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Ion channel mediated mechanotransduction in immune cells

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Abstract

The immune system performs critical functions to defend against invading pathogens and maintain tissue homeostasis. Immune cells reside within or are recruited to a host of mechanically active tissues throughout the body and, as a result, are exposed to varying types and degrees of mechanical stimuli. Despite their abundance in such tissues, the role of mechanical stimuli in influencing immune cell function and the molecular mechanisms responsible for mechanics-mediated changes are still poorly understood. The recent emergence of mechanically-gated ion channels, particularly Piezo1, has provided an exciting avenue of research within the fields of mechanobiology and immunology. Numerous studies have identified roles for mechanically-gated ion channels in mechanotransduction within various different cell types, with a few recent studies in immune cells. These initial studies provide strong evidence that mechanically-gated ion channels play pivotal roles in regulating the immune system. In this review, we discuss characteristics of ion channel mediated force transduction, review the current techniques used to quantify and visualize ion channel activity in response to mechanical stimuli, and finally we provide an overview of recent studies examining the role of mechanically-gated ion channels in modulating immune cell function.

Keywords

adaptive immunity; innate immunity; macrophage; mechanotransduction; Piezo1; T cell

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Author Contributions

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Introduction

The immune system consists of specialized cells that play a critical role in preserving homeostasis, as well as protecting against invading pathogens. Immune cells function in tissues throughout the body, exposing them to varying types and degrees of mechanical stimulation [1]. When they are first recruited into tissues from circulation, immune cells are exposed to an increase in fluid shear stresses upon adhesion to the endothelium, and then mechanical stretch when extravasated into the vessel wall [2,3]. Once in the tissue, cells can experience a variety of mechanical environments, for example cells within bone tissue experience significantly higher stiffness compared to those within muscle [4]. This mechanical environment is dynamic and can also change with the development and progression of pathological conditions. Hypertension enhances fluid shear stresses and mechanical stretch experienced by cells within blood vessels, whereas various disease conditions such as atherosclerosis, cancer, and fibrosis lead to tissue stiffening [2,3,5–7]. In addition to these examples, there are many other types of mechanical environments to which immune cells are potentially exposed within the body (Figure 1). Unlike other cell types that mostly reside in their tissue-specific environment permanently, immune cells are unique as they are recruited to a variety of different mechanical environments which, in turn, influence their function and the overall immune response.

Recent studies have suggested that the mechanical environment tunes immune cell function. Macrophages and dendritic cells are innate immune cells that facilitate inflammation, wound healing, and antigen presentation – functions that have all been shown to be regulated by mechanical stimuli, both independently and in conjunction with soluble factors. In stiff environments, macrophages and dendritic cells exhibit enhanced lipopolysaccharide (LPS)-induced inflammatory responses [8–13]. When grown on surface grooves or micropatterned adhesive lines, macrophages elongate, align, and express markers associated with wound healing in the absence of external soluble factors [14,15]. Varying degrees of mechanical stretch have also been shown to influence macrophage morphology, enzymatic activity, and inflammatory/healing activation states [16–19], as well as dendritic cell activation and T-cell priming [20]. In the adaptive immune system, mechanical forces have been implicated in the regulation of molecular interactions involved in antigen recognition and transmigration [21], T cell differentiation [22,23], and $CD8^+$ T cell killing [24–26]. Forces are required for T cell receptor (TCR) and major histocompatibility complex (MHC) interaction [27,28], B cell receptor (BCR) activation [29–31], immunological synapse (IS) formation [32], and integrin activation [24–26]. Moreover, in response to external mechanical cues, such as stiff environments, T cells exhibit enhanced cell spreading, motility, and activation [33–35]. The ability of immune cells to sense their mechanical environment is crucial in regulating cell behavior as well as modulating disease development and progression.

Immune cells express a variety of mechanoreceptors on their cell surface, including integrins and ion channels, which sense and transduce external mechanical stimuli. Integrins are well known mechanoreceptors that connect the cytoskeleton to the extracellular matrix (ECM). In response to mechanical stresses, integrins cluster and facilitate cytoskeletal remodeling and the activation of various signaling pathways [36]. They are key mechanosensors in immune cells and have been shown to regulate motility, phagocytosis, and activation [33,34,37–40].

In addition to integrins, mechanically-gated ion channels transduce mechanical stimuli into electrochemical activity which, in turn, activates ion-dependent signaling pathways [41]. Mechanically-gated ion channels are characterized by their ability to create mechanically regulated pore-forming subunits without requiring auxiliary proteins, and can confer mechanosensitivity in an otherwise insensitive cell [42]. Few channels meet these stringent requirements and include two-pore potassium channels (TREK-1, TRAAK-1, TRAAK-2) and some members of the transient receptor potential (TRP) family, such as TRPV4. More recently, the Piezo family of ion channels was identified, representing a major breakthrough in the field of mechanobiology [43]. In particular, Piezo1 is emerging as the body's global mechanotransducer, widely expressed and able to respond to a multitude of mechanical stimuli including cell membrane indentation, suction, laminar flow, cellular compression, deflection as well as mechanical properties of the substrate such as stiffness and nanotopology [43–49]. Piezo1, is a non-selective cation channel, permeable to Ca^{2+}, which elicits many downstream effects. The channel has already been shown to have extensive roles in physiology including vascular development, neural stem cell differentiation, red blood cell volume regulation, and cartilage force sensing [43,44,50–55]. Piezo2 has similar structure and electrophysiological properties compared to Piezo1, but as yet, functional roles of the channel are primarily described in the sensory system [56].

Several recent studies have explored the role of mechanically-gated channels in regulating immune cell function, showing an essential role for these channels in numerous physiological and pathological processes involving the immune system. In this review article, we will discuss ion channel mediated force transduction, overview the current techniques and advances used to quantify and visualize ion channel activity in response to mechanical stimuli, and review current studies examining the role of mechanically-gated ion channels in modulating immune cell function.

