in a bull's eye shape consisting of supramolecular activation clusters (SMAC) in the central (cSMAC) and peripheral (pSMAC) regions [45]. While the cSMAC contains activating and inhibitory receptors and is devoid of actin, the pSMAC establishes adhesion through adhesion molecules such as LFA-1, CD2 and MAC-1 [31]. These events spark a signaling cascade that leads to F-actin polymerization and accumulation at pSMACs [31], all of which are important for lytic granule polarization and degranulation through the NKIS.

For lytic granules to degranulate and effectively lyse target cells, the F-actin at the NKIS must be pervasive and permissive, viz., dynamically regulated. Various studies showed that contractility [46], actin retrograde flow [16] and even actin disassembly [47] at the NKIS are required for lytic granule secretion. Indeed the activating NKIS has higher actin retrograde flow [16]. On the contrary, slower actin retrograde flow at the inhibitory NKIS allows β-actin and the SH2-domain-containing protein tyrosine phosphatase-1 (SHP-1) to interact and result in SHP-1 activation [16] (Fig. 2). In addition, the activating and inhibitory signals from receptor ligand ligation at the cSMAC likewise regulate the density of actin, LFA-1 and talin-1 around the cSMAC [31]. Studies have shown that inhibitory ligands can suppress the spreading response of actin even if it was already initiated by activating signals [48,49]. The detailed mechanism on how NKIS transmits signals through a series of biochemical scaffolds has been well-documented previously [31,45,50]. However, information on the nanoscale spatial requirements of small GTPases in the regulation of F-actin dynamics has remained elusive. It is well known that small GTPases regulate actomyosin dynamics [51,52] and how dynamic regulation of small GTPases during NKIS formation and microtubule rearrangement is important. Coronin 1A, for instance, was demonstrated to activate Rho/Rac GTPases [53]. In NK cells, coronin 1A plays an important role in deconstructing synaptic cortical actin to create a permissive F-actin network for degranulation of the lytic granules. [47] How Rho/Rac GTPases and coronin 1A cross-regulate the F-actin network warrants future investigations.

Although actin dynamics at the NKIS of the effector (NK) cell is important, actin remodeling in target (cancer) cells can surprisingly contribute to immune evasion. In a study with human cytomegalovirus (HCMV) infected fibroblast cells, lesser mature immune synapse formation and lesser actin polymerization were observed in target cells, which impaired NK cytotoxicity, and this was a consequence of impaired WAVE2 signaling [54]. Although this study focused on HCMV-infected fibroblast cells as a target [54], it should be noted that the presence of HCMV has been suggested to be a plausible neoplastic cancer-causing factor in humans. This was demonstrated by its presence in >90% of common tumour types while absent in normal tissues [55]. Hence, an additional layer of the target cell (e.g. cancer cell)-induced actin remodeling in NK cells should be considered when accounting for immune evasion *in vivo*.

Based on the above observation, enhancing actin polymerization in target cells should conceivably promote the formation of NKIS and thereby enhance NK cytotoxicity. Nevertheless, an *ex vivo* study with primary human NK cells suggested otherwise [56]. The rate and extent of actin accumulation in target (breast cancer) cells inversely correlated with the overall susceptibility to NK-cell mediated lysis, especially for highly metastatic MDA-MB-231 breast cancer cells [56]. Functionally, the study also associated actin accumulation at the NKIS with an enhanced presentation of inhibitory ligands (e.g. HLA-A, -B, -C and PD-L1) on the target cell surface and reduced cytotoxic protease granzyme B production; importantly this was the same with the less metastatic cancer cell lines which displayed enhanced actin accumulation [56]. *In vivo,* epithelial to mesenchymal transition (EMT) involves cancer cells undergoing active cytoskeleton remodeling for morphological adaptations [57]. Hence, the observation that more metastatic cancer cells tend to build up a defense against NK cells by extensive actin accumulation, which may explain how some immune therapies fail. Nevertheless, the central theme revolves around the dynamic modulation of actin responses in both effector (NK) and target (cancer) cells.

2.1.1.2. Actomyosin – regulation of transcription factor/cofactor localization. The linker of nucleoskeleton and cytoskeleton (LINC) complex provides structural support to the nucleus and is made up of proteins within the inner and outer nuclear membranes [58]. These proteins relay intracellular contractile force and can deform the nucleus and mechanically open up the nuclear pore to allow passive diffusion of proteins into the nucleus [39]. Some of these proteins include transcription factors /cofactors such as YAP [39,59], twist1 [60], snail [61], GATA2 [62], NFκβ [63] and Eomes [23]. The review by Bouzid et.al, provides detailed structural and functional insights to the LINC complex [58].

Recently, it was shown that NK cells undergoing anti-cancer activity *in vitro* was able to induce the nuclear localization of transcription factor, Eomes, in response to enhanced intracellular contractility [23]. This caused an early phase increase in NK cytotoxicity [23]. Nevertheless, a gap exists in understanding how some proteins that can be biochemically regulated (e.g. by kinases) respond to cell force. For instance, although contractility was shown to induce YAP activation [39], it is unclear whether the cytoplasmic dominant mutant YAP (YAP5SA) can also be 'force-induced' into the nucleus. Furthermore, it has been shown that N-cadherin adhesion in mesenchymal stem cells could reverse the force-induced YAP nuclear localization [64]. In NK cells, the inhibitory receptor, KLRG1, binds to all three classical cadherins (E-, N- and R-) to inhibit NK cytotoxicity [65]. Hence, whether and how cellular adhesion in NK cells affects force-induced transcription factor /cofactor localization differentially, awaits further exploration.

2.1.1.3. Actomyosin – role in NK cell motility, infiltration and adhesion. In many 2D models, cell migration is initiated by actin protrusions known as lamellipodia and filopodia. The lamellipodial protrusion is dependent on the force generated by actin polymerization and other diverse factors, including actin-associated proteins (e.g. ARP2/3, WAVE, formins), which regulate this process [66]. The Rho and Rac small GTPases must also be spatially activated for effective NK cell migration [67]. On the other hand, some NK cells are present as circulating peripheral blood mononuclear cells; thus, begging the question on how non-adherent

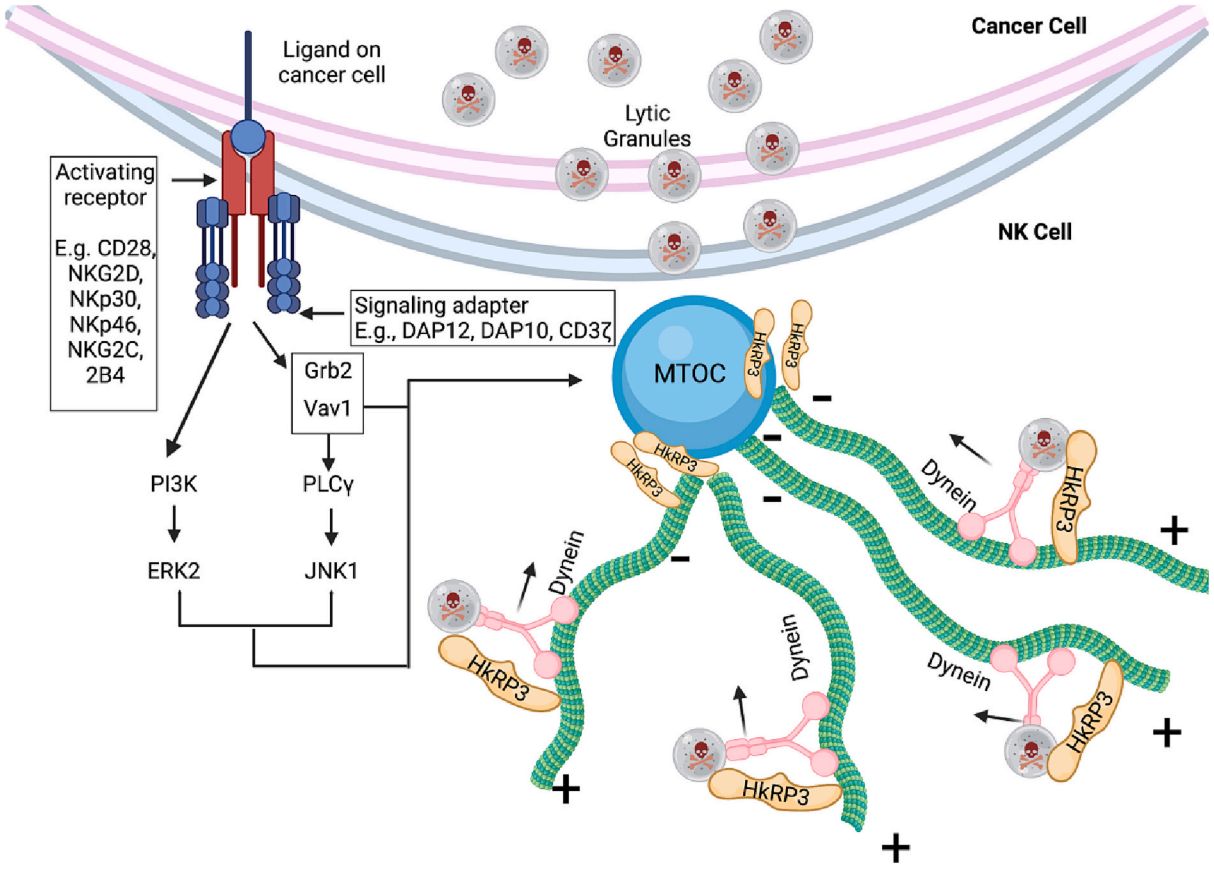

Fig. 3. NK activating signals stimulate the JNK and ERK pathways to signal MTOC polarization, while microtubule-highway directs lytic granule trafficking. Many NK activating receptors share a common signaling pathway to signal the polarization of the MTOC. Stimulation of activating receptor (e.g. NKG2D) results in the binding of signaling adaptor molecule (e.g. DAP10) and activates two pathways: (1) the PI3k → ERK2 and (2) the PLCγ → JNK1 pathways. PLCγ also requires the biochemical scaffolding intermediates, 'Grb2 and Vav1'. The polarization of MTOC towards the NKIS is coupled to transport of lytic granules towards the MTOC via minus end-directed dynein motor proteins on microtubules. Hence, the microtubule serves as a 'highway' for directed delivery of lytic granules to target cells and avoids 'bystander' killing. The HkRP3 interacts with the dynein motor complex and microtubules, and it is required for clustering of lytic granules around the MTOC towards the cancer cell target. Refer to Section 2.2.1 for details.
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cells lacking focal adhesion, might gain traction in its migration towards innate immune defense and trans-migrate/extravasate the endothelial barrier to reach their target tissue.

