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Dissecting cellular mechanics: Implications for aging, cancer, and immunity

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ABSTRACT

Cells are dynamic structures that must respond to complex physical and chemical signals from their surrounding environment. The cytoskeleton is a key mediator of a cell's response to the signals of both the extracellular matrix and other cells present in the local microenvironment and allows it to tune its own mechanical properties in response to these cues. A growing body of evidence suggests that altered cellular viscoelasticity is a strong indicator of disease state; including cancer, laminopathy (genetic disorders of the nuclear lamina), infection, and aging. Here, we review recent work on the characterization of cell mechanics in disease and discuss the implications of altered viscoelasticity in regulation of immune responses. Finally, we provide an overview of techniques for measuring the mechanical properties of cells deeply embedded within tissues.

1. Introduction – the role of mechanics in cell biology

Mechanical deformability of the cytoplasm of adherent cells is a critical cellular property associated with important cellular and subcellular processes. For example, during the wound healing response, migrating cells at the wound edge significantly increase the stiffness of their cytoplasm to enable dendritic filamentous actin (F-actin) assemblies to produce net protruding forces against the plasma membrane [1]. The translocation of organelles including the nucleus, endoplasmic reticulum, and mitochondria, is partly regulated by the local viscoelastic properties of the cytoplasm [2–5]. The intracellular viscosity of the growth cone also regulates axonal elongation of neurons [6]. These studies highlight the complex, dynamic and anisotropic nature of cells, which must respond in both space and time to the biochemical and biophysical cues presented by the cellular microenvironment to persist, differentiate, and migrate [7–9]. Changes in the mechanical properties of cells often correlate with disease states such as cancer [10,11], infection [12,13], and laminopathy (genetic disorders caused by mutations in the nuclear lamin gene *LMNA*) [2,4]. Moreover, embryonic fibroblasts derived from mouse models of progeria (premature aging) and muscular dystrophy display a significantly more compliant (*i.e.* more deformable) cytoplasm than wild-type controls [14]. These cells also display a poor resistance to shear forces and an impaired ability to migrate during wound healing [15]. During healthy aging, the cytoplasm of human dermal fibroblasts stiffens, a change accompanied by a significant increase in the traction forces exerted on the surrounding matrix [16,17].

Rheology is the study of how materials deform (strain) under the application of force (stress). In cell mechanics, stress is generated internally by cytoskeletal contractions or externally by hemodynamic and interstitial flow, or by interactions with neighboring cells and tissues. Stress is given as units of force per unit area. The relationship between stress and strain in typical elastic, viscous and viscoelastic materials is illustrated in Fig. 1. The cytoplasm of mammalian cells is known to exhibit viscoelastic-like behaviors. A more detailed explanation of rheology and cellular viscoelasticity can be found in Wu et al. (2018), Wirtz (2009) and Suresh et al. (2007) [11,18,19] and A summary of commonly used methods for the measurement and interrogation of cell mechanical properties can be found in Table 1. Young's modulus (*E*) and shear modulus (G) are common parameters used to characterize the rheological responses of cells. Young's modulus is measured by applying a uniaxial stress perpendicular to one of the surfaces of the sample to

Abbreviations: AFM, atomic force microscopy; AID, activation-induced demainase; APC, antigen presenting cell; BCR, B cell receptor; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; FA, focal adhesion; FDC, follicular dendritic cell; FN, fibronectin; GC, germinal center; IS, immunological synapse; MHC, major histocompatibility complex; MMPs, metalloproteinase; MRI, magnetic resonance imaging; MSD, mean-squared displacement; pMHC, peptide major histocompatibility complex; SHM, somatic cell hypermutation; TCR, T cell antigen receptor; TFM, traction force microscopy; VDJ, variable diversity joining

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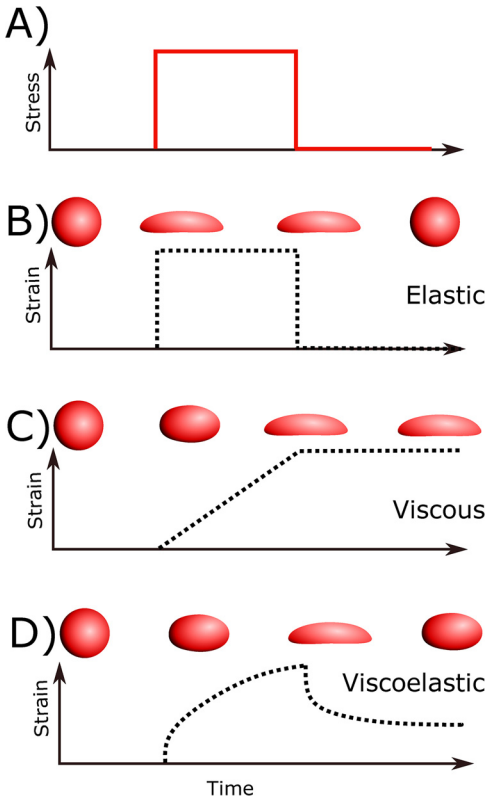