Ion channel mediated force transduction and signaling

Ion channels are pore forming subunits that allow for the passage of ions across the cell membrane. When cells are at rest, a combination of passive diffusion and active ion transport maintain an electrochemical gradient across the cell membrane. Activation of ion channels allows for the flow of ions down this gradient. Ion channels are diverse, and their activation is tightly regulated through specific chemical or physical stimuli dependent on the gating mechanisms of the channel. Mechanically-gated ion channels are activated via mechanical forces thought to be sensed through ion channel and cytoskeletal interactions (force-through-filament model) and/or directly through mechanical tension in the cell membrane (force-through-lipid model) [57–59].

Many mechanically-gated ion channels can be activated via multiple mechanical stimuli, e.g. membrane indentation, membrane stretch, shear flow, and hydrostatic pressure, all of which ultimately result in changes in membrane tension (Figure 2). The Piezo1 channel, which has been shown to gate via membrane tension [60], can detect an impressive array of mechanical cues including those listed above. Interestingly, Piezo1 does not require an intact cytoskeleton for gating [47,61], however it can also activate in response to cytoskeletal forces [48,50]. We previously showed that on stiff substrates, Piezo1 displays Ca^{2+} flickers

in the absence of any externally-applied mechanical force. Reduction of cellular traction forces either by seeding cells on a soft substrate or pharmacological inhibition of traction force generation reduced Piezo1 Ca^{2+} flicker activity [48,50]. However, it is important to note that cytoskeletal proteins themselves can modify membrane tension. For instance, the cortical cytoskeleton inhibits the membrane from stretching in response to mechanical manipulation, thus providing a mechanoprotective effect on Piezo1 [61]. Additionally, it has recently been shown that membrane tension can propagate rapidly in membrane blebs devoid of cytoskeleton, but is unable to propagate in intact cells [62]. Cell membrane tension is heterogeneous across the cellular membrane [62] and it is likely that cellular traction forces generate local increases in membrane tension that activate the Piezo1 channel. Alternatively, it is also possible that traction forces are conveyed to Piezo1 through a direct physical interaction between the channel and the cytoskeleton. The full picture of Piezo1 mediated force transduction is yet to be uncovered, but it is clear that the interplay between the channel and the cytoskeleton is an important regulator. Regardless of the nature of mechanogating, the net result of Piezo1 activation is the influx of ions, such as Ca^{2+}. These ions are diffusive in the cytoplasm and can therefore also mediate long range signals from localized mechanical events.

Ca^{2+} is an important second messenger molecule that regulates intracellular signaling pathways in immune cells. Increases in intracellular Ca^{2+} are generally thought to enhance LPS-induced macrophage inflammatory activation [63,64]. Moreover, Ca^{2+} has been identified as a key regulator in the activation of the inflammatory transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), since cell permeable Ca^{2+} chelators reduced the translocation of NFκB into the nucleus [63]. The ability of Ca^{2+} to regulate NFκB localization is thought to be due to the activity of Ca^{2+}-dependent proteases known as calpains, which are involved in the degradation of IkB, a key regulatory protein that inhibits NFkB activation [65,66]. Pharmacological inhibition of calpains prevented IkB degradation resulting in reduced NFkB - mediated inflammation [65,67]. Healing activation in immune cells is also dependent on calpains, with increased degradation of the pro-healing transcription factor signal transducer and activator of transcription 6 (STAT6) reported in the presence of increased intracellular Ca^{2+} or recombinant calpains [68]. In addition to macrophages, T cells have also been shown to rely on Ca^{2+} dependent pathways. In particular, T cell receptor (TCR) activation leads to increased intracellular Ca^{2+} that enhances the activity of calcineurin, a Ca^{2+}-dependent phosphatase that promotes the nuclear translocation of nuclear factor of activated T cells (NFATs) and subsequent T cell activation [69]. Despite these findings, the role of mechanical cues in regulating Ca^{2+}-dependent pathways remain unclear and is the subject of much interest. To better study mechanics mediated changes in ion channel activity, several key techniques have been developed.

Current techniques to quantify ion channel activity

The activity of mechanically-gated ion channels can be measured using various techniques, each with advantages and disadvantages. While patch clamping is often considered the gold standard, more recent microscopy-based methods have been developed and allow for the coupling of channel spatial information with function under more physiological conditions.

In this section, we will briefly review the methods that are commonly used to evaluate ion channel activity and provide some examples of studies utilizing each technique.

Patch Clamp

The patch clamp technique has been used to study ion channel activity for over 40 years and allows for quantitative electrophysiological measurements of ionic currents in single cells, isolated cells, or within tissues. Different configurations of the technique can be used to study the activity of very few channel molecules in a membrane patch while manipulating the intracellular or extracellular environments (cell-attached, inside-out excised, outside-out excised), or channels over the entire surface of the cell (whole-cell) (Figure 3). Traditional patch clamp techniques consist of the application of suction to a recording pipette that is in contact with a cell membrane, thus establishing a seal between pipette and membrane, electrically isolating the membrane patch [70]. Electric current generated by ion flux through the patched membrane enters the pipette and can be detected via an electrode, connected to a highly sensitive amplifier. When considering mechanically-gated ion channels, it is important to note that the patch configuration itself can exert mechanical forces onto the channels. For instance, Piezo1 inactivates quickly in cell-attached but slowly in outside-out patches due the resting tension experienced by the patch in cell-attached mode [60].