Since NK cells circulate against blood flow to reach their targets [25] (infected cells or cancer cells), the non-adherent rear of the cell, known as the uropod, plays an important role in polarity maintenance and conjugate formation [68]. The adherent front converges signals from various receptors such as the β1 and β2 (LFA-1) integrins and chemokine receptors (e.g. CXCR4, CCR5 and CXCR3) [69,70]. Conceivably, the engagement of surface receptors induces changes in actin dynamics, which has been shown to be important for NK migration though up-regulating high-affinity state β2 integrin [13]. In NK cells, β2 integrin also promotes phosphorylation of Fyn and Pyk-2 tyrosine and enhance Wiskott-Aldrich syndrome protein (WASp) activity [13]. Patients suffering Wiskott-Aldrich syndrome (an X-linked disease that mainly affects males with symptoms that include thrombocytopenia, eczema, recurrent infections, and small-sized platelets), with mutations in the *WASP* coding gene display reduced *in vitro* migration of NK cells [13] and this is associated with impaired NK cell adhesiveness to tumor necrosis factor-α (TNFα)–activated human umbilical vein endothelial cells [13]. Furthermore, WAS patients have defective NK cell actin polymerization, which attributes to reduced *ex vivo* NK cytotoxicity [14].

There is a positive feedback loop for LFA-1 activation to induce 'outside-in' signaling to foster actin polymerization [71] (Fig. 2). The cell surface receptor, ICAM-1, binds to LFA-1 to induce talin redistribution to the LFA-1 ligation site [72]. Subsequently, talin, along with vinculin, recruits Arp2/3. Simultaneously, talin with vinculin also activates the PIP2 to recruit WASp and activate Arp2/3 [72]. In concert, these processes lead to actin polarization for NK cells to elicit their cytotoxicity. Separately, LFA-1 engagement of ICAM-1 on target cells results in phosphorylation of Vav1, a guanine nucleotide exchange factor for the GTPase Rac, and the Src family kinase (e.g. Fyn) is responsible for this phosphorylation [73,74]. Phosphorylated Vav1 is able to act as a Rho GEF for small GTPases (e.g. Rho, Rac, Cdc42) to reorganize the actin cytoskeleton [75]. VCAM-1 expression on target cells also facilitates NK actin reorganization by binding to the β1 integrins on NK cells and rapidly activates Cdc42 and WASp activation and association with Fyn and Pyk-2 tyrosine kinases [13] (Fig. 2). It is beyond the scope of this review to describe in detail the roles of NK cell integrins in regulating phenotypic and functional identity of NK cells. Nevertheless, we recognize the importance of integrins in NK mechanotransduction and refer readers to the review by Shannon and Mace [76] which provides an excellent starting point to address this.

While adhesion happens on the surface of NK cells, the resulting actin reorganization has far-reaching consequences on NK cell contractility. The actomyosin complex generates the 'push' and 'pull' forces in cells and is present in the pSMAC and actomyosin arcs [31]. Various human NK cell-related diseases, such as May-Hegglin anomaly, a disease characterized by the presence of Döhle-like bodies (small rods) in leukocyte cytoplasms, are heterozygous for a C5797T mutation in MYH9, and are

associated with mutations in myosin II [31,77–79], implicating the importance of regulating actomyosin-mediated contractility in NK cells. Indeed, a case study of four patients with May-Hegglin anomaly affecting NK cells showed reduced cancer-killing abilities *in vitro* [79]. It will be interesting to investigate whether different biochemical or mechanical cues induce differential cellular responses through the adhesion proteins, which will offer a novel layer of regulation of NK activity.

2.2. Microtubules- a highway to provide direction

Microtubules are formed by protofilaments composed of alpha and beta tubulin heterodimers [80]. Individual GTP-bound tubulin polymerizes at the rapid growth end, known as the 'plus-end' [81]. The other end, known as the 'minus end', anchors the tubulin to the MTOC and membrane organelles such as the Golgi apparatus [81]. The microtubules have been extensively studied in the context of regulating NK cytotoxicity. The main focus of attention has thus far lie in their role in delivering lytic granules to the NKIS [31,82–85], and in the MTOC, which regulates NKIS polarization and cytotoxicity [31,50,82,86,87].

Dynein and kinesin are two microtubule-associated proteins (MAPs) motor proteins known to transport cargo along the microtubules. While dynein is minus end-directed [88], kinesin motor protein transports organelles along a microtubule towards the plus end [85]. To better appreciate how microtubule structure and dynamics regulate NK activity, we first describe the sequential events following tumorigenesis or infection.

2.2.1. The microtubule-associated proteins in NKIS – directed killing of target cells

To induce directed delivery of cytolytic granules to target cells, the F-actin needs to accumulate at the NKIS to facilitate MTOC polarization [31]. Many NK cell surface activating receptors signal the polarization of the MTOC and cytolytic granules to the NKIS. Some of these activating receptors include NKG2D [89,90], NKp44 [90], NKp30 [91] and NKG2C [91]. Mechanistic studies have suggested that the activation of JNK and ERK2 by the activating receptors is responsible for MTOC polarization [91,92]. More specifically, the engagement of activating receptors simultaneously induces Src family kinase activation of phosphoinositide-3 kinase to activate ERK2 and phospholipase PLCγ, which in turn, activates JNK [91]. While the MTOC polarizes towards the NKIS, lytic granules converge and mobilize towards the MTOC via minus end-directed dynein motor proteins [85] (Fig. 3). The microtubule-directed trafficking of lytic granules is important as dynein inhibition could lead to nondirected degranulation and bystander killing of target cells [84]. Hence, the microtubules form a 'highway' to ensure precise direction for lytic granules to move towards the NKIS to lyse target cancer cells. In addition, the Hook-related protein 3 (HkRP3) interacts with the dynein motor complex and microtubules [93]. The depletion of HkPR3 impairs NK cell cytotoxicity, which was attributed to aberrant MTOC polarity and impaired clustering of lytic granules around the MTOC [93].

Various adaptor and scaffold proteins have been identified to bind and regulate microtubule dynamics to influence NK cytotoxicity. For instance, proline-rich tyrosine kinase-2 (PYK-2/RAFTK) colocalizes with MTOC at the uropod of migrating natural NK cells [94]. Upon receptor activation, PYK-2, paxillin, and MTOC translocate from the uropod to the cell-cell contact area. However, overexpression of PYK-2 was shown to inhibit NK cytotoxicity, implying the scaffolding function of PYK-2 that may deregulate the balance of signalling complexes [94]. Interestingly, PYK-2 shares significant sequence homology to the focal adhesion kinases (FAK), although FAK was reported to be almost absent from NK cells [94]. Hence, it is unsurprising that Fyn-dependent phosphorylation or autophosphorylation of PYK-2 at Y402 is equivalent to Y397 FAK phosphorylation, which can lead to binding and activation of SH2 domain-containing proteins such as Src kinases [95]. Functionally, PYK-2 activation can result in microtubule dependent polarity through CLIP-170 as well as WASp and Arp2/3 dependent actin remodeling [96].

Finally, there are some regulatory proteins that co-regulate actin and microtubules. For example, Vav1, which is a Rho GTPase guanine nucleotide exchange factor, is a critical signalling mediator downstream of DAP10 in NK cells [97]. It was shown that Vav1 is essential for F-actin polymerization at the NKIS and the polarization of MTOC towards target cells, which requires microtubule dynamics [97,98]. Hence, how the spatio-temporal specificity of signaling proteins respond to biochemical and mechanical cues to corroborate actin and microtubule dynamics awaits further exploration.

While microtubules work to directionally deliver lytic granules to target cells, a multiplex of biochemical and biophysical events could happen simultaneously. The microtubule stabilizing reagent, paclitaxel, which alters microtubule dynamics by promoting microtubule assembly and inhibiting depolymerization [99,100] was surprisingly shown to increase NK cytotoxicity [101,102]. However, a conflicting report documented that paclitaxel down-regulates various adhesion molecules on both NK and target cells [103]. These observations suggest that apart from the delivery of lytic granules and MTOC polarization, microtubule dynamics can be regulated by other signaling pathways. Yet such conflicting evidence warrants further studies to verify the mode(s) of action of anticancer compounds on microtubule-mediated NKIS. Furthermore, there are limited studies on the roles of posttranslational modification of microtubules in immune cell biology. It was shown that microtubules are stabilized by de-tyrosination after T cell receptor engagement [104] and de-tyrosinated microtubules are also known to increase cytoskeletal stiffness [105]. Hence, how microtubule post-translational regulation may affect NK cell mechanical properties also awaits further investigation.

2.3. Intermediate filaments- less abundant but important

The intermediate filaments can extend throughout the nucleus into the inner nuclear membrane and are typically 10 nm thick filaments comprised of proteins encoded by more than 70 conserved genes [106]. Furthermore, the conserved genes are developmentally regulated in a tissue-specific manner. To date, intermediate filaments are classified into five types, comprising type I and II keratins, type III desmin and vimentin, type IV neurofilaments and type V lamins [106]. Together, these five types of intermediate filaments mediate cytoskeletal crosstalk to transduce signals from the cell surface to the nucleus.

The expression, type and abundance of intermediate filaments are dynamically and developmentally regulated. For example, the nuclear B-type lamin is expressed in all embryonic stages, whereas A-type lamins are turned on during differentiation [107]. While lamin A/C is highly expressed in macrophage and dendritic cells, the inactivated T cells, B cells and NK cells barely express detectable levels [108]. Hence, much less is known about how different types of intermediate filaments and their abundance are dynamically regulated to control immune cell function.