Fig. 1. Viscosity, elasticity, and viscoelasticity describe a material's response to stress over time. Here we show the representative response of an object under each of the aforementioned conditions. (A) For the purpose of this example, stress is given as a step function, which increases instantaneously and is applied at a constant rate, before instantaneously decreasing. (B) Perfectly elastic objects undergo immediate deformations under the application of stress. (C) Perfectly viscous objects undergo time-dependent deformations in response to stress. Viscous objects do not reverse their deformation in response to the withdrawal of stress. (D) Viscoelastic objects, such as cells, display both instantaneous and delayed deformations in response to the application of force. Deformations are only partially recovered under the cessation of force application.

deform it either by compression or by extension. On the other hand, the shear modulus is measured by applying a force parallel to the surface of the sample. Of note, common methods used to measure cell mechanical responses, such as atomic force microscopy (AFM), usually involved more complex deformation than either uniaxial extension/compression or simple shear.

An essential feature of Young's moduli measurements, especially for biological samples, is that the volume of the sample is not necessarily conserved during deformation. Poisson's ratio represents the extent to which a sample changes its volume, or equivalently the relationship between changes in the vertical dimension and the two orthogonal dimensions. A ratio of 0.5 between the two dimensions is observed in samples maintaining constant volume under stress. A Poisson's ratio of less than 0.5 indicates that the sample has lost or gained volume during compression or extension, respectively. For shear deformations, the volume is maintained during force application [20]. Commonly used techniques for measuring Young's modulus in biological samples include atomic force microscopy (AFM) and optical stretching. Particle tracking microrheology and magnetic twisting cytometry (MTC) [21] are commonly applied to measure the shear modulus (Table 1) [18]. Of note, an assumed value for Poisson's ratio is required to measure Young's modulus (E) or shear modulus (G) using techniques such as AFM. Several techniques, which derive frequency-dependent stress or strain measurements, are used to determine the dynamic modulus. These methods include magnetic twisting cytometry (MTC), parallel plates, and particle tracking microrheology; and directly measure the viscoelastic response of cells based on storage modulus and loss modulus. Methods such as AFM, optical stretcher, and parallel plates can be used to determine other viscoelastic properties like creep, which is characterized by an increase in strain with a decrease in strain rate (Fig. 1d).

In principle, different rheological measurements can be inferred from one another if certain assumptions about the material measured are held valid. For example, Young's modulus is related to the shear modulus by the expression $E = 2G\ (1 + \mathrm{n})$, where n is Poisson's ratio. However, this relationship is strictly valid for isotropic materials in the limit of small strain. Cells, however, are not isotropic (*i.e.* the local mechanical properties of cells may not be identical in all directions of observation) and accurate rheological measurements require finite, sometimes large strains to obtain reliable force data. Accumulating evidence from rheological studies of mammalian cells suggests that they can display poroviscoelastic properties at the force range of 2–5 nN [22]. This complex behavior is described by the sum of the viscoelastic properties of the cytosolic biopolymer network and the poroelastic properties that allows water movement within this network. AFM studies have also shown that cellular viscoelastic responses can be non-linear at force range of 20–700 pN [23]. Given that cellular viscoelastic responses are thus non-linear, estimates of moduli from primary data should be interpreted with caution [22–24].

Moreover, Poisson's ratio cannot be assumed to be a constant on the timeframe of nanoscale measurements. A porous material is poroelastic when fluid within it can move in and out due to pressure applied to the material. This is likely the case for living cells, which express aquaporin water channels and ion channels [20,25]. Poroelastic properties can lead to confounding results in the calculation of a sample's mechanical properties [26,27].

A large body of work now shows that cells in a disease state have significantly different physical properties from those under healthy homeostatic conditions. Molecular lesions, whether caused by genetic mutations, epigenetic modifications or infection, can result in aberrant cellular functions and an altered biophysical state. In this review, we address the role of cell mechanics in human disease and aging and discuss recent studies highlighting the importance of cellular rigidity and viscoelasticity in maintaining healthy immune homeostasis. Finally, we describe recent technological advances that allow for mechanical measurements in more physiologically-relevant 3D, multicellular systems.

2. The role of cell mechanics in human diseases

In the past few decades, a large number of studies have revealed that changes in cell and nuclear mechanics can be hallmarks of human diseases; particularly metastatic cancer, cardiovascular disease, inflammation, laminopathies, host-microbe interactions in infectious diseases, and frailty in aging. Here we highlight some critical findings that have associated cellular mechanics with human diseases and aging.

