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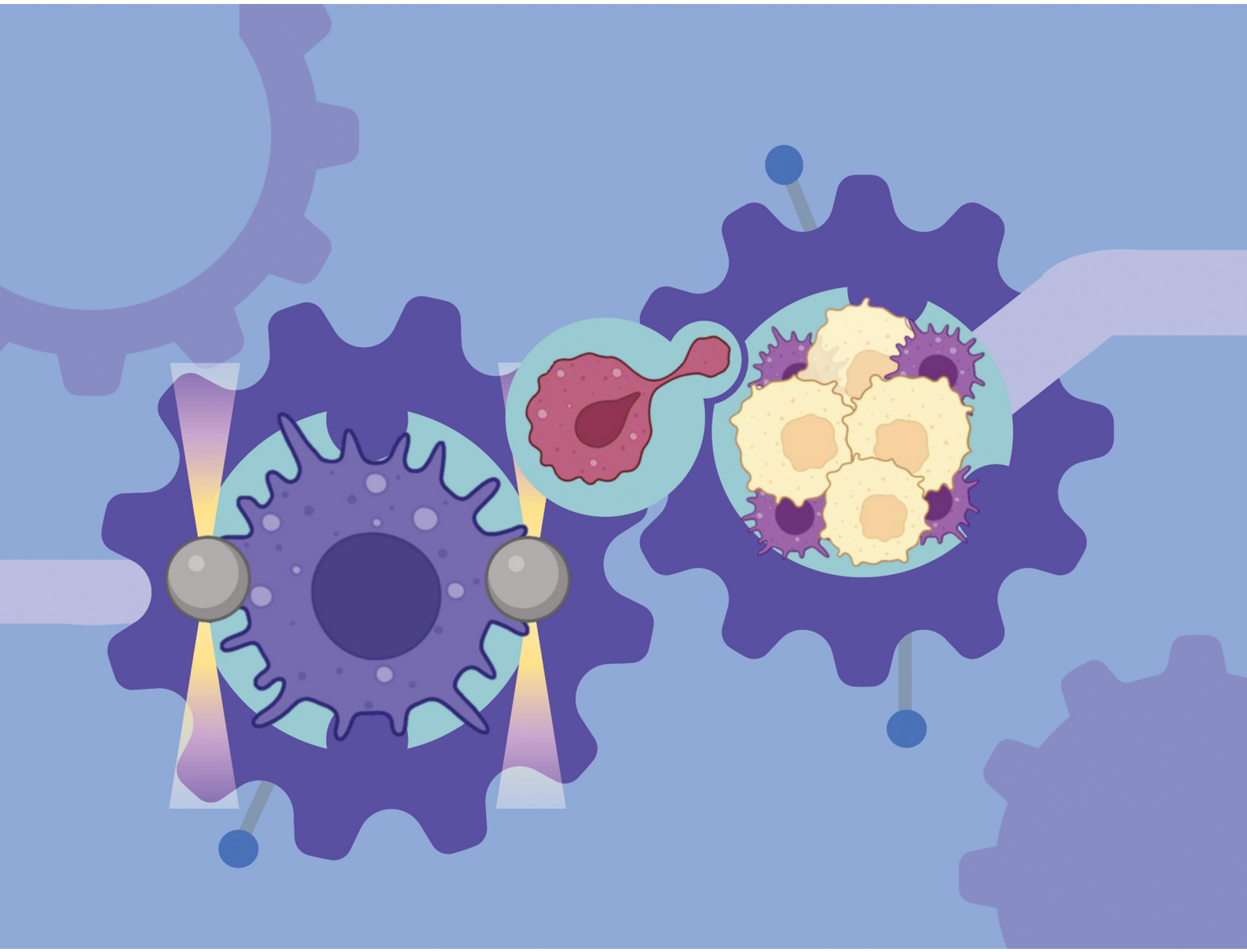

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PERSPECTIVE
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Single-cell analysis of innate immune cell mechanics: an application to cancer immunology

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The innate immune system forms a crucial defense mechanism of the human body against infections and foreign objects, orchestrates wound healing, and restores tissue homeostasis after resolving the insult. Furthermore, innate immune cells launch adaptive immune responses against tumors and mediate phagocytosis of cancer cells and cytotoxic tumor killing. They may also be exploited by cancer cells to promote angiogenesis and metastasis. Although the life cycle and function of innate immune cells require a continuous remodeling of their mechanical properties, data on the biomechanics of different innate immune cell subtypes are scarce. Recent advances in single-cell force spectroscopy have facilitated the exploration of immune cell mechanics at phyiological temperature, aiding in filling this knowledge gap. In this article, we discuss the existing data on the mechanical properties of innate immune cells and provide the first single-cell mechanical characterization of tumor-associated macrophages. The presented data on tumor-associated macrophages showcase the new technical possibilities for decoding immune cell mechanics in cancer, further contributing to our understanding of innate immune cell behavior in disease contexts.