Although research on the role of intermediate filaments in NK cells is hitherto limited, studies in other immune cell types have shed some light on the possible roles of intermediate filaments in regulating NK cell activity. For instance, it was shown that expression of A-type lamin is increased during T-cell activation to enhance F-actin polymerization [109]. Whether the expression of A-type lamin is increased to facilitate NKIS formation during NK activation or not, remains unknown. Furthermore, a recent study identified CD56 bright NK cells (activated NKs) to display higher levels of A-type lamin [110], suggesting that transcriptional or translational control may exist in NK cells to regulate lamin subtype expression to influence NK cytotoxic action.

2.3.1. Intermediate filaments in target cells conversely enable recognition of NK cells

Although there is a lack of understanding on the roles of intermediate filaments in NK cells, some studies have suggested that intermediate

Table 1
Common inhibitors and activators of NK cytoskeleton.#

Inhibitor	Target	Mechanism	Selected Applications
Cytochalasin B/D	Actin	Shortens actin filaments by blocking monomer addition at the fast-growing end of polymers	[14,16,136–142]
Latrunculin A/B	Actin	Sequesters G-actin and prevents F-actin assembly	[83,141,143–148]
Wiskostatin	Actin	Selectively inhibits neuronal WASP (N-WASP)-mediated actin polymerization in vitro	[13,148–152]
CK-666	Actin	Inhibitor of the actin-related protein 2/3 (Arp2/3) complex	[46]
Swinholide	Actin	Sequesters and stabilizes actin dimers to induces actin depolymerization	[146,153]
Blebbistatin	Myosin	Reversible inhibitor of non-muscle myosin II	[23,46,79,154]
Nocodazole	Microtubule	Nocodazole binds to β-tubulin and disrupts microtubule assembly/disassembly dynamics	[23,145,155,156]
Vinblastine	Microtubule	Depolymerizes microtubules and inhibiting microtubule assembly	[157–159]
Colchicine	Microtubule	Binds to the ends of microtubules to prevent the elongation of the microtubule polymer	[160]
Forchlorfenuron	Septin	Dampens septin dynamics and induces the assembly of abnormally large septin structures	[34]
Y-27632	ROCK	Y-27632 inhibits both ROCK1 and ROCK2 by competing with ATP for binding to the catalytic site	[148,161,162]
Activator	**Target**	**Mechanism**	**Selected Applications**
Jasplakinolide	Actin	Rapidly stabilizes pre-formed actin filaments and inhibits their disassembly in vitro. Also induces polymerization of actin monomers into F-actin in vivo.	[16,46,141,148,153,155]
Paclitaxel (Taxol)	Microtubule	Binds to the β-tubulin subunits of microtubules resulting in stable microtubules.	[101–103,158,163]

filaments in target cells can conversely affect NK cytotoxicity. For example, in NK cell-mediated lysis of infected human monocytes, vimentin expression on the cell surface serves as a ligand for the NKp46 receptor and this facilitates NK cell-mediated lysis of the target cell [33]. On the other hand, vimentin expression in cancer cells seems to make cancer cells more resistant to NK killing by upregulation of actin filament accumulation at the NKIS [111]. Although the study did not identify how vimentin regulates actin filaments [111], it is known that vimentin has a close structural relationship to actin, and vimentin regulates actin organization and contractility [112,113]. Furthermore, in support that actin accumulation in cancer cells negatively impacts NK cytotoxicity, research has shown that actin cytoskeletal remodeling drives breast cancer cell escape from NK cell-mediated cytotoxicity [56].

Altogether, the study of intermediate filaments in NK cells is limited, partly due to the low expression of intermediate filaments in NK cells. Nevertheless, it is clear that the intermediate filaments in target cells can: (i) act as a ligand for NK receptors or (ii) modulate target cell cytoskeleton dynamics to evade NK killing. Moving forward, more studies should be performed to investigate how intermediate filaments in target cells might modulate NK cell cytotoxicity.

2.4. Septins- the final checkpoint for lytic granule release

Finally, we review the fourth member of the cytoskeleton family, septins. All septins are GTP-binding proteins of 30–65 kDa that polymerize into hetero-oligomeric protein complexes and form filaments. Septins can also interact with other cellular components such as membranes, actin filaments and microtubules [114]. Two unique features of septins are its capacity to form ring structures and the non-polar nature of septin filaments that do not undergo dynamic turnover like actin or microtubules [114]. In yeasts, septin localization at the cortex is responsible for the maintenance of cell polarity [115]. Consistently, in eukaryotes, septin colocalization with microtubules is important for polarity maintenance [116]. All these observations tantalize consideration for future manipulation of septin to increase directed NK cell lytic granule release for its cytotoxicity.

2.4.1. Septins- regulator of immune cell cortex stiffness

Septins regulate many aspects of the adaptive and innate immune system. SEPT2 and SEPT11 accumulate at the phagosomes in macrophages to regulate phagocytosis [117]. Disruption of the septin cytoskeleton causes excessive membrane blebbing [118] in T lymphocytes which affects their motility [119]. It was also shown that septin-deficient T-cells display decreased cell cortex rigidity to facilitate the formation of membrane protrusions [120,121].

Unlike conflicting results regarding NK cells treated with microtubule inhibitor, paclitaxel [102,103], recent evidence demonstrated that genetic or pharmacologic stabilization of septin filaments resulted in the inhibition of NK cell cytotoxicity [34]. As shown by Phatarpekar et al. [34], septin surprisingly does not accumulate at the immune synapse as profoundly as actin filaments, and the ability of NK cells to recognize and kill NKG2D ligand expressing cells was drastically reduced. Interestingly, septins do not seem to affect the convergence of lytic granules or MTOC polarization to the immune synapse. Instead, septin interacts with the lytic granule fusion machinery and is required for granule membrane fusion by sustaining SNARE protein STX11 and STXBP2 interactions [34]. The finding that septin mainly accumulates at the cell cortex is consistent with the understanding that septin filaments regulate cell shape and cortical rigidity [122]. Septin assembly is reported to affect T-cell rigidity [119], which is shown to increase upon the formation of immune synapse [123]. Therefore, it will be of interest to delineate whether septin regulates NK cell rigidity. Moreover, septin is known to promote F-actin crosslinking [124] and interacts with DOCK8 GEF in NK cells [125], which activates Rho proteins [126]. Hence, it is possible that septin-mediated NK cell rigidity may be a biophysical marker for NK activation.

[#] With decades of research on testing cytoskeletal-related drugs, there is abundant literature; however, here we provide a representative number of papers without intentions to ignore the other valuable studies in the literature.

Similar to intermediate filament dynamics in target cells, septin expression in target cells also affect NK cell function when NK-target cells interact. For example, it was shown that SEPT2-depleted endothelium facilitates migration of NK cells [127]. Although the study attributed this to decreased VE-cadherin mediated junctional integrity [127], it is possible that decreased cell stiffness facilitated NK transendothelial migration. The regulation of target cell stiffness is of translational importance. NK-mediated anticancer activity occurs either through direct tumor cell interaction or communication with other immune cells or non-immune stromal cells in the TME. Migratory metastatic tumor cells are known to be softer than normal cells [19,20] and the capacity for NK cells to infiltrate solid tumor cells decreases with disease progression [128]. Hence, an understanding on how septins might regulate target and NK cell stiffness may have therapeutic and translational value.

2.5. The cytoskeletal components crosstalk to regulate immune functions

Biochemical and structural crosstalks between cytoskeletal components are evident in various immune cell types. The small GTPases are essential 'place' and 'pace'-makers in regulating cytoskeletal dynamics for cancer and immune cell functions. After microtubule destabilization in breast cancer cells treated with the microtubule destabilizing agent, nocodazole, the activation of Rho GEF, GEF-H1, fine-tunes actin dynamics [67]. On the other hand, GEF-H1 signaling enhances cross-presentation of tumour antigens to CD8 T-cells via dendritic cells [129]. Furthermore, Cdc42-interacting protein-4 (CIP4) links actin to microtubules in activated NK cells to regulate NK cytotoxicity by facilitating MTOC polarization [130]. The Ras superfamily of small GTPases, Rap1a and Rap1b, regulate cell proliferation, differentiation and adhesion in hematopoietic cells [131]. It was shown that NK cells express Rap1b as the major isoform [132]. Rap1b is indispensable for NK cell polarization, spreading and MTOC formation [132], all of which require coordination between actin and microtubule. For instance, Rap1 regulates the actin-severing protein cofilin-1 to remove existing F-actin to allow re-organization for branched actin in B and T cells [133]. On the other hand, Rap1b colocalises with the scaffold protein, IQGAP1, to facilitate sequential phosphorylation of B-Raf, C-Raf, and ERK1/2 [132]. IQGAP1 is a scaffold protein associated with F-actin and the microtubule plus-end binding protein, CLIP-170/ CLIP1 [134]. Hence, it is not surprising that activation of Rap1 small GTPase cascade simultaneously induces actin-reorganization in NK cells (through Rap1-cofilin and/or Rap1-IQGAP1) for spreading and MTOC formation for the delivery of cytotoxic granules to target cells. Nevertheless, it is unclear how selective NK activating receptors (e.g. NKG2D and Ly49D) can transiently activate Rap1b [132] and how NK cells regulate the balance of actin severing by cofilin and MTOC length through Rap1 regulation. It was suggested, however, that Rap1b could indirectly regulate Cdc42 and Rac1, via Vav1, which leads to improper tubulin oligomerization and MTOC formation [132].

Apart from biochemical regulation by actin and microtubule-associated proteins, direct structural crosstalks exist too. It was shown and proposed that microtubule irradiation from MTOC at the immune synapse can anchor to the periphery of actin ring to support MTOC polarization to the T-cell immune synapse center [135]. Therefore, it is conceivable that structural and biochemical crosstalks exist between different cytoskeletal components in NK cells. It would be pertinent to identify and translationally apply these crosstalk events to design therapeutic drugs targeting multiple pathways. However, understanding how the cytoskeletal network affects basic fundamental NK biology is essential. Table 1 summarizes drugs that target the cytoskeleton and highlights some studies in NK cells that have utilized these drugs.