2.1. Cell viscoelasticity in cancer

For metastatic cancer of epithelial origin (or carcinoma) to occur, tumor cells must invade and migrate through the stromal matrix, intravasate through the endothelium of blood vessels, survive the shear forces of blood flow, successfully re-attach to blood vessel walls, colonize a distal site, and be reactivated following dormancy, all while avoiding immune surveillance. The ability of cancer cells to succeed in each of these steps and advance towards the formation and growth of a secondary tumor depends, in part, on the physical interactions and mechanical forces between cancer cells and the microenvironment [28].

As cancer cells metastasize, they deform their shape to squeeze

Table 1
Summary of methods to measure the mechanical properties of cells.

Technique	Active	Passive	2D	3D	Characteristic measured	Technology requirements	Notes	References
Atomic Force Microscopy	x		x		Young's modulus, cell adhesion	AFM and cantilever tips	Cantilever tips can vary result outcomes; aqueous environment can be challenging	[132,133]
Traction Force Microscopy		x	x	x	traction forces; cell adhesion	Confocal microscope; microbeads	Can be computationally intensive, particularly in 3D.	[128]
Substrate/Gel Compaction		x		x	Traction forces	Commercially available substrates	Substrate stiffness can be varied to influence cellular responses	[134,135]
Optical Stretcher	x		x		Cell deformability; shear forces; cell viscoelasticity	Microfluidic system; brightfield microscope	High-throughput method of interrogating cell biophysical properties	[32,136]
Parallel Plates	x	x	x	x	Young's modulus; Stress and strain relaxation over time.	Microfabricated plates	Can be used to interrogate single cells and cell aggregates.	[137–140]
Cell Monolayer Rheology	x		x		Cellular adhesion; viscoelasticity	Standard rheometer	Can be used to quantify average measurements from multicellular samples	[141]
MRI		x		x	Translational forces; torque	Small animal MRI scanner	Can be used to quantify cell migration in 3D system. No single cell resolution.	[130]
Oil Droplet		x		x	Tensile and compressive forces	Confocal microscope	Alterations to droplet composition change range of force detection	[127]
Molecular Force Probes		x	x		Traction forces; Young's modulus	Confocal or standard fluorescence microscope	Can be implemented to measure cell-cell interactions. Range can be varied by altering sequence of probe.	[68]
Particle Tracking Microrheology		x	x	x	Shear modulus; viscoelasticity	Confocal microscope	Can be used to study viscoelastic response of cells embedded in 3D matrices	[10]
Magnetic Twisting Cytometry	x		x	x	Viscoelasticity	Confocal microscope	Used to quantify high-resolution structural changes	[21]
Micropipette Aspiration	x	x	x		Cortex elasticity and viscosity	Brightfield microscope	Stiffness measurements depend on theoretical models	[142]
Micropore Filtration		x	x		Viscoelasticity	Microfluidics; microfabrication facilities	Can mimic *in vivo* environments; can be prone to clogging	[143]
Microfluidics Flow Systems	x	x	x		Shear forces; viscoelasticity	Microfluidics; microfabrication facilities	High-throughput; downstream cells may be influenced by biochemical cues	[136]
Microfabricated Post Array		x	x		Traction forces; cell adhesion	Microfabrication facilities	Spacing of micropillars can influence cell phenotypic response	[50]
Substrate Stretcher	x		x		Frequency response ; cell and ECM viscoelasticity	Substrate stretcher device	Measurements of global cell mechanics and ECM;	[144]
Micromechanical sensors		x	x		Traction forces, cell mass (*via* spring Constant of cantilever); viscoelasticity	Microfabrication facilities	Measurements in aqueous solutions can dampen mass detections	[145,146]
Acoustic Trapping	x		x		Young's modulus; viscoelasticity	Brightfield microscope	Less damaging than optical tweezers and avoids cell contact of AFM	[147]
Deformability Cytometry	x		x		Viscoelasticity	Brightfield microscope, microfluidics system	High-throughput measurements of cell biophysical properties	[148]
Optical Tweezers	x		x		Viscoelasticity	Steerable laser system with inverted light microscope	Can be used to measure the effect of small-scale forces in living cells, but lasers can cause phototoxicity	[149]
Laser Ablation		x		x	Tensile forces	Steerable laser system	Can be used to infer tensile forces in 3D tissue systems	[150]

through the fibers of the extracellular matrix (ECM) and confining spaces formed by other cells (*e.g.* cells of blood and lymphatic vessels) and bones. This process depends on the dynamic regulation of the cytoskeleton, a dense cytoplasmic meshwork composed of three types of filamentous proteins: F-actin (filamentous actin), microtubule, and intermediate filaments. Reorganization of actin filaments is critical for the enhancement of epithelial-like cancer cell motility, a process known as epithelial-mesenchymal transition (EMT) [29]. The ability of a cell to deform is related to its viscoelastic properties, including its viscous modulus, elastic modulus and relaxation time [30–32]. As such, there has been increasing interest in how the mechanical properties of metastatic cells differ from those of non-metastatic or non-transformed cells. One prevailing hypothesis is that metastatic cells are more deformable, which enhances their motility through the basement membrane, endothelium, and dense matrices.