Modifications to the patch-clamp technique have allowed for the study of mechanical forces in regulating ion channel activation. The most common methods rely on suction pulses with a high speed pressure clamp or indentation of the cell membrane with a blunt glass pipette, both of which stimulate mechanically-gated ion channels [43]. Fluid flow, which exerts shear stress mimicking the flow of blood, can also be applied using the outside-out or whole-cell configurations [71]. While these techniques have provided critical insight into channel activity, they rely on the application of mechanical forces to the dorsal surface of the cell. As a result, the techniques provide little knowledge of mechanosensitive ion channel activation at the cell membrane-substrate interface, which contains a number of key molecules essential for adhesion and mechanotransduction in various adherent cell types [72]. To address this shortcoming, whole-cell patch clamp recordings have been obtained in cells cultured on elastomeric pillars, where mechanical stimuli applied through the deflection of individual pillars results in mechanosensitive channel activation [73]. More recently, novel electromechanical systems that utilize patch clamp recordings have been developed to study the role of mechanical stretch in regulating ion channel activity [74]. However, these approaches, which are performed in the whole-cell configuration, do not provide spatial information regarding the activation of the channels and also dialyze the intracellular compartment of the cell, making it hard to study the regulation of channel activity in native cellular conditions. Furthermore, these approaches have focused on transduction of externally-applied mechanical forces, and patch clamp based approaches are not optimally suited for studying channel activation by internal, cell-generated forces which also activate channels.

In summary, patch clamp electrophysiology can facilitate the rigorous quantification of biophysical properties of mechanically-gated ion channels including open probability,

conductance, and force required to open the channels, all of which have provided novel insights into mechanically-gated channel activity. However, a drawback to this method is that it requires specialized equipment and training, and is labor intensive [75]. Additionally, the method does not provide information on the spatial distribution of channel activity within the cell or allow measurement of channel activity under native physiological conditions.

Fluorescent Indicators

Several indicator-based techniques have been developed to study the flux of ions across cells. Among these, cell permeable chemical calcium indicators, such as Fura-2 and Fluo-4, that change fluorescence on binding Ca^{2+} are highly popular and reliable [76]. These indicators have been extensively used to study changes in cytosolic Ca^{2+} within immune cells in response to ion channel activation, including mechanically-gated ion channels [63,64]. In addition to chemical Ca^{2+} indicators, genetically encoded Ca^{2+} indicators (GECIs) based on the Ca^{2+} indicator GCaMP or FRET-based biosensors have been developed, opening up new lines of investigation. GCaMPs, which consist of Ca^{2+}-dependent binding protein calmodulin (CaM) and Ca^{2+}/CaM-binding motif M13 from myosin light chain kinase (MLCK) as well as a circularly permutated green fluorescent protein, rely on Ca^{2+}-dependent interactions between CaM and its binding motifs to alter the protonation state and thus fluorescence of the fluorescent protein [77]. These sensors can be engineered to be genetically expressed in specific cell types and can also be targeted to specific compartments of the cell, making it feasible for both *in vitro* and *in vivo* imaging. In comparison to organic Ca^{2+} indicators, GECIs generally have improved cytosolic dispersion and no leakage. One example of a GECI is Salsa6f, a protein consisting of tdTomato fused to the Ca^{2+} indicator GCaMP6f by a V5 epitope tag, allowing for precise ratiometric quantification of intracellular Ca^{2+} signals [78,79]. The Salsa6f probe provides several additional advantages for tissue imaging including: tracking cells in the absence of Ca^{2+} elevations when the GCaMP6f fluorescence is very dim; ratiometric imaging to eliminate motility artifacts; convenient single-wavelength (920 nm) femtosecond excitation for two-photon microscopy, signal to noise >20; stable expression in unperturbed cells; restriction to cytosol; sensitivity in cytosol from 100–1000 nM; no dye loading or leakage artifacts. Implemented in a transgenic mouse line for Cre-dependent expression, Salsa6f enables Ca^{2+} signaling to be specifically monitored in a wide variety of cell types [78–83]. Both chemical Ca^{2+} indicators and transgenic Ca^{2+} indicators can be used in multiple assays such as flow cytometry, fluorescence microscopy and with fluorescent plate readers. An advantage of chemical Ca^{2+} indicators over currently available transgenic Ca^{2+} indicators is their faster kinetics which allows imaging of fast Ca^{2+} events with higher fidelity. However, newer generations of GECIs will likely circumvent this limitation.

Several studies have utilized GECIs to study Ca^{2+} signaling in various different cell types. For example, Salsa6f was used to detect changes in cytosolic Ca^{2+} activity following TCR crosslinking *in vitro*, during motility of T cells *in vitro* and in the lymph nodes, Th17 cell Ca^{2+} activity in the spinal cord, and in response to the Piezo1 agonist Yoda1 [78–80]. Transgenic Ca^{2+} probes can also be used to study changes in Ca^{2+} activity in response to external mechanical forces. Salsa6f was used to detect and measure spontaneous Ca^{2+}

signals driven by Piezo1 activity in macrophages cultured on different stiffness substrates using confocal microscopy [83]. In addition, using a FRET based Ca^{2+} biosensor, Kim et al. showed that mechanical force exerted on fibronectin coated beads by optical laser tweezers promotes Ca^{2+} influx through the TRPM7 ion channel in human mesenchymal stem cells [84]. Moreover, high speed total internal reflection fluorescence microscopy (TIRFM) can be used to evaluate mechanics-mediated changes in Ca^{2+} activity at higher sensitivity compared to confocal microscopy. TIRF generates a high signal to noise ratio by specifically imaging a 100-nanometer range at the cell-substrate interface, permitting high resolution visualization of this region. Another advantage of TIRF is that as imaging is restricted to the cell-substrate interface this facilitates the investigation of cell-generated traction forces and ion channel activity. TIRFM imaging revealed the millisecond-scale Ca^{2+} flicker activity of Piezo1 elicited by cellular traction forces in adherent cells at the cell-substrate interface on stiff substrates [48,50].