2.6. NK heterogeneity and its effects on NK mechanotransduction and clinical implications

Many studies have shown that NK cells are heterogeneous and are represented by different subpopulations. This issue is extremely significant due to the fact that NK cell subpopulations demonstrate specific molecular features and may differ in immune activities which lead to variable clinical outcomes. Therefore, information about the mechanobiology of NK cells and the execution of approaches to increase their functions should bear in mind NK cell heterogeneity. A total of six distinct stages have been described to account for NK cell maturation in the bone marrow and lymph node [164]. It is out of the scope of this review to narrate in detail the developmental stages and the associated NK phenotypes. Abel et al. reviewed in detail the development, maturation and clinical utilization of NK subpopulations [164]. Nevertheless, it is important to discuss the common NK subtypes in circulation and those in tissues, and how their phenotypes affect NK mechanotransduction. In general, human NK cells are CD56 positive and CD3 negative [165]. The two major subpopulations are CD56 ^{bright} CD16 $^{dim/-}$ and CD56 ^{dim} CD16 $^{+}$, respectively [165]. Recently, it was also found that a rare percentage of NK cells are CD56 negative and this subpopulation depends on antibodies for cytotoxicity [166]. Nevertheless, here we discuss how two common markers of NK cells, CD56 and CD16, may affect or can be affected by NK cytoskeletal components.

Most NK cells in the peripheral blood are CD56 ^{dim} and are considered more mature than the CD56 ^{bright} NK cells in the secondary lymphoid tissues [164]. It is now known that NK cells acquire CD56 ^{bright} phenotype prior to down regulating CD56 expression to become CD56 ^{dim} [164]. Transition into CD56 ^{dim} NK cells is also associated with the expression of immunoglobulin superfamily member CD16 (FcγRIII) receptor that facilitates NK antibody-dependent cellular cytotoxicity (ADCC) [167]. Functionally, CD56 ^{dim} NK cells are considered more cytotoxic against cancer and their CD56 ^{bright} counterparts are known to be potent producers of inflammatory cytokines [168]. As discussed above, Pky2 is a scaffold protein interacting with β1 integrin, paxillin, and other protein tyrosine kinases and is required for NK cytotoxicity [95,96]. Furthermore, Pyk2 is required for MTOC polarization. Interestingly, it was found that CD56 knockout in NK cells abrogated Pyk2 phosphorylation at the NKIS and was associated with reduced NK cytotoxicity [95]. It was also suggested by the authors that CD56 functions to facilitate localization of Pyk2 at sites of potential activation autophosphorylation or NK polarization [95]. What remains unknown are the mechanistic details on how the brightness/ abundance of CD56 affects Pyk2 activation and actin accumulation at the NKIS. Understanding how CD56 affects actin accumulation is key to NK activation because actin polymerization is necessary for the polarization of CD2, CD11a, and CD11b to the pSMAC, as well as of perforin-containing granules to the cSMAC at the activating NKIS [160]. Furthermore, a dense intracellular actin network will inevitably result in a more rigid cell cortex that may affect NK function.

NK cell anti-tumor cytotoxicity, through ADCC, can enhance the aggregation of CD16 IgG receptor on NK cells which are interacting with IgG-opsonized tumor cells [169]. Nevertheless, development of resistance to therapeutic antibodies still results in decreased therapeutic efficacy. Several mechanisms may account for the decreased efficacy and are discussed by Capuano et.al [169]. Interestingly, several key surface receptors and intracellular proteins involved in the dynamic regulation of NK cytoskeleton are rendered inactive due to persistent ligation of CD16 on human NK cells. In one study, CD16 ligation on primary human NK cells by the anti-CD20 monoclonal antibody, rituximab, resulted in NKG2D activation tolerance, hypoactivation of Vav1 and persistent recruitment of SHP-1 [170]. As mentioned above, Vav1 is essential for F-actin polymerization at the NKIS and the polarization of MTOC towards target cells, which requires microtubule dynamics [97,98]. Likewise, NKG2D and SHP1 both regulate the NK cytoskeleton. Hence, whether pharmacological or genetic perturbation of NK cytoskeletal stability

and/or instability can affect NK ADCC, remains an open question that warrants future studies. Indeed, the binding of NK cells to target cell through CD16 rapidly activates the small GTPase Cdc42, which is known to interact with WASp protein that regulates Arp2/3 for actin branching [171]. WAS patients have impaired natural cytotoxicity due to perturbation and reorganization of cytoskeletal in NK cells [171].

Having discussed how CD56 and CD16 expressions can affect NK mechanobiology and cytotoxicity, it is apparent that clinical trials based on these molecular targets consider the heterogeneity of NK cells in the patient. For instance, in patients with chronic viral infection, an upregulation of CD56 negative and CD16 positive NK cells was found [166]. How then, would antibody-based therapies benefit these patients? Perhaps genome directed stratifications in clinical trial design and enrolments could help to make personalized medicine even more targeted. The vast amount of possible mutations in critical molecular pathways necessitates a myriad of therapeutic agents to target them. These mutations can be identified by next generation sequencing (NGS) to explain how pathways are bypassed or suppressed in unique NK subpopulations. For instance, if CD56 is important to facilitate localization of Pyk2 at sites of potential activation autophosphorylation or NK polarization [95], then in CD56 negative NK cells what are the genomic features that help to bypass this to still allow NK cells to perform its cytolytic capabilities? Furthermore, genetic heterogeneity developed over time at the intratumoral level plus the waiting time for NGS results, would still present major obstacles for precision medicine to materialize.

3. Mechanical cues affect NK cell activity

Biomechanical cues constantly shape homeostasis and pathophysiology of tissues and cells, even at embryonic stages of development. Although the study of mechanotransduction began in the late 1800s [172], a classical experiment by Discher's group[134] in 2006 demonstrated that mesenchymal stem cells commit to lineages specified by matrix rigidity [173]. This finding prompted studies on mechanical cues in pathophysiological processes, which led to possibilities of applying mechanical transduction to direct /redirect pathophysiological processes. For example, the mechanical activation/ inactivation of transcription factors and cofactors [174], the mechanical regulation of epigenome landscape [175], and even the involvement of mechanical cues such as ultrasound waves to kill tumour cells, were promoted [176]. Therefore, the second half of this review endeavors to consolidate our understanding of how mechanical cues affect NK biology and the potential translational opportunities thereof.

3.1.1. Modulation of NK cell rigidity

The rigidity of both the target cell and NK cell have potential translational implications. More than 20 years ago, it was shown that NK cell stiffness directly affects resistance to its passage through tissues [177,178]. With a diameter larger than the blood vessel but with low shape compliance, the NK cell has immense difficulty to extravasate. In the study, the authors showed that reducing NK cell rigidity by modulation of cytoskeleton organization could facilitate NK infiltration into tissues [177]. However, reducing the rigidity of NK cells could also compromise its immunotherapeutic efficacy [177]. From these observations, we may envisage that modulating NK rigidity could pose a double-edged sword. Indeed, intracellular contractility is linked to higher rigidity, and it is required to promote nuclear localization of various NK effector genes (e.g. Eomes [23,179] and NF-κB [63,180]). However, a rigid NK cell will have difficulties reaching the target site. Therefore, achieving the balance between rigidity and softness (and maneuverability) of the NK cells is critical. It is plausible that the immune defense system and the tumor microenvironment would negotiate such a measure-countermeasure to achieve anti- /pro- tumorigenesis. Therefore, it is important that future research on NK cell rigidity should consider the status of its target cell rigidity (see Section 3.1.2).

Interestingly, a common cell growth supplement, IL2, routinely added to maintain and expand primary NK and NK cell lines in culture, was shown to increase NK cell rigidity [181], which was maintained for up to 96 h after IL2 withdrawal [181]. Therefore, caution needs to be exercised on common observations of NK cell activity *in vitro,* which may not entirely recapitulate in the *in vivo* dynamic environment due to the altered biophysical parameters of NK cells. Unbiased transcriptomic and proteomic data [182] will provide clarity on genes that are affected by modulation of NK rigidity, and this will enable us to identify pathways that need to be considered during *ex vivo* therapeutic manipulations prior to adoptive transfer into recipients.

3.1.2. Substrate rigidity regulates NK activity

The findings on substrate rigidity on NK activity generally supports the view that stiffer substrates enhance NK cell cytotoxicity [15,16,183]. These studies have direct translational relevance. Cancer cells are relatively softer than normal cells [19,184] and are especially so for metastatic cancer cells, which enables it to migrate [185]. Hence, finding ways to sensitize NK cells to softer target matrices could have translational potential to overcome the NK inhibitory signal sent from target cancer cells. Conversely, ways to prevent softening or reverse softening of primary tumour to pre-empt metastatic tumourigenesis during EMT may aid immunotherapies.

Human NK cells seeded on a stiff substrate (142 kPa) has been shown to secrete higher amounts of granzyme A and B, FasL, granulysin, and IFNγ compared to NK cells seeded on soft 1 kPa 2D flat substrates [15]. Since circulating NK cells might encounter circulating tumour cells, Friedman et al. [15] then fabricated cell-sized spherical beads of soft (9 kPa), medium (34 kPa) or stiff (254 kPa) substrates, and consistently showed that stiffer beads promoted NK cell spreading and degranulation. Nevertheless, information is lacking on how curvature sensing can compound to stiffness sensing of spherical substrates.