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1. Innate immunity

Cells of the innate immune system arise from hematopoietic stem cells in the bone marrow. Once matured, they are released from the bone marrow to the bloodstream, from where they can home to tissues until the body mobilizes them to cope with infection, trauma, or cancer.[1–3] Innate immune cells detect molecular cues, such as changes induced by microbial infections and inflammatory mediators like cytokines and chemokines, and then launch adaptive immune responses while mounting their effector responses, such as phagocytosis for

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macrophages, and cytotoxicity for natural killer cells.[3] Research into immune cell signal transduction by applying the tools of biochemistry, molecular biology, and genetics has identified networks of secreted ligands, cell surface receptors, intracellular signaling pathways, and transcriptional regulators that coordinate various aspects of cell behavior. There is a growing realization that the mechanical properties of a cell add an extra layer of regulation to innate immune cells.[4]

It has become increasingly evident that the mechanical properties of a cell can offer intrinsic biophysical cues for determining physiological cell state transitions. The link between mechanical properties and cell function is particularly obvious in innate immune cells as they need to undergo extensive morphological changes during endothelial transmigration and phagocytosis, display rapid movement through blood vessels and tight interstitial spaces, strongly adhere under shear flow, and form highly dynamic interfaces with other cells.[4–9] How mechanical forces are involved in regulating essential cellular processes such as cell-surface receptor activation, cell migration, intracellular signaling, and intercellular communication, in various immune cell types, has been the focus of numerous excellent reviews.[4,7] Although the link between the mechanical properties of an innate immune cell and its function might seem obvious, data on the basic mechanical properties of various innate immune cell subtypes (at physiological temperature) are scarce and for diseased states lacking almost entirely. Several important early studies have described the mechanical behavior of an innate immune cell (mainly neutrophil) as a ''fluid-filled bag with an elastic cortical envelope''.[10,11] Recent advancements in single-cell force spectroscopy have played a pivotal role in unraveling immune cell mechanics and investigating the ''cortical shell-liquid core'' concept in greater depth. In this perspective, we discuss the existing data on the mechanics of innate immune cells, in particular monocytes and macrophages, and explore how current technology can be applied to study immune cells associated with cancer.

2. Advances in single-cell force spectroscopy

Progress in single-cell force spectroscopy (SCFS) techniques enables substantial advances in our understanding of the tight relationship between mechanics and function in innate immune cells. This, in turn, contributes to the emergence of the field of mechanoimmunology or immunomechanics. When performing SCFC experiments, biophysicists generally consider two readouts: cell elasticity, referring to the stretchiness of cells, and viscosity, referring to the fluidity of cells. Together, these parameters determine the mechanical phenotype of a cell. On one hand, the application of mechanical probes commonly employed in cell biophysics is relatively new to immunologists, and, on the other hand, biophysicists have focused mostly on the mechanics of the simplest blood cell, the red blood cells (RBCs). RBCs are therefore among the best-studied cells in the field of cell mechanics, and the values of their mechanical properties are supported by a large body of experiments by different SCFS methods like optical tweezers (OT), acoustic force spectroscopy (AFS), atomic force microscopy (AFM), micropipette aspiration (MA), and other techniques, all giving consistent results.[12–19] Brief descriptions of commonly employed SCFS techniques are provided in Box 1. Despite the fact that the application of SCFS techniques in RBC studies has led to many important findings in hemorheology and improvement in understanding disease,[20–28] data on the mechanical behavior of RBCs at biomedically relevant temperatures was lacking till very recently, mainly due to technical limitations. Motivated by this gap in knowledge, we have recently initiated a single-cell mechanics research program to characterize the mechanics of red and white blood cells by utilizing several complementary SCFS techniques, including AFS, OT, and AFM, in a temperature controlled system to mimic physiological and medically relevant conditions. Indeed, using RBCs as a model, we demonstrated that cellular mechanics are highly temperature-sensitive,[29] emphasizing the need to probe

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cell mechanics in temperature-controlled environments. The temperature dependency of the mechanical properties has also been suggested for neutrophils, with a demonstrated decrease in apparent viscosity as temperature increases.[10] Inspired by these observations, we recently moved into the direction of innate immune cell mechanics, specifically monocytes and macrophages, on which we will focus below.

Box 1

Single-cell force spectroscopy (SCFS) techniques

Cell mechanics rely on technical approaches that enable the quantification of mechanical properties at the single-cell level. Several of the most commonly used strategies are described briefly below.

Atomic force microscopy (AFM). AFM-based indentation is a widely utilized approach for quantifying the mechanical characteristics of adherent cells at subcellular levels. The atomic force microscope consists of a cantilever of known stiffness that applies a predetermined force or deformation onto an adherent cell or tissue at a specific speed. The resistance force resulting from the cell deformation is then measured by detecting the laser deflection and capturing it with a photodetector, from which the mechanical properties can be quantified.