In comparison to patch-clamping, the use of indicators coupled to microscopy-based methods provide a more physiological approach of analyzing Ca^{2+} activity through ion channels. A major advantage is that these experiments can provide spatial information on both channel localization and activity. Moreover, genetically encoded probes allow for visualization of activity within tissues as well as specific subcellular compartments, better recapitulating the mechanical environment experienced in physiological conditions. Despite these advantages, patch clamp techniques still provide a more quantitative approach to assessing changes in current and voltage, and hence are preferable for studying questions related to biophysical pore properties, such as ionic selectivity and inactivation. Therefore, it is important to note that both approaches provide distinct advantages and disadvantages and should be utilized in consideration with the specific scientific question under examination.

Pharmacological Tools

The development of several pharmacological agents has allowed for additional methods to study the activity of mechanically-gated ion channels. Applying mechanical force to study mechanically-gated ion channels more closely mimics their physiological context, however can be technically challenging. It also does not allow for sole activation of channels, having the potential to activate multiple mechanically-sensitive proteins at once. Therefore, pharmacological agents are also useful tools with which to study these channels. Activators of mechanically-gated ion channels include Yoda1, a specific agonist of Piezo1 which can activate Piezo1 channels in the absence of mechanical stimulation [85], and more recently Piezo1 activators Jedi1/2 have also been discovered [86]. Additionally, there are multiple agonists for TRPV4 including 4α-PDD [87] and GSK1016790A [88]. Inhibitors of mechanically-gated ion channels, however, are more limited. GsMTx4, derived from a spider toxin, inhibits excitatory stretch-activated ion channels, likely acting indirectly via the lipid membrane [89]. Moreover, many mechanically-gated channels are non-selective cation channels, which can be blocked by gadolinium chloride and ruthenium red, which also inhibit other channel families [90,91]. More specific antagonists exist for TRPV4 including HC-067047 [92] and RN-9893 [93] which has good oral bioavailability for use in *in vivo* studies. Dooku1, a Yoda1 analogue, can also be used as an antagonist of Yoda1-induced Piezo1 activity [94]. The use of these chemical tools can be applied in conjunction with all

methods used to study ion channel activity, and more specific pharmacological agents are likely to emerge over the next few years.

Mechanically-gated ion channels in immune regulation

Immune cells are exposed to varying degrees and types of mechanical stimulation, of which include hydrostatic pressure, environmental stiffness, shear stress, stretch, intercellular interactions, and mechanical cues resulting from biomaterial interactions (Figure 1). While numerous studies have identified the role of soluble or biochemical stimuli in regulating immune cell function the molecular mechanisms responsible for mechanics-mediated changes are less well understood. In this section, we will review studies that have specifically examined the role of mechanically-gated ion channels in cells of the immune system.

Innate immunity

The innate immune system offers a first line of defense, providing surveillance in tissues throughout the body. Macrophages are key cells of the innate immune system that are recruited to tissues as circulating monocytes and whose function has recently been shown to be regulated by Piezo1. Solis, et al. initially found that murine monocytes and macrophages highly express Piezo1 when compared to other known mechanically-gated ion channels [95]. In contrast, Piezo1 expression was shown to be considerably lower in neutrophils, also cells of the innate immune system. In addition, they found that Piezo1 is critical in sensing and transducing cyclic hydrostatic pressure, a mechanical stimulus experienced by cells within the lungs. Using bone marrow derived macrophages (BMDMs) isolated from myeloid specific Piezo1 conditional knockout mice ($Piezo1\Delta^{LysM}$), the authors show that macrophages lacking Piezo1 have reduced inflammatory activation when exposed to cyclic hydrostatic pressure compared to cells isolated from control mice [95]. Moreover, cells lacking Piezo1 exhibited reduced expression of hypoxia-inducible factor 1α (HIF1α), a transcription factor that has previously been shown to promote inflammation [96,97]. HIF1α has also been shown to influence macrophage metabolic state through promotion of glycolysis metabolism which in turn is known to enhance inflammatory activation [96,98]. This suggests that mechanically-gated ion channels could regulate cell function through modulation of metabolic pathways, a potentially exciting new avenue of research within the emerging field of immunometabolism. Moreover, Solis et al. confirmed their findings *in vivo* and showed that Piezo1 within recruited monocytes and not tissue-resident alveolar macrophages was responsible for bacteria-induced lung inflammation and fibrosis through upregulation of HIF1α (Figure 4). The authors also suggest that activation of monocytes could potentially result from a combination of extravasation, which itself is known to activate monocytes [99], and exposure to cyclic pressures resulting from pulmonary recruitment.

Myeloid cell specific expression of Piezo1 was also shown to regulate cancer and polymicrobial sepsis. Aykut et al. show that Piezo1 signaling promotes expansion of myeloid-derived suppressor cells and pancreatic cancer progression (Figure 4). Using a pancreatic ductal adenocarcinoma (PDA) model, the authors show that inhibition of

Piezo1 through myeloid cell specific knockout mice or using GsMTx-4 significantly reduced tumor mass and myeloid cell expansion when compared to relevant controls. In contrast, administration of Yoda1 led to myeloid cell expansion and enhanced tumor mass. Similar findings were also reported using patient-derived organotypic tumor spheroids, thus extending the potential for therapeutic efficacy of Piezo1 modulation to humans [100]. In addition, Aykut et al. identify a role of Piezo1 in regulating polymicrobial sepsis, as myeloid cell specific Piezo1 conditional knockout mice were observed to have improved survival as well as reduced sepsis score, inflammatory marker production, and myeloid cell expansion when compared to controls [100]. Finally, this study mechanistically demonstrated that Piezo1 activity enhances histone deacetylase 2 (HDAC2) activity, which suppresses retinoblastoma gene *Rb1* via epigenetic silencing and prevents myeloid cell expansion [100–102]. Further evidence showing Piezo1 activation in response to pathological mechanical forces *in situ* could provide greater clarity on the role of mechanically-gated channels in modulating disease.