Interestingly, softer substrates inhibited MTOC polarization to the NKIS and resulted in unstable synapses known as the 'kinapse' [15]. Of note, LFA-1 engagement and talin recruitment to the NKIS was not inhibited in soft matrices. These findings again suggest that the protein downstream of talin signaling could be impaired in softer matrices. One possibility is the reduced recruitment of vinculin to the NKIS in a soft matrix. It was shown that vinculin-mediated crosslinking of integrin–talin complexes to the actin cytoskeleton facilitates actin polymerization and nucleation [186]. It is possible that either vinculin recruitment to the NKIS is hindered or the mechanical exposure of the vinculin binding site under force, is insufficient on a soft matrix. A similar finding was reported recently by the Barda-Saad group [187]. The SHP-1 protein conformation state was shown to regulate the phosphorylation of various actin-regulating proteins, such as Vav1 and Src substrates [188]. The interaction of SHP-1 with β-actin causes an open SHP-1 conformation that suppresses NK cytotoxicity [16]. Moving a step further, the group examined how activating and inhibitory signals can affect NK actin retrograde flow and SHP-1 activity. On a stiff substrate coated with inhibitory signals, SHP-1 assumes an inactive closed conformation at the NKIS [16]. However, under activating settings, soft and stiff substrate did not affect SHP-1 conformation [16]. One interesting observation in the study was that coating a soft substrate with an activating signal (e.g. CD28) could overcome the slow actin retrograde flow in NK cells [16]. This directly supports the presence of some 'inside-out' signaling that has yet to be discovered for NK cells. Indeed, another study demonstrated that adding PMA/ ionomycin (a common NK cell stimulant) rescued soft substrate-induced loss of NK cell cytotoxicity [15]. These observations point to a potential for restoring NK activity even when encountering soft targets like metastatic cancer cells.

EMT has been proposed to account for the softening of primary tumours and facilitate metastasis. During EMT, various transcription factors (TF) (e.g. TWIST and SNAIL) in cancer cells are known to be

dynamically regulated and contribute to cancer cell softening [57]. Hence, genetic manipulation such as targeted CRISPR [189] of these EMT/metastasis-promoting TFs may delay cancer cell softening to give NK cells a chance to 'sense' and eliminate the primary cancer cells. On the other hand, it should be noted that the tumour microenvironment is complex, and ECM stiffening often stimulates cancer cell stiffening through mechanically or biochemically-induced factors. For instance, stiffer ECM due to high collagen and fibronectin contents can trigger enhanced force application by tumour cells on the ECM, further stiffening the cancer cells [190]. Hence, multivariate methods to sensitize NK cells to a softer matrix should be considered to improve translational outcomes during immunotherapies.

As mentioned above, NK cells can restore their cytotoxicity on softer substrates upon chemical stimulations (e.g. by CD28 [16] and PMA/ionomycin [15]). If uncertainty revolves around modulating the TME and cancer cells, metabolic reprogramming of NK cells may be another option for NK cells to sense soft metastatic cancer cells. It is known that apoptotic cells are softer due to cytoskeletal degradation, and NK cells may avoid killing these cells to conserve energy [15], a process that may be recapitulated during NK-cancer surveillance. Aggravating the situation, limited nutrients and enhanced lipid metabolism by cancer cells can enrich TME with lipids [191], resulting in the accumulation of intracellular lipids in NK cells that increases PPAR (peroxisome proliferator-activated receptor)-mediated nuclear receptor signaling and inhibits NK cytotoxicity further [191]. Hence, metabolic reprogramming of NK cells through *ex vivo* NK genetic modulation may 'supercharge' NK cells to recognize and kill cancer cells. One example is the expression of the sterol regulatory element binding protein (SREBP) transcription factors that sustain the rate of glycolysis and OXPHOS in NK cells [192] and inhibition of intracellular cholesterol. [191] The possibility of metabolic changes of NK cells via PPAR-mediated regulation of NK activities and cytoskeletal dynamics needs critical assessment based on more targeted empirical evidence.

3.2. Hyperthermic treatment of NK cells enhances its function

Classical mechanotransduction study focuses on the involvement of the cytoskeletal network altered by extracellular matrix properties. However, it is known that various ion channels respond to exogenous stimuli such as temperature, curvature of the substratum and even voltage. These channels are collectively known as transient receptor potential (TRP) channels. In general, the TRP vanilloid (TRPV) channels are all heat-activated and can induce conformation changes [193]. Thus, we next focus on how heat/ hyperthermic treatment of NK cells can modulate NK activity.

Reports have implicated the occurrence of fever in improved cancer recovery [194] and studies now agree that elevated body temperature improves immune cell functions [195]. Experiments in mice showed that whole-body hyperthermia for 30 min at around 40 °C could improve NK cytotoxicity and reduce lung metastatic occurrence [196]. In another study involving eight healthy volunteers, a significant increase in NK cell lysis was observed after the subjects were immersed in a water bath at 39.5 °C for two hours [197]. However, exposure to higher temperatures of 42 °C for one hour almost completely abolished NK binding and cytolytic activities and this was not due to reduced cell viability [198]. Unfortunately, there are lesser mechanistic studies on how elevated temperature affects NK activity. One study showed that thermal stress resulted in clustering of activating NKG2D expression but did not change the overall surface level of NKG2D activating receptor [199]. Thermal stress also increased the expression of NKG2D ligand, MICA, on target cells [199]. Although the detailed mechanism remains to be explored, it is possible that NKG2D activation could modulate F-actin dynamics at the NKIS, which as mentioned above, is important for NK cytotoxicity. Indeed, Billadeau group [200] recently showed that NKG2D-DAP10 signaling promoted F-actin nucleation and NK cell-mediated killing.

3.3. Ultrasound treatment recruits NK cells to tumor sites

Somewhat related to hyperthermic treatment to activate NK cells, ultrasound has also been explored for more than 30 years [201]. The thermal effect of ultrasound is known as 'absorption', in which the mechanical energy is converted into heat energy [202]. One of the main advantages of ultrasound is its minimum invasiveness [203] compared to whole body hyperthermic treatment. Radiofrequency ablation (RFA) is a type of ultrasound therapy used clinically to treat cancer. The use of RFA to treat cancer evolved during the past few decades [201,204]. Since the cytoskeleton provides structural integrity to cells, it is not surprising that ultrasound will inevitably result in mechanical changes to cytoskeletal integrity and activation of biochemical pathways. Indeed, it was recently shown that ultrasound causes Piezo1 channel-mediated calcium entry to disrupt microtubules in breast cancer cells [176]. As mentioned in Section 2.5 GEF-H1, which is normally coupled to microtubules, is released and this enhances RhoA-Myosin IIA pathways. The activation of GEF-H1 after microtubule disruption was also observed in cancer cells treated with ultrasound [176]. However, what consequences will ultrasound have on NK cells?

With the exception of one study in hamsters [201], all others have shown an accumulation of NK cells in tumour sites exposed to ultrasound [204–206]. Information regarding the cytotoxic activity of NK cells accumulated at the tumour site, is lacking and conflicting. While a study more than 30 years ago showed that ultrasound suppressed NK cytotoxicity [201], a recent investigation demonstrated that ultrasound enhanced NK cell anti-tumour immunity by the upregulation of NKG2D and higher production of effectors, IFN-γ and TNF-α [207]. Although the detailed mechanism is lacking, drawing conclusions from the hyperthermic treatment of NK cells [200] (Section 3.2) and the activation of GEF-H1-RhoA-Myosin IIA in breast cancer cells [176], it is possible that ultrasound causes NK cell recruitment to the tumor site and dynamically reorganizes NK cell cytoskeleton.

Similar to a lack of mechanistic information on NK cytotoxicity related to ultrasound, detailed mechanistic studies on how ultrasound promotes accumulation of NK cells at tumour sites is scanty. One of the reasons why NK cell-mediated therapy for solid tumours is less effective is the inadequate homing of infused NK cells to the solid tumour sites due to tight junctional-complexes of the cancer cells. In advanced ultrasound therapies, ultrasound coupled to microbubbles is reported to induce vasculature permeability perturbations for immune cell delivery to tumour sites [208]. Details on how ultrasound impacts vasculature permeability is out of the scope of this review. However, Xu et al. have provided a comprehensive review on translational prospects of ultrasound and microbubbles for tumour immunotherapy [209] and Liu et.al reviewed how ultrasound-targeted microbubbles can remodel the microenvironment for immunotherapeutic effect [210]. Nevertheless, to our knowledge, there is no study thus far, associating ultrasound-induced vasculature/ cell junction disruption of cancer cells to NK cell accumulation at the tumour sites. On the other hand, one study showed an increase in CXCL3 ligand 1 expression in tumour sites after ultrasound treatment [206] and increased expression of CXCL3 ligand 1 is known to be a positive prognostic marker that is associated with increased attraction of immune cells, including NK cells [211]. Unfortunately, how ultrasound increased CXCL3 ligand 1 in the tumour is unknown. Prospective studies in these areas would provide useful understanding on how ultrasound therapy enhances NK cell recruitment to the tumor sites.

3.4. Other cancer treatment options affecting NK mechanobiology

While the sections above discuss direct mechanical cues affecting NK mechanobiology, it should be noted that *in vivo* pathophysiology is complex, and mechanical cues could result in biochemical responses and vice versa. Hence, other treatment options such as chemotherapy, targeted cancer therapy and immune therapy will ultimately result in

biochemical changes that affect NK response to cancer cells. Nevertheless, the intricate networks of signaling changes induced by these therapies can result in complex paracrine and autocrine responses leading to biochemical changes that may affect NK mechanobiology. Additionally, more detailed mechanistic studies in this area is required. Hence these therapies will only be discussed briefly in this review.

3.4.1. Chemotherapy

The alkylating agent, dacarbazine (DTIC), is one of the most widely used chemotherapeutic agent for treatment of metastatic melanoma and Hodgkin's lymphoma [212]. Despite inducing the formation of functional adducts within the DNA of cancer cells and halting cell division, DTIC was also shown to upregulate NKG2D ligands [213], which signals NKG2D receptor, resulting in enhanced killing of melanoma cells by NK cells and CD8 T-cells [213]. Similar observations were made for another chemotherapeutic reagent, temozolomide (TMZ), which is used to treat glioblastoma. As mentioned earlier, NKG2D-DAP10 in NK cells can generate F-actin at the NKIS, thereby promoting NK cell cytotoxicity [200] (see section 3.2). Hence, the question remains on whether repeated rounds of chemotherapy will reduce actin dynamics in NK and other immune cell types. A review by Zingoni et al. discussed different classes of chemotherapeutic agents in detail and provides a good summary on how NK cells are affected in their cytotoxicity towards different cancers [214].