Optical tweezers (OT). In OT, cells of interest are clamped between two optically trapped beads and subsequently stretched by displacing one of the trapped beads, while keeping the other bead stationary (Fig. 1). Multiple stretching cycles can be performed on a single cell at different values of velocity. From a stretching cycle, typical force–extension curves are obtained from which the mechanical properties can be calculated.

Acoustic force spectroscopy (AFS). In AFS, cells are confined between beads and the glass surface of the AFS microfluidic chip (Fig. 1). Acoustic forces are applied *via* a piezo element, which generates standing acoustic waves that push the beads up toward the acoustic node, instantaneously stretching the cells at approximately constant stress. From the resulting displacement–over–time curves, the mechanical properties of the cells can be quantified.

Micropipette aspiration (MA). MA is a technique that involves using sensitive negative pressure from a pressure-based controller to aspirate a cell into a micropipette. The cell is initially immobilized on the tip of the micropipette, and then suction is applied to draw the cell inside the pipette tube. To measure the distance traveled by the aspirated portion of the cell within the micropipette tube, the cell's movement can be tracked and monitored using a microscope. From the deformation displayed, the mechanical properties can be quantified.

Acoustic tweezing cytometry (ATC). ATC employs ultrasound pulses to apply controlled forces to individual cells using membrane-bound microbubbles. This technique enables the implementation of a creep test, the amount of deformation the cell undergoes over time while exposed to a constant force over

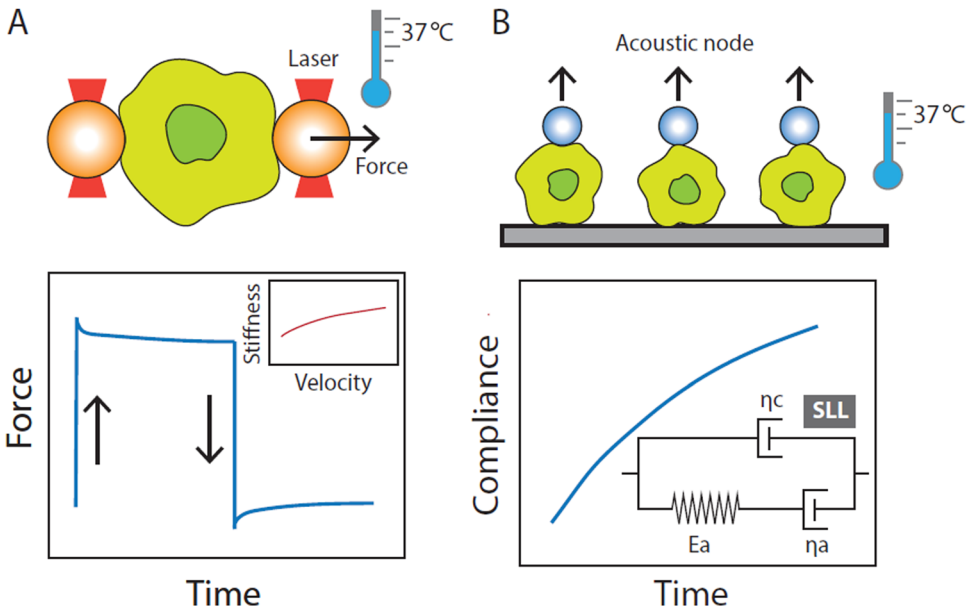

Fig. 1 Optical tweezers (OT) and acoustic force spectroscopy (AFS) experimental setups at physiological temperature (37 °C). (A) Schematic representation of the OT microfluidic chip containing an innate immune cell sandwiched between two optically trapped beads. The cell is stretched by displacing one of the trapped beads while keeping the other bead stationary. Multiple stretching cycles are performed in which the cell is pulled at a constant strain rate at different values of velocity. From the stretching cycle, we obtain typical force–extension curves from which the stiffness at each stretching velocity is determined. (B) Schematic representation of the AFS microfluidic chip loaded with innate immune cells. Cells are confined between beads and the surface of the chip. A standing acoustic wave drives beads to the acoustic node thereby stretching the cells. From the resulting displacement–over–time curves, we calculate the compliance over time. We then determine the mechanical properties of cells by fitting the standard linear liquid (SLL) model through the compliance-over-time curves. The SLL model places a spring and a dashpot (elastic modulus E_a and viscosity η_a) associated with the cell cytoskeleton in parallel with a background viscous medium ($\approx$ the cytosol) with viscosity η_c.

a specific period of time. From the creep response, cellular mechanical properties can be quantified.

Magnetic twisting cytometer (MTC). MTC exerts twisting stress on cells using functionalized magnetic microbeads attached to the cells. Specifically coated beads bind specific adhesion receptors on the cell surface and become tightly anchored to the cytoskeleton through focal contacts. The beads are then permanently magnetized in the horizontal plane of the cells and subsequently twisted in an oscillatory magnetic field causing them to rotate and align with the oscillating field. This rotational motion imparts a gentle mechanical torque onto the cell. From the mechanical torque and bead displacement, mechanical properties can be extracted.