We have recently shown that Piezo1 regulates macrophage sensing of stiffness *in vitro* and in the foreign body response to implanted materials *in vivo* (Figure 4) [83]. We found that macrophages cultured on stiff (280 kPa) substrates expressed more Piezo1 and exhibited enhanced Ca^{2+} activity compared to cells cultured on soft 1 kPa substrates. In the presence of liposaccharides (LPS) and interferon-γ (IFNγ), Piezo1 activation by Yoda1 led to enhanced inflammatory responses whereas Piezo1 depletion reduced inflammation. We also showed that Piezo1 is a mechanosensor of stiffness *in vivo*, since stiffness enhanced foreign body response was abrogated in myeloid cell specific Piezo1 depleted mice [83]. These observations suggest that myeloid cell Piezo1 plays an important role in regulating the foreign body response to stiff material implants. Stiffness has also been shown to influence Piezo1 activity in dendritic cells. Chakraborty et al. revealed that stiffness-mediated changes in yes associated protein (YAP) and Piezo1 activity influence dendritic cell inflammatory responses and metabolism [13]. Given recent reports elucidating the importance of YAP in the regulation of immune cell function and identifying Piezo1 as a transcriptional target of YAP signaling in cancer cells [8,13,103], this finding expands Piezo1 modulation to YAP signaling pathways in immune regulation. Moreover, demonstration of YAP nuclear-cytoplasmic localization by Piezo1 activity in neural stem cells further supports this idea [50].

In addition to cancer and the foreign body response, Piezo1 has been shown to influence the development and progression of cardiovascular diseases, such as aortic valve stenosis (Figure 4). Baratchi et al. show that stenosis or narrowing of the aortic valve promotes adhesion, phagocytosis, and inflammatory activation of monocytes derived from patient blood samples taken before and after transcatheter aortic valve implantation [104]. Through computational modeling and *in vitro* experimentation, the authors find that stenosis enhanced fluid shear stresses experienced by circulating monocytes, which promoted Piezo1-mediated Ca^{2+} influx, activation of CD11b (integrin α_M), and thus cell adhesion. The authors also show that macrophages differentiated from monocytes obtained from patients suffering from stenosis displayed enhanced phagocytosis and uptake of oxidized low-density lipoproteins (oxLDL) when compared to samples obtained post-treatment. This finding suggests that immune cells may retain memory of their mechanical environment,

which influences cell function even following a multiday *in vitro* differentiation process; however, whether this memory is due to Piezo1 signaling remains unclear.

Corroborating a role for Piezo1 in cellular uptake, Ma. et al. found that mice expressing a Piezo1 gain-of-function (GOF) mutation which slows channel inactivation, specifically in myeloid cells exhibited enhanced iron deposition in the liver and iron levels in serum (Figure 4) [105,106]. Mechanistically, the authors show that the Piezo1 GOF mutation in macrophages enhanced phagocytic activity resulting in increased red blood cell turnover and erythropoiesis. This, in turn, caused reductions in the level of hepcidin, a hormone that prevents the release of iron into the blood and is produced in coordination with red blood cell production, eventually leading to iron overload. While this study did not explicitly evaluate the effects of mechanical forces on channel activity, phagocytosis-induced membrane activity could indeed potentially lead to channel activation [107,108]. The role of other pathological mechanical stimuli, such as environmental stiffness, in regulating Piezo1-induced phagocytosis and iron metabolism may be of interest in future studies. Interestingly, this group found that a mild Piezo1 GOF mutation was present in a third of individuals of African descent, thus identifying a potential genetic risk factor that leads to increased iron levels in African Americans [106]. Piezo1 clearly plays a role in mechanotransduction within the innate immune system, and although only a few studies currently exist, there will likely be more in the near future.

While Piezo1 has been the focus of this section, there are other ion channels that can regulate innate immune cell function in response to mechanical stimuli. These largely consist of the transient receptor potential (TRP) family of ion channels. For example, in macrophages the TRPV4 ion channel promotes LPS-stimulated phagocytosis of *E. coli* particles on stiff substrates [109]. Moreover, Dutta et al. show that global knockout of TRPV4 suppressed bleomycin-induced fibrosis of the skin and reduced inflammatory activation in macrophages [110]. Mechanically-gated ion channels are critical molecules involved in sensing and transducing a variety of mechanical stimuli within innate immune cells which modulates the development and progression various pathological conditions.

Adaptive immunity

Unlike cells of the innate immune system, adaptive immune cells - including B, T and NK cells - are non-adherent or circulating cells that are recruited to various tissues through the process of homing and transmigration, where they recognize soluble or membrane bound antigens and ligands leading to their activation [111–113]. Immune cells experience a range of complex mechanical cues during these processes and a role for mechanical forces has been described for a host of T and B cell functions [21,22,25,27–30]. However, the molecular mechanisms that link specific mechanical forces to biological functions remain poorly understood. Despite the vast literature on the role of Ca^{2+}, K^{+}, and Cl^{-} ion channels in lymphocyte function [114,115], considerably less is known about the role of mechanically-gated ion channels.

Fundamental to the activation of adaptive immune cells is their exquisite ability to detect and discriminate antigens through B and T cell receptors. In the case of T cells, this involves recognition of peptides presented in association with major histocompatibility

complex (MHC) molecules expressed on the surface of antigen presenting cells by T cell receptors (TCR). MHC-TCR interactions lead to the formation of an immunological synapse (IS)- a dynamic macromolecular structure on the plasma membrane resulting from the rearrangement and clustering of signaling molecules driven by cytoskeletal changes, and culminating in the activation of a host of signaling pathways. While the biochemical details of antigen-receptor interactions are fairly well understood, the role of mechanical forces in fine-tuning these interactions has been recognized more recently. For example, dynamic cellular mechanical activities present within T cells and/or antigen presenting cells (APCs) could act upon the receptor ligand bond thus mechanically stimulating both cell types [116]. As a result, the TCR complex itself is thought to be mechanosensitive [117,118]. In addition, TCR mediated activation is also known to promote traction force generation and Ca^{2+} influx in these immune cells [119,120].