3.4.2. Targeted therapy

Targeted cancer therapy is often referred to as the foundation of 'precision medicine'. The common targeted cancer therapeutic agents are Ras-targeted and kinase-targeted therapies [215]. Paradoxically, targeted therapies can sometimes have off-target effects on immune cells; and these effects can be immune-stimulatory or immune-inhibitory depending on the cancer type and combination of tumour and drug(s) used [216]. For instance, treatment of hematological malignancies with tyrosine kinase inhibitor (TKI), imatinib, enhanced the expression of peripheral blood NK cell activating receptors (NCR and NKG2D). On the other hand, the MEK kinase inhibitor, PD3025901, suppressed surface expressions of activating receptors and reduced the anti-tumour activity of *ex vivo* cultured NK cells from melanoma patients [216]. Much focus has been placed on studying the expression of activating and inhibitory surface receptors, proliferation and cytotoxicity of NK cells in patients undergoing targeted therapies. To our knowledge, unfortunately, there are no studies linking targeted therapy to NK cell mechanobiology, e.g. cytoskeletal remodeling, integrin conformational changes and migration, all of which are relevant to how NK cell utilizes mechanoforce to 'sense' its target. From our discussion above, the formation of NKIS, and the engagement of cell surface receptors can result in cytoskeletal changes important in NK mechanobiology. Hence, it will be pertinent to study cytoskeletal responses to targeted therapies and chemotherapeutic agents.

Anti-angiogenic targeted therapy drugs are also at the forefront of anti-cancer therapy development. $BRAF^{V600E}$-mutant melanoma and many solid cancers produce immunosuppressive and pro-angiogenic factors such as IL6, IL10 and VEGF [217,218] into the tumour microenvironment (TME). In one study, NK cells have been shown to increase adhesion to VEGF coated matrigel and endothelial cells [219]. Furthermore, VEGF is known to stimulate actin reorganization and migration of endothelial cells via Akt kinase [220]. Hence, it seems counterintuitive that although NK cells may migrate and adhere in the TME due to VEGF, the overall cytotoxicity of NK cells against tumour decreased. Nonetheless, the complex TME harbors an array of immunosuppressive cytokines and other immune cell types (e.g. regulatory T cells), and the interaction of soft cancer cells with NK cells may contribute to reduced NK cytotoxicity. In addition, different cell types may respond differently to VEGF stimulation. For example, VEGF was shown to impair the motility of human dendritic cells [221]. Next generation targeted cancer therapies could combine anti-cancer (e.g. anti-angiogenic drugs) and pro-NK (e.g. immune checkpoint inhibitor drugs) to overcome resistance to targeted therapies. This leads to our discussion of immune therapies with a focus on NK cells and the potential contribution to NK mechanobiology to 'sense' the target.

3.4.3. Immune therapy

Immune therapies with NK cells are promising platforms for elimination of cancer cells. The ability to perform *in vitro* activation, expansion and genetic modification of NK cells prior to adoptive transfer, would allow the healthcare industry to overcome cancer and drug resistance. Cytokines, autologous NK cells, allogenic NK cells and CAR-NK cells are 'pioneers' in NK cell treatment [222]. However, there are no detailed studies on how these therapies are directly attributable to changes in NK mechanobiology. Hence, this review will briefly discuss some aspects of immunotherapies with inference on how immune therapies may affect NK mechanobiology.

As mentioned in section 3.1.1, the prolonged *in vitro* incubation of NK cells with the common cytokine supplement, IL2, can result in highly rigid NK cells. Interestingly, IL2 and IL21 can synergize to enhance human NK cell cytotoxicity, which is associated with increased production of perforin, granzyme B, CD69 and a slight increase in NKG2A expression [223]. Inferring from these two observations, viz., IL2 increases NK cell rigidity and IL21 with IL2 together promotes slight increase in inhibitory NKG2A expression, we propose that a combinatorial treatment of isolated NK cells *ex vivo* with interleukins, can sensitize NK cells to prevent pre-mature exhaustion and maintain cytoskeletal dynamics for cytotoxic effector functions. While NK exhaustion is in part caused by chronic exposure to activating stimuli during viral infection, tumorigenesis, and prolonged cytokine treatment [224], a persistent and intense actin response can be envisaged at the NKIS and within the cell cortex in protractedly activated NK cells, which may result in rigid NK cells. Rigid NK cells may initially display enhanced contractility favorable for NK-mediated cytotoxicity. However, as discussed above in section 3.1.1, rigid NK cells do have their fair share of problems. The slight increase in NKG2A inhibitory receptor in IL2 and IL21 treated NK cells [223] may prolong the functional life span of NK cells to facilitate dynamic regulation of cytoskeletal components. After all, it is known that NKG2A can inhibit NK cells by disrupting actin network at the NKIS [225]. The review by Chu et al. provides an update on cytokine treatments for NK immune therapy [222].

While allogenic and autologous NK cell treatments are currently being explored for clinical use, mechanistic studies on how these treatments affect NK cell mechanobiology is lacking, albeit important. Inhibitory receptors on donor allogeneic NK cells do not recognize HLA-1 on recipient cells, thus cancer cells can be removed by allo-reactive cancer cells. Again, 'mis-engagement' of inhibitory receptors may promote dynamic regulation of cytoskeletal components through modulating SHP-1 responses. SHP-1, as mentioned earlier, can be regulated by actin dynamics and it is inhibitory to NK cytotoxicity [16]. Much more mechanistic and imaging studies are needed to evaluate the effects of allogeneic and autologous transfer of NK cells on NK mechanobiology.

3.4.4. Radiotherapy

Radiotherapy remains as a mainstream treatment for cancer and can be used in combination with chemotherapeutic agents. Much focus has been on how radiation affects T cell immune response and only recently, mechanistic studies on how radiation affects NK cells directly and indirectly has gained pace. High-dose ionization radiation (HDIR) is detrimental to healthy cells as it induces DNA damage and it causes cellular senescence in cancer cells. Conversely, some studies showed improved NK cytotoxic responses with low-dose ionization radiation (LDIR) [226,227], facilitated by p38-MAPK and IL2 signaling pathways [226,227]. However, how these pathways affect the mechanobiology of NK cell remains unknown. In general, studies have suggested that radiotherapy can promote migration and retention of NK cells to cancer/tumour sites [228,229] and causes cancer cell membrane tension and

composition modulations [230].

Walle et al. showed that during radiotherapy, NF-κB and mTOR pathways in pancreatic, melanoma and prostate cancer cells orchestrated the released of the chemokine, CXCL8, causing the directional migration of $CD56^{dim}$ NK cells [228]. Interestingly the regulation of NF-κB and mTOR pathways with radiotherapy suggested the activation of a senescence-associated secretory phenotype that has been attributed to the progression of EMT [231]. Hence, activation/ inhibition of EMT could present a double-edged sword in immunotherapy. A review by Castriconi et al. beautifully summarized the molecular mechanisms of directed migration and retention of NK cells in human tissues [232].

While it seems that radiotherapy may promote NK migration, adhesion and retention to tumour sites, which improves survival against cancer, Tuomela et al. showed that radiotherapy can transiently induce membrane tension and composition changes in cancer cells that results in resistance to pore-forming perforin attack by NK cells [230]. In respect of this, the authors have suggested that the timing and dosage of radiotherapy is crucial for better outcome of combined radiotherapy and immune therapy. A 72-h period following radiotherapy was deemed the golden period that cancer cells are susceptible to NK cells [230]. Interestingly, this study also showed similar effects comparing cell cycle arrest drugs and radiotherapy. Furthermore, the use of a microtubule inhibitor, nocodazole, induced cell cycle arrest suggesting that cytoskeletal integrity is key to NK-dependent cytotoxicity [230]. Hence, whether chemotherapeutic and targeted therapy drugs that arrest cell cycle will elicit similar membrane changes on cancer cells (and NK cells) as a result of cytoskeletal rearrangements, warrants further investigations.

3.5. Mechanically-induced nuclear deformation in immune cells

The discussion of mechanical cues in NK and target cell stiffness in the previous sections relate to a central theme of forces within the cell. It may be envisaged that mechanical cues during physiological processes like NK transmigration, can alter intracellular organelles. Such outcomes may include the deformation and restoration of the nucleus which may induce changes to the 3D genome organization and regulation of gene expressions for immune activity and cytotoxicity.

Although research on NK cell nuclear deformation and its impact on genomic activities is limited, there are some observations made with other immune cell types of the common lymphoid lineage (e.g. T [233] and B [234] cells and dendritic cells [235]) that may partially represent NK cells. Proposed in 2012 and 2013, T-cells activated by beads coated with surrogated antigens resulted in the repolymerization of cylindrical actin cortical layer from the beads towards the nucleus [233]. The actin layer pushes and retains the nucleus at a fixed position and ultimately deforms the nucleus and the plasma membrane [233]. The same researchers also demonstrated that T-cell activation induced nuclear deformation (elongation) correlated with gene expression changes in CD69 expression, which was shown to be dependent on ERK and NF-κB signalings [236]. Interestingly, as an early T-cell activation marker, CD69, has also been shown to be indispensable for NK cell-mediated killing of resistant targets [237]. Hence, during the transmigration of NK cells into tightly confined TME, there may be opportunities for NK cells to upregulate CD69 expression. However, more work is required to define the spatial and temporal requirements for altering NK surface markers during transmigratory events.