Particle-tracking microrheology (PTM). PTM collects and analyzes the distribution of spontaneous movements of fluorescently coated microspheres embedded in the cytoplasm of live cells to spatially map its local viscoelastic properties. From the bead trajectories, the mean square displacements of beads are computed which are transformed into mechanical parameters, describing the local viscosity and elasticity of cytoplasm.

3. Innate immunity from a biophysical perspective

The life of an innate immune cell is intensely physical, requiring a continuous remodeling of its mechanical properties. To illustrate this, we follow the physically perilous journey of a monocyte along its way to an inflamed tissue (Fig. 2). Inflamed tissues release potent inflammatory mediators that drive monocytes out of the bone marrow (or other reservoirs such as the spleen) and into the circulation to be rapidly transported to their destination.[30] An example of such a chemical signal is the chemokine CCL2, a strong chemoattractant for monocytes and a powerful initiator of inflammation.[31] Using a combination of AFS and OT (see Fig. 1 for experimental setup), we have recently shown that CCL2 exposure *in vitro* causes primary human monocytes to stiffen, as well as induces a more fluid-like phenotype.[32] These very finely tuned chemokine-induced alterations in the mechanical properties of monocytes may help their switch to a migration-competent state and potentially their accumulation at sites of infection. During their transport in the blood, monocytes have to strongly deform to pass through the narrowest capillaries and bifurcations. This process depends on a finely tweaked cortical tension combined with a high cell viscosity that governs the fast transit through the vascular network while preserving cell integrity.[33] While approaching the site of inflammation, monocytes move closer to the endothelium, establishing contact and eventually creating adhesions when they reach the low-flow postcapillary venule environment. Initially, these adhesions are transient and weak, mediated by selectins, causing the monocytes to roll along the endothelial surface.[34,35] However, they eventually come to a halt and firmly attach by forming stronger integrin bonds in regions where activated endothelial cells exhibit high concentrations of chemokines on their luminal surface. Subsequently, monocytes extend cellular protrusions and commence crawling atop the endothelial cells, prompting them to find a path through the endothelium and into the underlying

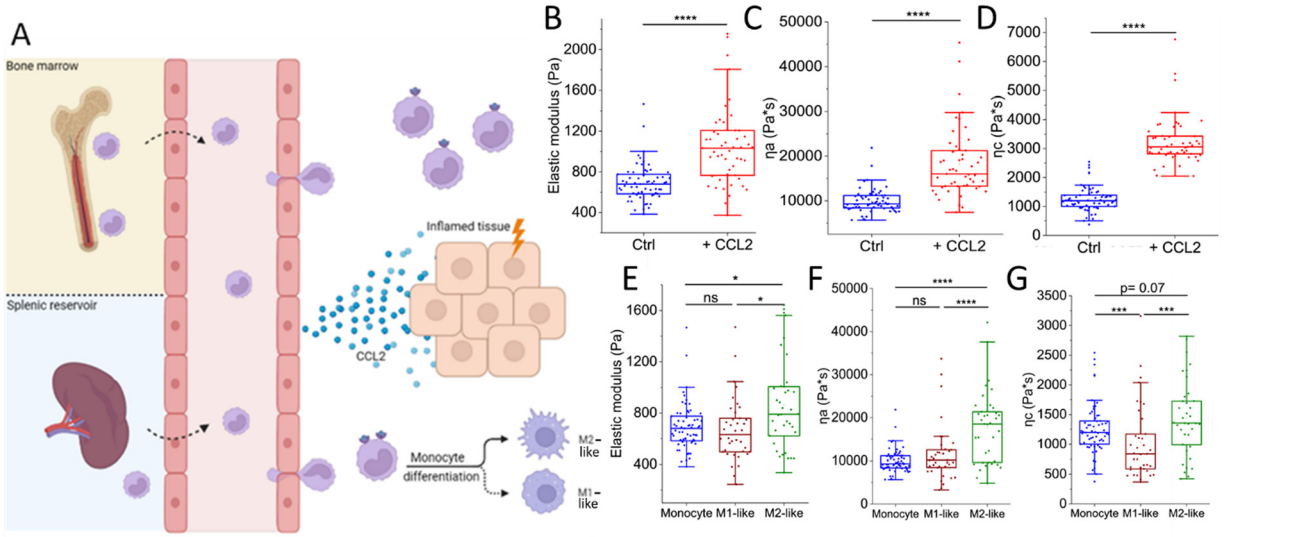

Fig. 2 The intense physical life of a monocyte on its way to an inflamed tissue. (A) Inflamed tissues release chemokines such as CCL2 that drive monocytes out of the bone marrow and spleen into the circulation. Upon binding CCL2, monocytes switch to a migration-competent state, reflected by an increase in (B) stiffness (elastic modulus) and viscosity (C) η_a and (D) η_c. Then, monocytes transmigrate through the endothelium during which they undergo extensive (squeezing) mechanical alterations. Upon entering the tissue, monocytes differentiate into either M1-like or M2-like macrophages. M2-like macrophages are (E) stiffer and (F) and (G) less fluid-like than their precursors, whereas M1-like macrophages and monocytes exhibit similar mechanical properties at physiological temperature (37 °C). Macrophage experiments included 38 M1-like and 38 M2-like macrophages collected from 4 donors. Monocytes (ctrl: n = 63, +CCL2: n = 51) were collected from 3 donors. Graphs (B)–(D) are re-plotted from ref. 32 and graphs (E)–(G) are re-plotted from ref. 32 and 46.