The earliest indication of a role for Piezo1 in T cell function *in vivo* came from a study by Ma et. al. demonstrating that T cell specific expression of a gain-of-function (GOF) mutation in Piezo1 confers partial protection in a mouse model of cerebral malaria [105]. While the study did not investigate the specific T cell subsets involved or the mechanism by which Piezo1 might facilitate protection, a role for Piezo1 in CD8+ T cells was inferred based on the established role for parasite-specific CD8+ T cells in pathogenic disruption of the blood-brain barrier [121,122]. It should be noted that the use of *Cd2-Cre* to express Piezo1 GOF mutation in lymphocytes does not preclude a role for Piezo1 in NK cells, which are known to express CD2 [123], and are involved in the pathogenesis of cerebral malaria [124].

Two recent studies have directly addressed the role of Piezo1 channels in TCR-induced T cell activation. Liu et al found that siRNA mediated knockdown of Piezo1 results in reduced TCR activation in human T cells [125]. Early signaling events downstream of TCR activation, which include ZAP70 phosphorylation, Ca^{2+} signaling, and CD69 expression, were impaired in Piezo1 deficient CD4+ and CD8+ T cells in response to bead immobilized anti-CD3 and anti-CD28 antibodies leading to diminished T cell proliferation after 4 days of activation. In contrast, activation of Piezo1 with Yoda1 enhanced TCR activation in cells exposed to soluble anti-CD3 and anti-CD28 antibodies which do not activate T cells on their own, thus suggesting that Piezo1 activation circumvents the need for immobilized antibodies to achieve optimal TCR activation [125]. Mechanistically, Liu et al. found that Ca^{2+} influx through Piezo1 activates calpains, Ca^{2+} dependent proteases. The proteolytic activity of calpains has previously been shown to be critical in reorganization of the actin cytoskeleton within T cells, which is essential for stabilization of immunological synapses and effective TCR activation [125–127]. Based on these findings, Liu et al. postulate that Piezo1 mediated Ca^{2+} influx is essential to cytoskeletal remodeling and optimal TCR activation in human T cells, although mechanistic details are currently lacking and warrant further study.

Jairaman et al. [revised manuscript submitted] used transgenic mice with T cell specific deletion of Piezo1 (*Cd4-Piezo1*$^{-/-}$ mice) to investigate the role of Piezo1 in CD4+ T cell function in the context of experimental autoimmune encephalomyelitis (EAE), an autoimmune model of neuroinflammation. Subsets of CD4+ T cells play a vital role in shaping the course of EAE-effector Th1 and Th17 cells drive EAE induction and progression, while regulatory T cells (Treg cells), which are recruited later during EAE

exert inhibitory effects on effector T cells leading to disease remission [80]. Surprisingly, Piezo1$^{-/-}$ CD4+ T cells did not show any impairment in TCR-induced Ca^{2+} signaling, T cell proliferation and Th1 and Th17 cell polarization *in vitro* in response to plate-bound as well as bead-immobilized anti-CD3 and anti-CD28 antibodies (Figure 5). These findings are different from Liu et al, and may reflect the differences in knockdown strategy or possibly the differences between mouse and human T cell biology. Homing and motility of Piezo1$^{-/-}$ CD4+ T cells were shown to be indistinguishable from WT cells, consistent with the fact that T cell development and seeding into secondary lymphoid tissue is normal in *Cd4-Piezo1*$^{-/-}$ mice. Yet, *Cd4-Piezo1*$^{-/-}$ mice show partial protection against EAE, with reduced numbers of Th1 cells and increased numbers of Treg cells in the draining lymph node (DLN) 10 days after injection of $MOG_{35–55}$ peptide. Treg cells were also increased in the spleen and spinal cord of *Cd4-Piezo1*$^{-/-}$ mice at 24 days after EAE induction. Interestingly, the study found that specifically under Treg polarizing conditions *in vitro*, Piezo1$^{-/-}$ CD4+ T cells show increased expansion into Treg cells and show enhanced TGFb signaling which is critical for Treg differentiation (Figure 5). These *in vitro* findings mirror the higher number of Treg cells in the secondary lymphoid organs and spinal cord of *Cd4-Piezo1*$^{-/-}$ mice induced with EAE. Consistent with a role for Piezo1 in Treg cell function, Treg-specific inducible deletion of Piezo1 led to partial EAE protection in *Foxp3*$^{eGFP\text{-}Cre\text{-}ERT2}$ *Piezo1*$^{fl/fl}$ mice. Several key questions remain about how Piezo1 functions in T cells: what mechanical stimulus activates Piezo1 in T cells; how is Piezo1 differentially regulated in Treg cells; what is the mechanistic link between Piezo1 and TGFb signaling; are there negative regulatory mechanisms that inhibit promiscuous Piezo1 activity during T cell function?

Piezo1, due to its ability to influence T cell function, has also been explored as a potential therapeutic target or for its role in regulating various diseases. For example, Pan et al. describe a method whereby Piezo1-expressing T cells sense and transduce ultrasound stimulation, applied remotely and noninvasively, to control cell transcriptional activity and chimeric antigen receptor (CAR) protein expression for antigen recognition to target tumor cells [128]. The authors find that cells deficient in Piezo1 have reduced Ca^{2+} influx in response to stimulation when compared to control cells, thus confirming the ability of Piezo1 to sense and transduce ultrasound stimuli. The resulting Ca^{2+} influx is known to activate calcineurin, a Ca^{2+} dependent phosphatase, which activates the nuclear factor of activated T-cells (NFAT) transcription factor [69]. NFAT translocation to the nucleus activates an engineered NFAT response element which allows the expression of designed target genes that help to kill tumor cells [128].