Measurements of cell mechanical properties have consistently shown that cancer cells are softer than their healthy counterparts and that this increased cellular compliance (*i.e.* deformability) correlates with an increased metastatic potential; though the underlying mechanism for such mechanical softening remains to be fully described. To date, increased cellular compliance has been observed in breast cancer [33], colorectal cancer [34], ovarian cancer [35], kidney cancer [36] and melanoma [37]. More compliant cancer cells are often noted to have a more disorganized cytoskeleton. However, it is important to note that these conclusions stem from *ex vivo* analysis of tumors or in vitro analysis of cell lines; it remains to be validated in more physiologically relevant microenvironments.

2.2. Cell viscoelasticity in an immunological context

Molecular level biophysical properties have been studied in the context of immune cell activation [38,39]. Knowledge of these properties has been leveraged for therapeutic design [40]. However, cellular mechanics has been largely overlooked in the context of the immune system. A few recent studies have shown that immune cells are capable of sensing and responding to the physical properties of their environment or target cell. Overall, these studies have demonstrated that there are built-in mechanical response programs in immune cells that allow them to react to intercellular and microenvironmental mechanical cues [41–44]. Below we summarize recent findings in three immune cell subsets: T cells, B cells, and dendritic cells.

2.2.1. T cells

T cells are a critical component of the adaptive immune response; they mediate antigen-specific killing of cancerous or infected cells or release a variety of immune effector molecules that license other cells to do so. Each T cell expresses a unique variant of the T cell antigen receptor (TCR), which is selected during thymocyte development to recognize antigens presented in the context of Major Histocompatibility Complex Class I (MHC-I) or MHC-II in the case of CD8+ or CD4 + T cells, respectively. Given the current rise in prominence of adoptive T cell transfer [45] and Chimeric Antigen Receptor T (CAR-T) cell therapies [46], a deeper understanding of the mechanistic underpinnings of cytotoxic T lymphocyte (CTL)-mediated tumor cell killing is crucial to the advancement of cell-based therapies. Studies of the immunodeficiency disease Wiskott-Aldrich Syndrome have identified remodeling of the actin cytoskeleton as critical to T-cell activation [47]. Indeed, more recent work has shown the role of the actin cytoskeleton in re-ordering the molecular organization of signaling molecules at the cell-cell interface, both in the T cell and its target [48,49].

Recently, Huse and colleagues used the OT-I T cell system to investigate the role of mechanical forces in CTL-mediated target cell killing [50]. On recognition of cognate pMHC, $CD8^+$ T cells re-polarize toward the target cell and release cytotoxic granules containing perforin and Granzyme B [51]. By using a cell micromanipulator, Basu et al. were able to show that T cells initially exert a pushing force on peptide MHC (pMHC)-loaded beads, followed by a period of prolonged pulling and engulfment of the target. These findings correspond well with previous work showing that the immunological synapse (IS) is sustained by retrograde flow of actin toward its center [52,53]. Intriguingly, it was then found that CTL killing efficiency depended on the viscoelastic properties of the target cell. Cognate antigen-loaded B16 melanoma cells were more resistant to killing by T cells when grown on a soft hydrogel substrate (E = 12 kPa) than when grown on a rigid substrate (E = 50 kPa). Furthermore, disruption of the target cell cytoskeleton with Latrunculin A, which blocks F-actin polymerization and lowers membrane tension, resulted in decreased CTL killing efficiency. Treatment with the myosin II inhibitor blebbistatin, which increases membrane tension, resulted in increased killing efficiency of target cells by CTLs.

It has been proposed that by altering the ECM through enhanced contractility, tumor cells can increase stiffness of the microenvironment [54]. In this scenario, if the tumor cells themselves became more rigid, such an alteration to the ECM would be expected to make them more susceptible to CTL-mediated killing. However, as discussed before, metastatic cells tend to be more compliant than their healthy counterparts, a characteristic correlated with invasiveness [55,56]. Equally, changes to the ECM have been proposed to result in the formation of a fibrous physical barrier that prevents immune cell infiltration [57]; shedding light on a complex relationship between cancer cells, immune cells, and the overall architecture of the tumor microenvironment. Given Basu et al.'s findings, this could imply that metastatic cells are less prone to CTL-mediated killing, a finding that warrants further investigation and provides a new angle for therapeutic intervention. Equally, regulation of membrane tension by intracellular pathogens could constitute a mechanism of immune evasion [58].