tissue.[35–37] Extravasating monocytes undergo extensive mechanical alterations, helping them to squeeze through the narrow gaps in the endothelial barrier. Inside the affected tissue, monocytes differentiate into macrophages. It has previously been shown that during the course of differentiation, macrophages exhibit distinct mechanical properties compared to their precursor cells.[38] Irrespective of the macrophage type, macrophages stiffen and behave less fluid-like than monocytes. However, monocyte differentiation into macrophages occurs complementary with the acquisition of a functional phenotype that depends on microenvironmental signals.[39] Traditionally, macrophages have been categorized into two major polarized phenotypes, M1 and M2.[40] *In vitro* activation using the T_h1-type cytokine interferon-gamma (IFNγ), granulocyte-macrophage colony-stimulating factor (GM-CSF), lipopolysaccharide (LPS) and/or Toll-like receptor 4 (TLR4) engagement gives rise to pro-inflammatory M1-like macrophages, whereas stimulation with macrophage colony-stimulating factor (M-CSF) or the T_h2 cytokines interleukin 4 (IL-4), IL-10, or IL-13 generates the anti-inflammatory M2-like macrophages.[41,42] It is important to note that *in vivo* macrophage polarization is not binary, but has a spectrum-like level of activation that cannot be defined by two extremes.[39,40,43,44] Since macrophages are highly plastic cells that are able to acquire different functional phenotypes, it is tempting to speculate that they may also adopt different mechanical phenotypes to accommodate these different and sometimes opposing functions.[45] To address this, we recently employed several single-cell force spectroscopy techniques to mechanically characterize human (*in vitro* GM-CSF-induced) M1-like and (*in vitro* M-CSF-induced) M2-like macrophages.[46] Indeed, M2-like macrophages exhibit a higher overall viscosity and appear to be stiffer than M1-like macrophages. M2-like macrophages have been shown to exhibit higher phagocytic capacity than M1-like macrophages and phagocytosis can lead to increased stiffness caused by elevated production of reactive oxygen species.[47] This stiffness hampers the ingestion of additional particles, as phagocytosis requires deformability as a prerequisite.[47–49] Consequently, there seems to be a threshold for phagocytosis that can be reached either by engulfing numerous small particles or a few larger ones.[50] In addition, the higher viscosity of M2-like macrophages presumably facilitates their migration in porous interstitial-like environments,[38] supporting the major function of anti-inflammatory macrophages; phagocytosis followed by efflux from the tissue.

In summary, it has become increasingly clear that monocytes and macrophage subtypes exhibit distinct mechanical phenotypes, which correlate with their diverse functions, and that immune triggers such as the chemokine CCL2 and cytokines GM-CSF or M-CSF can modulate this mechanical phenotype at physiological temperature. Besides monocytes and macrophages, a significant body of evidence exists on the mechanical properties of human primary neutrophils in physiological and diseased states (Table 1). However, single-cell mechanical data for other human innate immune cells, particularly natural killer cells, mast cells, eosinophils, and basophils are scarce. Table 1 presents an overview of studies that employed direct single-cell measurements to mechanically characterize primary innate immune cells, or indirectly by measuring morphological properties as a proxy for mechanics.

4. Resolving immune cell mechanics in cancer

The physical microenvironment also plays an important role in the modulation of mechanical properties. An example of such a microenvironment is the one surrounding a growing tumor. In the following section, we will explore the effects of the tumor microenvironment on the mechanical properties of macrophages therein.

Cancers evolve as complex ecosystems that consist of malignant tumor cells and numerous non-cancerous cells, such as immune cells, cancer-associated fibroblasts, endothelial cells, pericytes, and various additional tissue-resident cells that are embedded in a complex network of fibers and proteins known as the extracellular matrix (ECM).[51] The ECM provides a physical scaffold for cells and helps to regulate their behavior.[52] In the case of cancer, changes in the communication between cells and the ECM play a key role in tumor growth and spread. Cancer cells can alter the stiffness and composition of the ECM, creating a positive tumorigenic feedback loop that alters cancer cell mechanics and promotes their survival, proliferation, and invasiveness.[53] Altered mechanics have therefore been considered a hallmark of disseminating tumor cells: more than two-thirds of metastatic cells exhibit lower levels of stiffness.[54]