Conclusions and Future Perspective

The studies described here have all provided critical insight into the role of mechanically-gated ion channels in regulating important immune cell functions. These channels are activated in response to diverse mechanical stimuli within innate immune cells leading to enhanced expression of adhesion molecules, inflammation, and phagocytosis, all critical immune cell functions (Figure 4). In contrast, Piezo1 is shown to influence Treg expansion in adaptive immune cells (Figure 5); however, the role of mechanical stimuli in influencing channel activity and cell function remains unexplored. In addition, channel activity was also shown to have important functional roles in the development and progression of numerous

disease conditions *in vivo*. For example, Piezo1 exacerbated bacteria-induced lung fibrosis in response to hydrostatic pressures and its activity was also shown to enhance tumor growth [95,100]. In addition, Piezo1 was required for the enhanced foreign body response to stiff material implants compared to soft implants [83]. while Piezo1 was also shown to promote the expression of adhesion molecules and phagocytosis resulting in aortic valve stenosis or iron overload, respectively [104,106]. Moreover, Piezo1 activity was shown to enhance the severity of EAE. These studies have provided critical insight into the potential of Piezo1 and mechanically-gated ion channels in regulating immune cell function. However, numerous questions still remain, including the effects of mechanical stimuli in different disease contexts. Further studies of the role of mechanically-gated channels in modulating immune cell responses to varying mechanical environments will likely improve our current understanding and provide novel molecular targets for immune regulated diseases.

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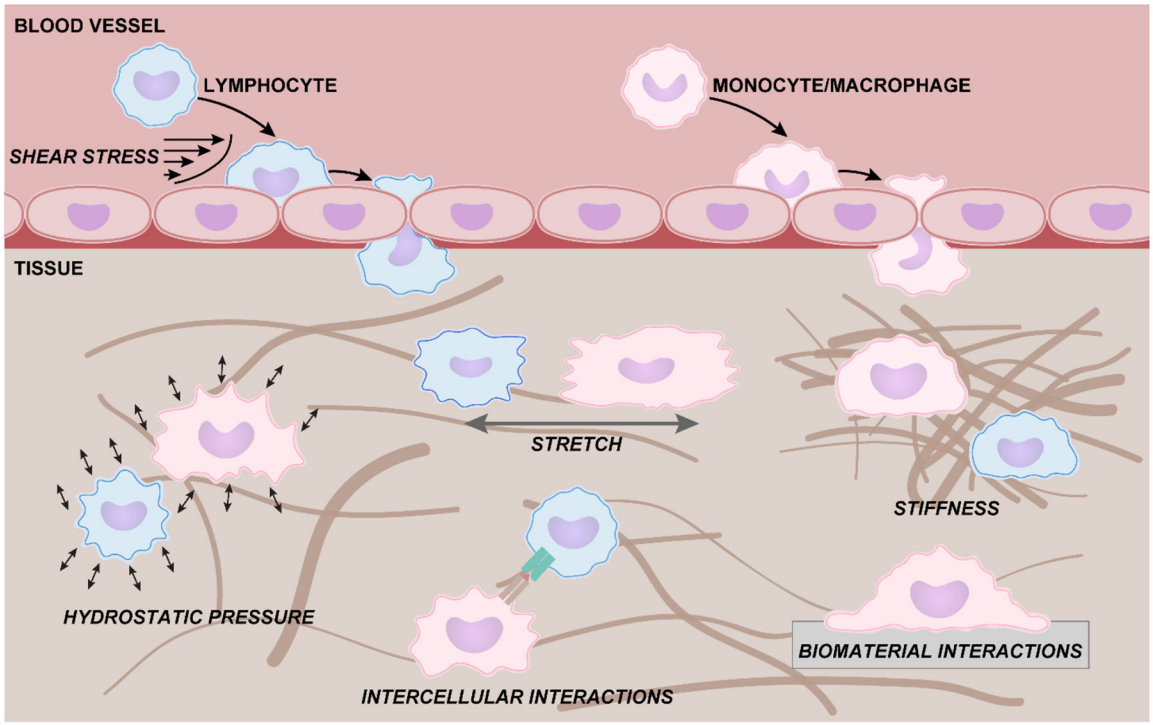

Figure 1: Common mechanical stimuli experienced by innate and adaptive immune cells.
Schematic illustrating the different mechanical stimuli experienced by immune cells as they extravasate through blood vessels and adhere to mechanically active tissues or foreign implantable materials.