2.2.2. B cells

Along with T cells, B cells are important components of the adaptive arm of the immune system. B cells exert their role in the adaptive immune response through differentiation into antibody-producing plasma cells and through the production of diverse immunological signaling molecules that serve to direct and regulate the response to infection [59]. During pathogenic infection or tumorigenesis, B cells are exposed to 'foreign' antigens either through cross-presentation by professional antigen-presenting cells (APCs), such as dendritic cells (DCs), or through encounters with soluble antigens present in the interstitial fluid. These antigens are recognized through the B cell receptor (BCR), which is comprised of membrane-bound Immunoglobulin domains noncovalently associated with the transmembrane signaling heterodimer Iga/Igb (CD79a/b) that provides the immunotyrosine-based activation motifs necessary for BCR-mediated signaling and B-cell activation [60]. B cells undergo VDJ (Variable, Diversity and Joining) recombination resulting in an expansive BCR repertoire [61]. On activation, B cells migrate through germinal centers in secondary lymphatic organs where they undergo proliferation, somatic cell hypermutation (SHM) mediated by the enzyme AID, and immunoglobulin class-switching. Within germinal centers, B cells that have undergone SHM are then selected in a Darwinian manner for clones expressing high-affinity versions of the BCR. Clones expressing low-affinity BCR undergo apoptosis [62].

Several recent studies have shown that the BCR acts a mechanosensor, a hypothesis proposed following work by Neuberger and Batista on the mechanism of antigen extraction by B cells [63,64]. Further support for this hypothesis comes from studies showing that B cells are capable of exerting forces intercellularly through the IS [65,66]. B cells activation is strongly influenced by the context in which antigen is presented. Using a system whereby an antigen was presented to B cells on polyacrylamide substrates with variable Young's modulus, ranging from high stiffness to low stiffness (22.1 kPa to 2.6 kPa), Wan et al. showed that B cell activation was enhanced when antigens were presented on rigid substrates [67]. Antigen presented on the rigid

substrates drove increased BCR microcluster formation, which correlated with sites of increased tyrosine phosphorylation indicative of BCR signaling. In agreement with this finding, B cells activated on the rigid substrate also showed increased expression of the downstream activation marker CD69 [67]. Interestingly, selective pharmacological disruption of various cytoskeletal components indicated that B cell force-sensing utilized microtubule dynamics.

Furthermore, Spillane and Tolar (2016) determined that B cells preferentially internalize antigens *via* mechanical sampling, as opposed to chemical extraction through the release of lytic enzymes. When an antigen was presented on a flexible plasma membrane sheet, the ability of B cells to internalize antigens of different affinities was unaffected. However, when introduced on stiff planar lipid bilayers, B cells were less able to extract lower affinity antigens. This finding suggests that B cells engage in a molecular "tug-of-war" dictated by the bond rupture strength of the antigen tether. These experiments were performed using two DNA-based probes to measure the extraction mechanism and intercellular forces (Table 1) [68–70].

Under pathological conditions, B cells encounter antigens presented in the context of dendritic cells (DCs) and follicular DCs (FDCs), both of which function as professional APCs but fulfill different roles in conditioning the adaptive immune response. Using AFM, Spillane and Tolar probed the viscoelastic properties of DCs and FDCs, showing that FDCs have higher overall stiffness than DCs. This decreased viscoelasticity in FDCs promoted more stringent affinity-based sampling of antigens by B cells. Indeed, the viscoelasticity of flexible membranes has been shown to limit mechanical loading on molecular bonds [71]. These findings suggest that DCs may serve to initiate low-affinity antigen responses, whereas the more stringent antigen-extraction conditions presented by FDCs may help to select B cells for further affinity selection within GCs. Of note, no enzymatic liberation of antigen was detected when B cells were presented antigen by either DCs or FDCs, suggesting that antigen extraction by B cells may be purely mechanical in nature [70].

Overall, these findings illustrate how APCs must regulate their viscoelastic properties according to their functional requirements. By improving our understanding of APC mechanical properties and their regulation, we could better create APC-based therapeutic approaches to promote the generation of high-affinity B cell clones.

2.2.3. Dendritic cells

Dendritic cells serve as sentinels of the immune system by continually sampling, processing, and presenting antigens [72]. Under immune homeostasis, DCs continually traffic from tissues to the lymphatic organs *via* the lymphatics to promote immune tolerance [73,74]. However, in the presence of pathogen- or damage-associated molecular patterns detected through pattern recognition receptors and local inflammatory cues, immature DCs become activated and undergo maturation, taking on a highly migratory phenotype and upregulating expression of signaling molecules, such as MHC-II, CD80, and CD86, necessary for the activation of adaptive immune cells [75,76]. As mature DCs are highly migratory, they encounter a range of different microenvironments with different biochemical and biophysical properties. Although the biochemical signals governing DC function have been well examined, new studies are beginning to shed light on the biophysical properties governing DC phenotype. Immature DCs cultured on substrates of varying stiffness differentially regulate their expression of several C-type lectin receptors [41]. Interestingly, when cultured on compliant substrates ($E = 2$ kPa), immature DCs show increased expression of two C-type lectin receptors, MMR (Macrophage Mannose Receptor, CD206) and DC-SIGN (Dendritic Cell-Specific Intercellular Adhesion molecule-3-Grabbing Non-integrin, CD209) compared with DCs conditioned on a stiffer substrate ($E = 50$ kPa) [41]. In agreement with this finding, DCs cultured on soft substrates were more effective at taking up antigens.