Tumor-associated macrophages (TAMs) are the most abundant innate immune cell type in solid tumors. TAMs are often recruited from bone marrow-derived monocytes *via* the tumor cell-derived CCL2-CCR2 chemokine signaling pathway, but can also differentiate from resident macrophages.[55–57] Within the tumor microenvironment, tumor, and host cells are known to trigger macrophage polarization and hijack their inflammatory and wound-healing capacities to favor tumor progression.[58,59] In many solid cancer types, anti-inflammatory TAMs with M2-like properties are the predominant macrophage population, which has been directly correlated with poor prognosis and increased metastatic potential.[60–62] Whereas the alterations of cancer cell mechanics within the tumor microenvironment have been extensively studied,[63–65] they remain to be elucidated for the macrophages and other immune cells present therein.[66] This major shortcoming has mainly been due to the lack of methodologies to probe TAM mechanics. Here, we report preliminary data on the first single-cell mechanical characterization of TAMs at physiological temperature and discuss the technical possibilities offered by state-of-the-art technology and the challenges involved. We used single-cell optical tweezers to measure stiffness profiles of resident F4/80$^+$ macrophages freshly isolated from healthy mammary gland tissue from littermate control FVB mice and TAMs isolated from spontaneous mammary tumors from the *WapCre;Cdh1$^{F/F}$;PIK3CA-H1047R* genetically engineered mouse tumor model (FVB background), driven by the loss of E-cadherin and mutant PIK3CA.

Table 1 Overview of studies that employed direct single-cell measurements to mechanically characterize innate immune cells or to qualify their morpho-rheological properties. AFS-acoustic force spectroscopy, OT-optical tweezers, OS-optical stretcher, MA-micropipette aspiration, AFM-atomic force microscopy, RT-DC- real-time deformability cytometry, MF-microfluidics, MR- microplate-based rheometer, MTC-magnetic twisting cytometry, ATC-acoustic tweezing cytometry, DBC-dielectrophoretic biomechanical characterization, ND-CRF- non-dialyzed chronic renal failure, HD-hemodialysis, ARDS-acute respiratory distress syndrome

Innate immune cell type	SCFS technique	Experimental model	Conclusion
Neutrophil	OS	Human primary neutrophils (isolated from blood)	Complement product C5a increases neutrophil deformability[87]
		Human primary neutrophils (isolated from blood)	Mechanical deformation induces priming and rapid depolarization of neutrophils[88]
	MA	Human primary immature myeloblasts, promyelocytes, myelocytes, and neutrophils (isolated from bone-marrow)	The mature neutrophil is becoming progressively softer than its progenitors[89]
		Human primary neutrophils (isolated from blood)	ICAM-1-mediated priming causes neutrophils to stiffen[90]
		Human primary neutrophils from ND-CRF and HD patients (isolated from blood)	Neutrophil deformability decreases in patients with CRF[91]
		Human primary neutrophils (isolated from blood)	The first model for the passive rheological behavior of neutrophils[92]
	Cell poker	Human primary neutrophils (isolated from blood)	fMLP-mediated priming causes neutrophils to stiffen[93]
	AFM	Human primary neutrophils (isolated from blood)	Human primary neutrophils are six times softer than their precursors (HL60 cell-line)[94]
		Rat neutrophils (isolated from blood)	Adherence-mediated priming causes neutrophils to stiffen[95]
	RT-DC	Human primary neutrophils (isolated from blood)	Neutrophil priming induces stiffening in the first 1–5 min, followed by softening within 15–30 min post-priming[96]
		Sepsis patient-derived neutrophils (isolated from blood)	Neutrophils from patients with sepsis possessed an increased deformability[97]
		Psoriasis patient-derived neutrophils(isolated from blood)	CXCL16 and IL-8 soften neutrophils facilitating infiltration into skin[98]
	Micro-fluidics	Human primary neutrophils (isolated from blood)	Neutrophils exposed to serum from ARDS patients rapidly stiffen[99]
	MR	Human primary neutrophils (isolated from blood)	Neutrophils stiffen during phagocytosis. The observed stiffening is dependent on the size of the engulfed particles[100]
Monocyte	AFS, OT	Human primary monocytes (isolated from blood)	Characterization of monocyte mechanics at physiological temperature. The work reports elastic and viscous parameters, strain dependency of stiffness, and resolves the effect of CCL2[32]
	MR	Human primary monocytes (isolated from blood)	Characterization of elastic and viscous properties of monocytes, adhered to a fibronectin coated surface[101]
	MF	Human primary monocytes (isolated from blood)	Monocytes exposed to serum from ARDS patients rapidly stiffen[99]
	RT-DC	Systemic sclerosis (SSc) patient-derived monocytes (isolated from blood)	All three subpopulations of monocytes identified had higher deformation and cross-sectional area in SSc patients as compared to healthy controls[102]
	Cell poker	Human and rabbit primary monocytes (isolated from blood)	LPS exposure induced monocyte stiffening[103]
Dendritic cell	RT-DC	Human primary monocyte-derived dendritic cells (isolated from blood)	Maturation of dendritic cells leads to increased cellular stiffness and higher membrane fluidity[104]
	MR	Human primary monocyte-derived dendritic cells (isolated from blood)	Characterization of elastic and viscous properties of dendritic cells. Exposure to IFNγ or LPS stiffens dendritic cells. The opposite occurs for a TNFα + PGE2 cocktail[101]
	AFM	Mouse bone marrow-derived dendritic cells (isolated from bone marrow)	Dendritic cells stiffen during maturation, which enhances their ability to prime T-cells[105]
Macrophage	AFS, OT	Human primary monocyte-derived macrophages (M-CSF and GM-CSF stimulated) (isolated from blood)	Characterization of macrophage mechanics at physiological temperature. The work reports elastic and viscous parameters, strain dependency of stiffness, and resolves the difference between M1 and M2[46]
	MR	Human primary monocyte-derived macrophages (M-CSF stimulated) (isolated from blood)	Characterization of elastic and viscous properties of macrophages, bound to a fibronectin coated surface. Exposure to IFNγ or LPS stiffens macrophages. The opposite occurs for a TNFα + PGE2 cocktail[101]
	MTC	Human alveolar macrophages (isolated from lung tissue)	Macrophages stiffen upon exposure to LPS and IFN-γ. Macrophages stiffen when cultured on a rigid substrate and soften when cultured on a less rigid substrate[49]
	ATC	Mouse peritoneal macrophages (isolated from peritoneal cavity)	Macrophages with rod-shaped crystal-like drug inclusions were softer than macrophages that engulfed solid polyethylene microbeads[48]
Eosinophil	RT-DC	Human primary eosinophils from atopic and non-atopic subjects (isolated from blood)	Eosinophils from atopic subjects show a higher total area ratio than eosinophils from non-atopic subjects, comparable with basal priming. Eosinophil deformability and area were unchanged between the atopic and non-atopic eosinophils[106]