Actin dynamics can account for the force within the cell that deforms the nucleus. Despite such deformation resulting in differential gene expressions, it is now known that nuclear mechanics can alter chromosomal positions to activate/inactivate genes. Simply put, chromosomes have their 'territories' (chromosome territories, CT). Large chromatin loops that extend away from CT, and several regions within the CT are known to have higher probability of transcription factor activation [238]. For instance, CD8 gene expression during thymocyte development is accompanied by relocation of the locus outside its CT [238]. During NK cell transmigration into and within the TME, NK cells have been shown to be deformed and elongated. It is known that as endothelial cells elongate, their nucleus deforms and chromatin condenses towards the center resulting in reduced proliferation [239]. In another mouse embryonic stem cell study, the nuclear deformation provides a driving force for long-range migration associated with the reorganization of heterochromatins [240]. These observations suggest a conundrum that the transmigration of NK cells into confined areas (e.g. TME or lymphoid tissues) depends on a 'push' factor driven by nuclear deformation. Indeed, one study showed that Arp2/3-driven actin polymerization enables nuclear deformation of dendritic cells, which facilitated dendritic cell migration through complex environments [235]. A recent review by Kalulula et al., described the details of nuclear deformation and the associated functional consequences (including nuclear rupture; not discussed here) with a limited focus on immune cells [241]. Nevertheless, research on NK cell nuclear deformation and its consequences on NK transmigration, cytotoxicity regulation and exhaustion, is lacking and awaits further interrogation.

4. Advanced engineering approaches to study NK cell mechanobiology

The application of advanced materials, especially at the nanoscale levels, to interrogate mechanotransduction events in NK cell biology is a subject of intense research. Although NK cells offer 'off-the-shelf' solution to cancer immunotherapy, gene editing is still a technical hurdle that needs to be addressed for fast and efficient production of highly cytotoxic NK cells for immunotherapies [242]. This is readily appreciated since NK cells have endogenous pattern recognition receptors (e.g. TLRs) that detect and mount an immune response against foreign materials [45]. Hence, many researchers have envisaged the effective delivery of 'bio-packages' into NK cells to be a significant milestone in proving NK cell-based therapy as an alternative to CAR T-cell therapies. Furthermore, the mechanical properties of advanced materials (e.g. nanowire length and diameter) are under intense scrutiny.

While advanced materials and engineering techniques can be used to deliver 'bio-packages', it can also mimic *in vivo* tumour microenvironment (TME) even under *in vitro* conditions. Hence, developing innovative engineering approaches (e.g. microfluidic devices) to study NK cell behavior in relation to the TME and the relevant tissue mechanics is also an ongoing effort in the NK field. There are countless examples of advanced engineering approaches related to NK cells. These include but are not limited to microfluidics, microspheres, nanomaterials, quantum dots and fullerene materials. Saux and Schvartzman [243] provided an in-depth review on these engineering approaches. Here, we will focus on how microfluidics, microsphere and nanowires are applied to NK cell mechanobiology.

4.1. Microfluidics to investigate NK cell cytotoxicity

Two unique advantages of microfluidic devices are the requirement for ultra-low volumes of biofluids for rapid and easy analyses and more accurate representation of *in vivo* environments [244]. NK cell researchers have employed microfluidics devices to examine how the TME can affect NK cells. In one study, a tumour-on-a-chip model included breast cancer cells embedded in a 3D matrix, and a lumen at one end of the microchamber which mimicked the vasculature present in the tumour [245]. Similar to the *in vitro* observation reported by Wong et al. [23], rapid cancer killing was observed by NK-92 cells, and prolonged exposure to cancer cells resulted in NK cell exhaustion [245]. In a similar study, breast cancer cells grown in spheroids were embedded in 3D collagen hydrogel within the microfluidic device [246]. Apart from only accessing NK-alone-induced cell cytotoxicity, ADCC was also monitored and was consistently shown to induce cytotoxicity limited to spheroid surface [246].

Interestingly, it was observed that NK-92 cells (an NK cell line

undergoing clinical trial) penetrated tumour spheroid much faster than antibody penetration [246]. Hence, using this model, it is possible to study how the NK cell machinery 'force'-opens tumour cell-cell junction and migrates within the spheroid. However, both studies fell short of investigating how microenvironmental factors can contribute to NK cell cytotoxicity against cancer. It is known that the TME is hypoxic and maintains a low pH [45,247]. Hence, future studies may benefit from the advantages of microfluidic devices to alter the gradient of nutrients, oxygen and even acidity in the microfluidic device to better represent the *in vivo* conditions of the TME.

The TME is a reservoir of a myriad of stromal cells, including endothelial cells, fibroblasts, adipocytes, stellate cells and innate and adaptive immune cells, which elicit potent pro/anti-tumour activities. The NK-dendritic cell interaction is now recognized as an important component for NK cell migration [248]. One of the mechanisms is the production of chemokines that induce NK-cell migration [249,250] towards the TME. Hence, apart from studying NK-tumour interaction, investigations on the interaction between NK and other stromal cell types has been made possible with microfluidic devices. Hipolito et al. [251] demonstrated that using two microfluidic chips (D^3-Chip and T^2-Chip) have enabled NK cells to come into contact with mature dendritic cells more often than with immature dendritic cells. Interestingly, the branched protrusions from dendritic cells that exhibit certain morphological and mechanical features are also an important consideration to study NK-dendritic cell interaction [243].

Another prospect of using microfluidics is the possibility of investigating vaccine dendritic cells and their effects on NK cell and adaptive immune cells. Shin et al. reported a protocol to perform microfluidic assay for simultaneous culture of multiple cell types on surfaces or within hydrogels [252]. Vaccine dendritic cells can stimulate NK cytotoxicity and cytokine secretion. In a process termed dendritic cell editing, activated NK cells can eliminate uninfected and immature dendritic cells due to lower MHC expressions in immature dendritic cells. Along with enhanced killing of target cells by activated NK cells (due to vaccine dendritic cells releasing antigens), mature dendritic cells can activate T cells through antigen presentation [253]. However, with the help of advanced multi-cellular microfluidics, more pertinent investigations could be made on dendritic cell-NK interaction and especially so for detailed characterization of the best combination of NK/ dendritic cell subsets to yield optimal cell-cell interactions. For instance, in normal noninflamed lymph nodes, NK cells are $CD56^{bright}$ $CD16^{dull/-}$ and it was suggested to be less capable of activating dendritic cells [254]. On the other hand, $CD56^{dim}$ $CD16^{dull/-}$ NK cells were able to better activate dendritic cells [254]. How CD56 and CD16 levels on NK cells are affected by dendritic cells and their implications in conversely activating T cells need to be elucidated with more research.

4.2. Advanced materials & approaches- microspheres and nanowires to engineer NK cells

Advanced materials have been gaining attention in NK cell research because of their abilities to promote NK cell survival, proliferation, infiltration and even mimic dendritic cell activation [25,243,255–257]. Due to the immune-suppressive nature of the TME, NK infiltration and NK exhaustion have been long-standing problems associated with NK-based immunotherapy. Recently, Dan et al. [258] demonstrated the use of porous microspheres to encapsulate and protect NK cells from the complex immune-suppressive TME. The porous microspheres were mainly made up of alginate solution and poly(ethylene oxide), with diameters ranging from 250 to 700 μm. The porosity facilitated NK cell oxygen and nutrient exchange, resulting in continual proliferation and secretion of cytotoxic molecules from the encapsulated NK cells through the microsphere pores [258]. Thus, adoptive transfer of the microsphere embedded NK cells provides a localized concentration/ reservoir of NK cells within the TME. Overall, NK cells within the microspheres were found to robustly kill tumour cells *in vivo* owing to (i) sustainable release of perforin and granzyme, which pervades the TME to kill cancer cells and (ii) budding off of the NK cells from the microsphere to initiate contact-based killing of surrounding residual cancer cells and tissues [258]. Hence, it is possible in future, to directly inject microsphere-encapsulated NK cells into solid tumours to ascertain their anti-tumour effects. Nevertheless, a biological hurdle still exists with the highly immune-suppressive TME that may increasingly create an unfavorable environment (e.g. hypoxia and acidity) for NK proliferation.

Apart from microspheres, nanowires are also gaining attention among researchers using advanced materials to study NK cell biology. Three main schools of thought which prompted the use of nanowires of varying lengths and compliance came from observations that: (i) NK cells apply mechanical forces to probe target cells and to decide whether it should be attacked or tolerated [257], (ii) nanowires mimic the long dendrites of dendritic cells [257] and (iii) nanowires mediate efficient delivery of packages (e.g. siRNA) into immune cells [242].

In one study, it was found that NK cells can exert a 'pinching' force of about 10 pN on dense nanowires resulting in immobilization of the activating receptor ligand, MICA, which was required for NK cell degranulation [257]. However, the same group later showed that nanowires of ≈20 μm in length could induce NK cell activation even in the absence of antigen functionalization on the nanowires [255]. Nevertheless, the main conclusion from the studies was that nanowires were more efficient at stimulating NK activation compared to flat surfaces [255,256]. It was suggested that the nuclear translocation of NF-κB in response to nanowires was mainly responsible for the enhanced production of NK cytotoxic molecules. However, the exact molecular/ mechanical mechanism at the NK-nanowire interface that resulted in the transmission of signals into the cytoplasm, for NF-κB nuclear translocation, remains unclear. Since the nanowires in these studies were functionalized with NKG2D ligand, MICA [255], it is possible that the activation of DAP10 could affect actin remodelling and intracellular contractility to promote transcription factor nuclear localization. Going forward, it will be interesting to: (i) identify proteins (e.g. integrins) present on the NK cell surface that might pull against the nanowires to sense and exert a pinching force on the nanowires, (ii) ask whether similar mechanisms depending on tyrosine kinases identified in fibroblasts [259] exist in NK cell biology, and (iii) modulate the length and density of the nanowires, to regulate the topography of the nanowires to mimic professional antigen presenting cells such as dendritic cells. The roles of the branched projections in dendritic cell in modulating NK responses are still unclear. With the use of nanowires coated with dendritic cell MHC class I ligand, we propose future studies to ascertain whether antigen loading is a pre-requisite for dendritic cell activation of NK cells, and to seek insights into the outcome of the above-mentioned research strategies.