Migration of DCs through tissues depends on integrin receptors. As is reviewed elsewhere, integrins serve as mechanical signaling platforms to probe the biophysical properties of the ECM in a process termed 'outside-in signaling' [77]. Interestingly, expression of β_2 integrin seems to be at least partly regulated by substrate stiffness. Immature DCs cultured on stiff substrates (50 kPa) expressed more β_2 integrin than those on substrates of intermediate stiffness (12 kPa), though strangely expression was found not to be significantly different between cells grown on a substrate with Young's modulus of 2 kPa and cells grown on the 50 kPa substrate. This trend was repeated when examining CCR7 expression. Nevertheless, DCs matured on the softest substrate were found to be the most migratory in response to the chemoattractant molecule CCL21 [41].

Overall, the phenotypes and functions of dendritic cells are partially determined by the mechanical conditions of the microenvironment. By controlling the rigidity of the tumor stroma, cancer cells may create the conditions necessary for evasion of immune surveillance. More studies are warranted to provide a clearer understanding of the interplay between immune cells and the tumor stroma.

2.3. Cell viscoelasticity in aging

Changes in the mechanical properties of cells are a hallmark of the aging process [17]. Age-dependent increases in cell rigidity are accompanied by the onset of numerous diseases, including vascular degeneration [78], cardiac dysfunction [79], and cancer [11]. Accumulating evidence indicates that aging correlates with progressive changes to the mechanical integrity of cells and tissues and impaired response to mechanical forces [80–83]. The association between cell mechanics and aging is likely due to a multitude of cellular and subcellular processes that depend on the dynamic mechanical deformability of the cytoplasm [19], such as gene expression [84], the translocation and replication of organelles within the cytoplasm [85,86], the movement and biogenesis of mitochondrial bodies along cytoskeletal tracks [87], and cell polarization during wound healing [1]. These mechanical changes also regulate the ability of cells to migrate through 3D matrices and blood vessels [28]. AFM studies on adherent human cells including epithelial cells [80,88], fibroblasts [82], and cardiomyocytes have shown that they stiffen with aging [83]. Increased cell stiffness is not limited to the cytoplasm. One study also found increased stiffening at the cell edge as well as the perinuclear region [80]. Contrary to the aforementioned studies, others have shown that skin fibroblasts display cytoplasmic softening with age, as measured by AFM [89]. However, on the whole, data suggest that age-dependent cytoplasmic stiffening is a general trend for cells within the body [80]. Measurements of whole cell elasticity by optical stretching also shows increased cellular stiffening in suspended skin dermal fibroblasts with aging [81]. As well as fibroblasts, an association between membrane deformability and aging has also been observed in red blood cells [90]. It has been hypothesized that cellular mechanical properties are altered with increased lifespan as a result of age-dependent changes to the composition and organization of the ECM [81,88,91]. Technological limitations have made this hypothesis difficult to test in vivo, with most published studies coming from *ex vivo* samples. Hence validated biophysical biomarkers of aging remain elusive.

3. Tissue microenvironment and cell mechanics

To date, most cell mechanical studies have been conducted in tissue culture models or *ex vivo* samples in which tissue architecture and complex external biochemical cues are generally absent. By contrast, cells in situ are deeply embedded in tissues and in contact with the ECM and other cells. It has been consistently demonstrated that microenvironmental biochemical factors, such as cytokines and growth factors, as well as biophysical factors such as the stiffness and structure of the ECM have critical roles in regulating fundamental cellular processes such as cell motility [92–95], division [96], adhesion [97,98], and gene expression [99,100]. Work on actin dynamics has shown that cells

behave differently when placed in a 3D environment than when cultured on 2D substrates. This is important because measurements of cells in a 3D in vitro context are thought to better represent cell physiology in vivo.