Table 1 (*continued*)

Innate immune cell type	SCFS technique	Experimental model	Conclusion
Natural killer (NK) cell	MA	Human primary NK-cells (isolated from blood)	IL-2-activated NK-cells show a reduction in rigidity after thioglycolate treatment[107]
		Human primary NK-cells (isolated from blood)	Reducing NK cell rigidity by thioglycolate facilitates NK infiltration into tissues, but compromises its immunotherapeutic efficacy[108]
		Human primary NK-cells (isolated from blood)	IL2 exposure increases NK cell rigidity, which was maintained for up to 96h after IL2 withdrawal[109]

In order to probe the mechanical properties of macrophages from healthy mammary glands and TAMs from mammary tumors, we performed multiple stressing–destressing cycles, in which the cells were pulled and relaxed at a constant strain rate at different values of velocity (1, 5, and 10 μm s^{-1}). Examples of hysteresis loops of WT macrophages and TAMs at a stretching velocity of 10 μm s^{-1} are shown in Fig. 3B. From the slope of the linear ascending part of the stress–strain curve, the stiffness of the macrophages at each stretching velocity can be determined. Notably, only cells showing a distinguished hysteresis loop were included in the analysis. Possible reasons for not obtaining the characteristic loops are the improper attachment of the beads to the cells, cells that are in poor condition (*i.e.*, entered apoptotic phase), and off-centered beads during stretching. Average stiffness values for WT macrophages and TAMs at each stretching velocity are presented in Fig. 3C. Our data suggest that, for this particular tumor model, TAMs are stiffer than the corresponding tissue-resident macrophages. It has previously been shown that YES-associated protein (YAP), a mature nuclear transcription coactivator that responds to a variety of mechanical changes, senses the hardness of the surrounding matrix and stimulates macrophages to transform into the immunosuppressive M2 type.[67] In addition, *in vivo* and *in vitro* studies have shown that macrophages adjust their polarization state in response to surrounding mechanical stimuli.[68] As such, the involvement of YAP in the polarization of macrophages towards the immunosuppressive type would be interesting for future studies.

These experiments are technically difficult, in particular, due to the limited number of immune cells that can be assessed, and their inherent heterogeneity requiring high resolution to detect small changes in mechanical properties. Nevertheless, the presented data demonstrate that sensitive measurements of immune cell mechanics are achievable thanks to technological advancements.