Apart from providing mechanical cues to NK cells, nanowires can also be used to deliver biomolecules into NK cell cytosol. The high mechanical compliance of nanowires provides the robustness for it to be tuned into diameters and lengths of varying degrees [242] and allow for spontaneous penetration into cell membranes of various cell types (e.g. fibroblast, cancer and immune cells) [260]. The nanowires are usually made of silicon and prepared using chemical vapor deposition (CVD) methods [260]. Plasma-treatment of the silicon nanowires allow for deposition and co-deposition of biomolecules such as siRNA on the nanowires [260]. As demonstrated by Shalek et al. [242], different immune cells would require nanowires of varying lengths and densities. Being non-adherent, NK cells were suggested to require nanowires that were longer (2–3 μm), sharper (diameter < 150 nm), and denser (0.3–1 per μm^2) in comparison to larger adherent cells (e.g. macrophages). Nevertheless, these nanowires were proven to be effective in the delivery of siRNA into the cytoplasm of the cells without affecting their viability [242]. Nanowires have immense translational potential to deliver biomolecules into the NK cell cytoplasm. One obvious advantage is the direct delivery of biomolecules without the need for lentiviral/ retroviral transduction. Primary cells, especially that of NK cells, are

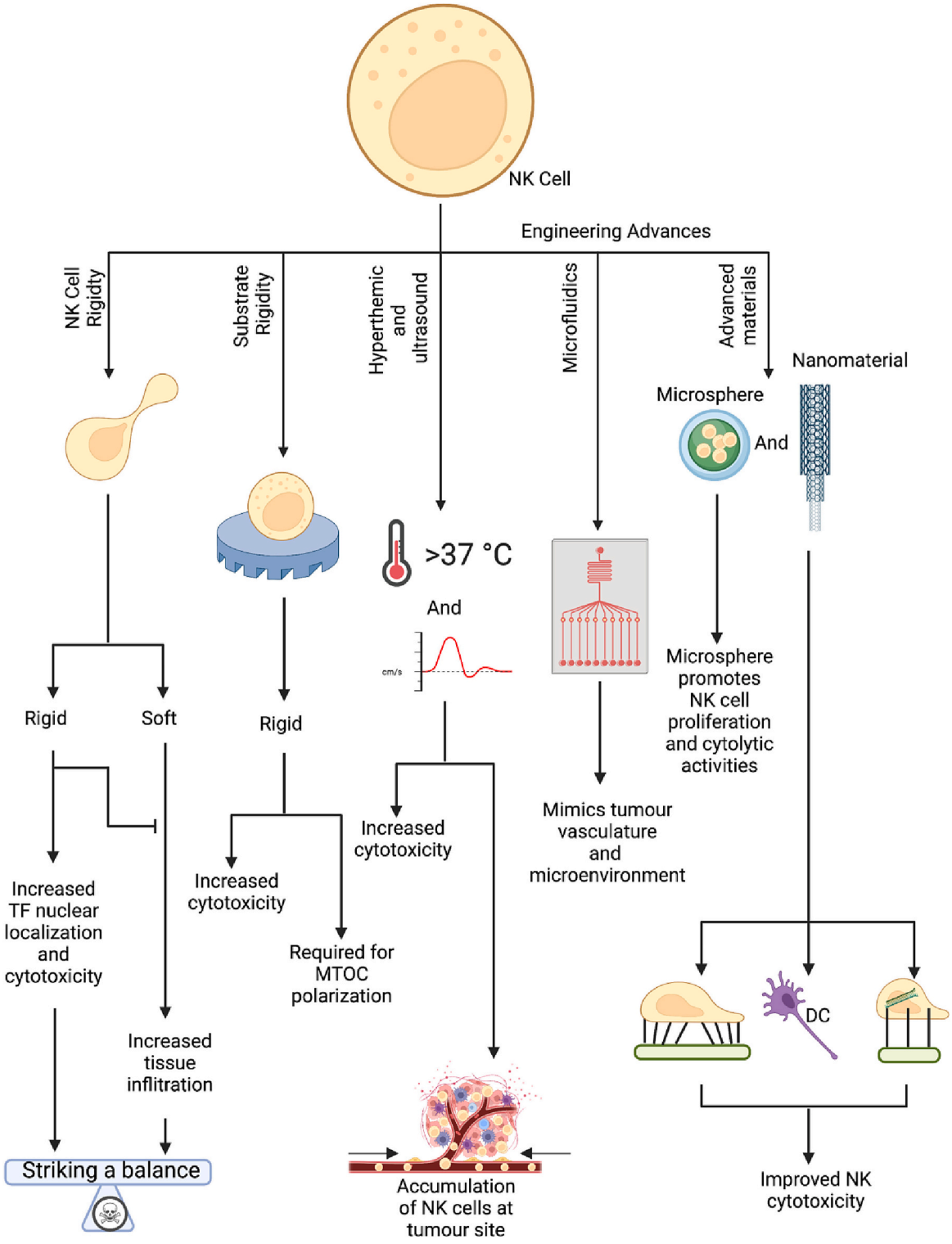

Fig. 4. Mechanical cues regulate NK cell biology. Biomechanical cues affect the cytotoxicity and accumulation of NK cells at target sites. The NK cell rigidity can be increased to promote cytotoxicity inducing TF nuclear localization. However, a stiff/ rigid NK cell will have difficulties in tissue infiltration. In general, a stiffer substrate has been shown to enhance NK cytotoxicity. Whole body/ organism hyperthermic treatment or minimally invasive ultrasound can increase NK cytotoxicity and induce accumulation of NK cells at target sites. Microfluidic devices can mimic the TME vasculature and provide a valuable tool to study NK-tumour cell interaction *in vitro*. Nanomaterials such as microsphere has been explored to protect NK cells from the harsh *in vivo* TME and promote proliferation of NK cells *in vivo*. Nanowires or varying lengths can enhance NK cytotoxicity, mimic dendritic cell (DC) dendrites, and even deliver bio-packages (e.g. siRNA) into NK cells. Refer to Section 4 for details.
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notoriously difficult to be maintained in the long term after viral transduction. Hence, delivery of biomolecules directly with nanowires will facilitate the uptake of genetic materials into NK cells.

5. Conclusion and future directions

This review started with providing an overall understanding of the cytoskeletal network in NK cells followed by a discussion on the mechanical cues that can affect NK biology. Importantly, most of the pathways are intertwined and should not be studied in isolation. For instance, the polarization of MTOC depends on actin accumulation at the NKIS [88] and actin accumulation at the NKIS depends on A-type lamin [109]. Each of these events are regulated by their corresponding pathways and networks of molecules. In mechanosensing, the actomyosin also plays a critical role in the transmission of cell force into biochemical signals [23,255]. Moreover, various biomechanical cues are intricately linked (Fig. 4). For instance, the rigidity of the cell matrix can be fine-tuned with the length of nanowires and the temperature of the micro-environment can be tweaked with ultrasound intensity. Hence, it is important to study NK cell biology at the systems level. Dhillon et al. [261] provided an excellent.

review on approaches to understand human immune system using systems biology. Furthermore, as we progress into the era of personalized medicine, systems biology will offer invaluable insights to inform the stratification of individuals with the same syndrome but with different underlying mechanisms and staging of the disease. In combination with next generation single cell spatio-transcriptomics, more subpopulations of NK cells in different tissues may be identified. How these new subpopulations of NK cells will affect therapeutic precision and how they are individualized in well-designed clinical trials would need further study. It is beyond the scope and a limitation of this review, to discuss in detail how NK mechanobiology might affect clinical trials, although it will be predictably significant to cancer-immunomodulation.

Many aspects of mechanosignaling in NK cell biology remain to be explored. For instance, the influences from cell confluency, shear stress, and compressive force on NK cells have thus far remained elusive. This review could serve as a starting point for scientists to appreciate the importance of dynamic cytoskeletal components in regulating NK cytotoxicity. For example, bearing in mind how the expression of surface activating receptors or internalization of antibody recognition receptor, CD16, could affect NK cytotoxicity through modulating cytoskeletal proteins. Nevertheless, researchers should not be contented with only phenomenon-driven research and ignore the mechanistic understanding of mechanosensing and mechanotransduction in NK cell biology. Employing the use of advanced bioengineering materials and approaches can provide insights into some of these unanswered questions. For example, it was shown that T cell stiffness is enhanced upon the formation of the immunological synapse [123]. Microfluidic device has been shown to be able to perform stiffness-dependent separation of cells [262]. Hence, microfluidic chips may aid in the characterization of NK cell stiffness upon NK activation.

Furthermore, an uncharted but important aspect in mechanosensing is the role of force on NK cell nuclear morphology and gene expression. For NK cells to reach into the tumour mass, it must infiltrate the cell-cell junctions between the tumour cells. Inevitably, NK cells which are squeezed through tight junctions will have its nucleus deformed in the process. It has been shown in fibroblasts, that cell geometry induces differential degrees of intermingling between specific chromosomes resulting in specific regulation of gene expression [263]. The stability and duration of chromosome intermingling seems to depend on biomechanical and biochemical factors. While cell geometry and confinement on micropatterns can induce chromosome intermingling [263], RNA polymerase and transcriptional activation are enriched at chromosome intermingling regions observed during embryonic stem cell differentiation on fibronectin coated dishes [263,264]. Hence, the stability and duration of intermingling should also depend on physiological cues, viz., activated/inactivated signals during physiological differentiation or permanently activated/inactivated in transformed cancer cells. Therefore, it will be of interest to leverage on the potential of biochemically (e.g. ECM coating) and biophysically (e.g. channel size) well-defined microfluidic devices to deform the NK nucleus, and examine global genomic changes in NK cells with altered nuclear geometries.

This review has provided an overview of the potential of mechanotransduction in NK cell biology. Detailed mechanistic as well as systems level understanding of NK cell biology during and after mechanotransduction is needed and warrants future studies to 'force' NK cells to sense and immunomodulate infected and cancer cells.

Author contributions

D.C.P.W and J.L.D wrote the manuscript. All authors commented on the manuscript.

Declaration of Competing Interest

The authors declare no competing interest.

Data availability

No data was used for the research described in the article.

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