In a 3D ECM, cell-matrix adhesion and signaling pathways are distinct from those required for 2D migration [94,95,101–103]. Migration on 2D collagen-coated dishes is largely driven by actin assembly and actomyosin contractility [104,105]. The same cells in a collagen-abundant 3D ECM display dendritic pseudopodial protrusions that rely both on actomyosin contractility and microtubule dynamics [106,107]. Further, 3D cell migration is tightly associated with the expression of metalloproteinases (MMPs) [106], which are dispensable in 2D migration, and the physical properties of the 3D matrix [28,107,108]. As a result, cell migration patterns are fundamentally different on a 2D substrate in comparison to cells deeply embedded in a 3D matrix [91]. Recent studies further show that cells cultured under spheroid conditions exhibit complex spatiotemporal cellular migration profiles (including collective migration) as well as distinct polarization patterns of the F-actin cytoskeleton [109].

Focal adhesions are mechanical linkages between the cellular cytoskeleton and its surrounding ECM. They also play a distinct role in regulating cellular processes for cells embedded in the ECM. Each focal adhesion is comprised of tens of structural and signaling proteins [110]. Through loss-of-function studies, a myriad of focal-adhesion proteins, including talin, vinculin, FAK, p130Cas, and zyxin, have been implicated in the regulation of 2D cell migration, mechanosensation, and mechanotransduction. Clustering of FA proteins enhances cell adhesion to the ECM and promotes signaling between the matrix and the cell, but in the context of migration, the relationship between FA protein clustering and cell speed is non-linear [111]. In 2D, FA shape and number are not good predictors of cell migration; instead cell speed and persistence both increase and then decrease with the size of FAs [112]. Systematic depletion of FA proteins and assessment of cell behavior in a 3D matrix paints a different picture [113,114]. For instance, the depletion of zyxin decreases cell migration on dishes, but it induces remarkable periodic migratory excursions in a 3D matrix. Indeed, there is no correlation between 2D cell speed/persistence and 3D cell speed/persistence when FA proteins are depleted. Likewise, reducing the level of $\alpha_5\beta_1$ integrin expression on the surface of breast carcinoma cells using a shRNA-mediated approaches only modestly reduced migration on 2D FN-coated surfaces, but had a significant effect on migration in 3D collagen-fibronectin gels [115].

Together, given that cellular functions can change dramatically in response to tissue microenvironments, integrating the physiologically-relevant microenvironment factors in model tissue systems in vitro can potentially advance our understanding of mechanical properties of the cell in contributing to disease progression in vivo.

4. Mechanical measurement for cells deeply embedded in a tissue

To better replicate the 3D environment, several groups have used reconstituted extracellular matrices, organoid models, and tumor spheroid culture techniques. These methods have allowed for more physiologically relevant analysis of cellular mechanical properties. More discussion on 3D ECM models can be found in a recent review [116]. Here, we discuss recent approaches that can be used to measure the mechanical properties of cells deeply embedded in 3D tissue structures.

4.1. Measurement of the viscoelasticity of deeply embedded cells

Though many methods can probe the mechanical properties of live cells (see Table 1 for an overview), most of these techniques require a direct physical contact between the cell surface and the mechanical probe of the instruments (*e.g.*, the cantilever of the AFM, or the glass micropipette for the micropipette suction approach). When cells are partially – but not fully – embedded in an ECM, the mechanical properties of cells can be computed with an AFM-based approach [117]. Microfluid-based systems cells such as the optical stretcher also cannot probe the viscoelastic properties of cells when embedded in tissues or ECM matrices since it required the cells to be in suspension.

Particle tracking microrheology (PTM), sometimes referred to as nanorheology, has been used to study the mechanical properties of endothelial and cancer cells fully embedded in a 3D matrix [118,119]. PTM has been applied to measure cellular responses to changes in environmental forces. For example, serum-starved 3T3 fibroblasts, rapidly reorganize their F-actin network following deformations due to applied shear flow forces, resulting in cytoplasmic stiffening [120]. This mechanism was determined to occur *via* Rho-kinase-mediated signaling as ROCK inhibition abrogated cytoplasmic stiffening under shear conditions. Similar particle tracking-based approaches have also been used to study the dynamics of membrane microdomains and the entry of viral particles into cells [121–123].

The principle of PTM is to observe the movements of sub-micron particles that are injected into the cytoplasm and infer the viscoelastic properties *via* a generalized Stokes-Einstein analysis of the mean-squared displacements derived from bead movements. The particles are typically injected either with ballistic injection [10] or microinjection [124]. Trajectories of these beads can then be tracked *via* standard fluorescent microscopy followed by imaging analysis.

Compared to other mechanical measurement methods, viscoelastic properties of cells measured by particle tracking microrheology do not rely on direct contact with the cells or tissues. Therefore, PTM can be applied to cells that are deeply embedded within tissue structures and 3D hydrogels. The small bead size and sufficiently long duration of microrheology experiments ensure that inertial forces acting on the beads are negligible. If it is also assumed (and verified) that the beads are not actively transported within the cell, then the only two types of forces acting on the beads are: 1) small random forces due to the random bombardment of water molecules generated by thermal energy and 2) counteracting frictional forces due to the movement of the beads. The mean squared displacement of the bead over a given time period can be used to estimate the viscoelastic properties of the cell. Details of this process and the necessary calculations are reviewed by Wirtz [19].