5. Future perspectives

Over the last decade, a conceptual framework has emerged for measuring and interpreting the mechanical properties of immune cells. However, the field is still in its infancy. Although various studies have provided a useful approximation of the mechanical behavior of innate immune cells by utilizing *in vitro* cell lines, they do not fully recapitulate *in vivo* immune cell

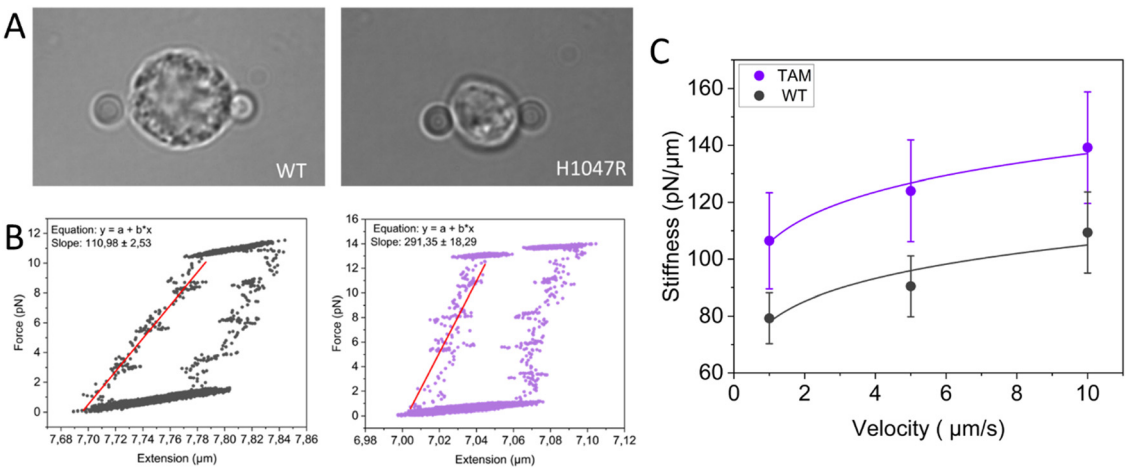

Fig. 3 Probing the mechanical properties of TAMs using OT. (A) Microscopic image of a freshly isolated macrophage from healthy mammary glands (left) and TAM (right) trapped between two 4 μm silica beads. (B) Typical hysteresis loop of a macrophage from healthy mammary glands (Mice: n = 3, total cells: n = 10, left panel) and TAM (Mice: n = 4, total cells: n = 9, right panel) during a stressing–destressing cycle at a stretching velocity of 10 μm s^{-1}. (C) The power law fits ($y = ax^b$) reveal a significant shift in stiffness to higher values (p-value < 0.05), while the overall shape remains similar, indicating that both macrophage types respond similarly to increased strain.

physiology and functionality.[38,69–78] We have started to unravel the mechanical properties of monocytes and macrophages and the potential link to their immune functions under physiological conditions and in cancer. These studies can help us better understand the function of innate immune cells in health and disease. It has recently been shown that mechanical markers can be used for tracking disease progression and treatment in various tissues.[79,80] Such approaches may also be utilized as future possibilities in the field of mechanoimmunology. For instance, the mechanical properties of immune cells may provide us with insights into how these cells cope with challenging circumstances, such as the aberrant deposition and organization of ECM protein that results in increased matrix stiffness, which is often observed in the mammary tumor microenvironment.

The mechanical characterization of innate immune cells could enable the development of mechanical biomarkers that can be used to monitor innate immune activity in both healthy and diseased states. To achieve this goal, a step in the right direction was taken by Toepfner and colleagues, who were able to detect human disease conditions by single-cell morpho-rheological phenotyping of blood.[81] Real-time deformability cytometry, a novel high-throughput method for the continuous mechanical characterization of single cells, allowed them to identify all major blood cells in a single drop of blood and characterize their mechanical alterations in several disease conditions.

It must be noted here that macrophages, orchestrators of virtually all major diseases, do not circulate in the blood. The approach introduced by our lab, therefore, brings us a step closer to mechanically characterizing all innate immune cells (both circulating and tissue-resident) that impact disease progression, as we have shown for TAMs (Fig. 3). Future studies using a combination of organ-on-chip modeling and SCFS could circumvent the need for specifically isolating the relevant cells of interest, preventing the potential of inadvertent cell change.[82,83]

In addition to diagnostics, we hypothesize that mechanical phenotyping opens avenues for mechanics-targeting therapies in which external stimuli could be employed to program innate immune cells toward desired mechanical phenotypes. For instance, if we could solely change the mechanical properties of cells with minimally altering any other process, then we could soften and reprogram otherwise rigid M2-like TAMs to M1-like macrophages and see whether that halts tumor growth through enhanced tumor cell killing. Undoubtedly, cell mechanics is intricately linked with numerous other processes, making it a challenging task to identify specific factors that solely influence mechanical changes. In fact, mechanical cues experienced by immune cells are translated into biochemical signals through the process of mechanotransduction, which, through multiple signaling pathways, impacts crucial cellular functions including metabolism.[4,7] We have recently described how mechanical cues, sensed through the cytoskeleton or distortion of the cell and organelles, can induce metabolic changes in the cell and how the resulting alterations in metabolism, in turn, can feed back to regulate the mechanical properties of cells and tissues.[84] Advances in analytical technologies are enabling studying metabolism at the single-cell level,[85] and have been recently applied to innate immune cells too.[86] Future endeavors should therefore incorporate correlating mechanical information from a cell to other data including multi-omics.

Conflicts of interest

K.E.d.V. reports research funding from Roche/Genentech and is consultant for Macomics, outside the scope of this work.

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