In addition to PTM, there are new non-invasive optics-based methods for measuring mechanical properties, such as Brillouin microscopy, which can extract mechanical information about cells and tissues at sub-micrometer resolution [125,126]. However, this method does not provide a direct measurement of the Young's modulus of cells.

4.2. Measurements of mechanical stresses for deeply embedded cells

Using biocompatible, cell-sized fluorocarbon oil droplets, Campás et al. were able to infer anisotropic mechanical forces active in 3D tissue structures based on measurements of the droplet's deformation [127]. The droplets were fluorescently labeled allowing for visualization within the cellular environment and modified with integrins or cadherins to be able to interact with cells in a defined biological context. By embedding the droplets in 3D cell aggregates consisting of either fluorescently labeled tooth mesenchymal cells or mammary epithelial cells and modelling the droplet deformations observed in Z-stack confocal imaging sections, the researchers were able to calculate maximal anisotropic stress values of $\sim 1.5\,\mathrm{nN/\mu m^{2-2}}$ and $\sim 3\,\mathrm{nN/\mu m^{2-2}}$ for the two cell types, respectively. Traction force microscopy studies of matrix embedded fibroblasts have reported similar values of $2\,\mathrm{nN/\mu m^2}$ [128]. Pharmaceutical-induced disruption of the actin-myosin network was shown to induce relaxation of the oil droplets into spheres. This technique was further applied to an in vivo system; integrin-coated microdroplets with lower interfacial tension were injected into the mandibles of embryonic mice. Using the same formula, it was shown that the average value of the maximal anisotropic stresses generated in

their system was ~1.6 nN/mm^2, comparable to values observed in 3D cell aggregates. This fluorescent oil droplet technique provides a new tool for studying multicellular mechanical properties using an in vivo system, though the authors caution that this method has limitations in accounting for pressure changes caused by large-scale tissue flow, which has been shown to occur during embryogenesis [129].

One key challenge facing the field of mechanobiology is the measurement of individual cells or select clusters of cells embedded within living tissues. Confocal and multi-photon microscopy have a limited penetrance when it comes to visualizing cells within complex multicellular structures due to scattering of light by tissues. This presents a technical challenge for those looking to measure cell mechanics within a living organism. To investigate collective cell migration, a key process in embryogenesis and tumor metastasis, Koretsky and colleagues applied a time-lapse MRI-based approach to observe cell migration under physiologically relevant 3D conditions [130]. By embedding cells within a 3D collagen matrix, they were also able to circumvent another common problem of microscopy; that is, the interaction of cells with the rigid glass coverslip. Using this approach, Chen et al. observed cell migration in unlabeled MDCK cells over a period of 30 h. However, a major caveat of the MRI-based approach is that only large clusters of cells have the necessary iron content to be observed by the scanner. By correlating the deformation of the ECM, Chen et al. attributed remodeling of the matrix to the collective action of MDCK cell clusters, although this was not always the case, as they pointed out that competing cell aggregates may cause equal and opposing deformations to the cell matrix resulting in less-than-expected changes to the collagen microenvironment. Combining this approach with new cell compartment technology may yet provide a unique tool for studying the collective biomechanical forces of cells aggregates within a physiologically representative 3D system [131].

5. Conclusion and future directions

Decades of research have contributed to our understanding of the mechanical processes underlying healthy tissue homeostasis and disease-causing alterations. It is becoming clear that a cell's physical properties are determined by the complex interplay of cell-intrinsic and cell-extrinsic factors derived from the microenvironment.

In this review, we have highlighted the role of cell mechanical and biophysical properties in disease and aging, but also the implication for these same properties in the maintenance of immune homeostasis. Numerous studies have now shown that cancer cells appear to be softer than their healthy counterparts, a characteristic that is associated with increased invasiveness and metastasis [11]. Conversely, preliminary studies on the biophysical properties of aging have indicated that tissue samples from an older cohort tend to be stiffer than those of samples taken from younger patients [17]. Further to this, we have discussed how immune cells sense and respond to these mechanical properties and how these may lead to different cellular outcomes. We are only just beginning to tease out the mechanisms underlying the dynamic nature of cellular mechanics.

Traditionally, measurements of a cell's viscoelastic properties have relied on techniques such as AFM and optical or magnetic tweezers, which cannot easily be applied to tissue embedded samples, thus raising concerns as to their physiological relevance. As we have discussed, cells are sensitive to the physical features or their environment and can alter their own physical characteristics in response to these cues. As we move forward, advances in rheological technology will allow us to interrogate the mechanical features and functions of cells embedded within 3D tissues and, in time, within living organisms.

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