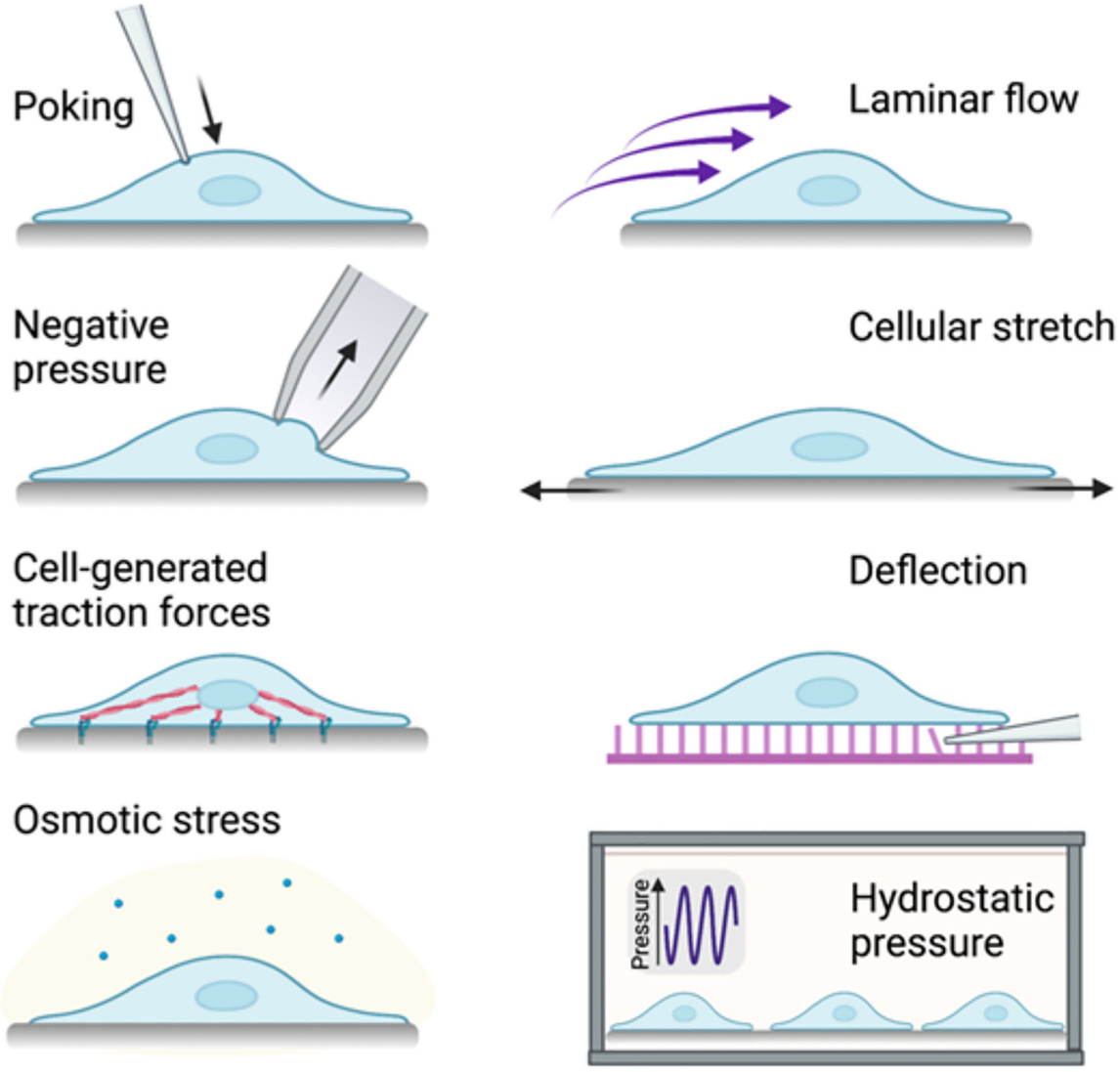

Figure 2: Techniques to activate mechanically-gated ion channels.

A variety of techniques have been developed to study mechanically-gated ion channels, as depicted above. These often aim to mimic the vast mechanical stimuli cells are subject to *in situ*. The channel activation mechanisms shown here can be used in conjunction with methods that record channel activity, such as patch clamp or imaging. They can also be combined with molecular techniques to measure the downstream effects of channel gating.

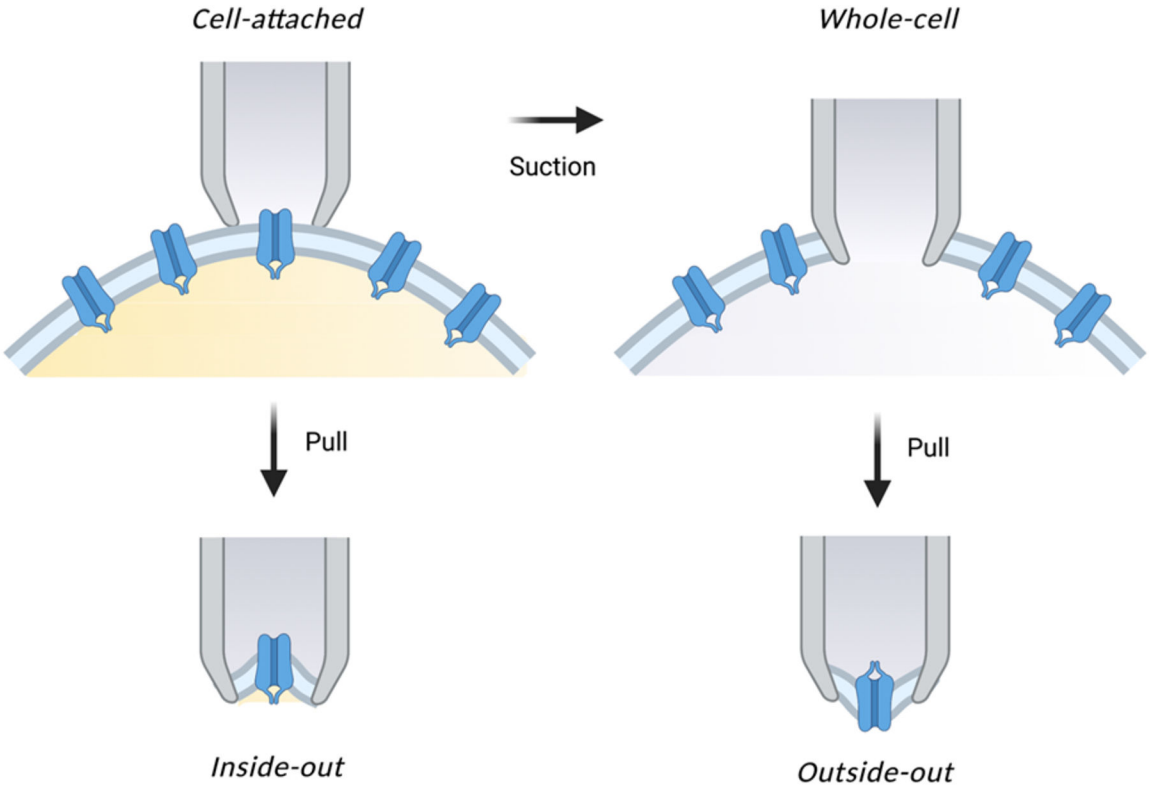

Figure 3: Patch clamp configurations.

In the *cell-attached* configuration, the pipette is sealed to a small patch of the cell membrane and the activity of one or several ion channels within the membrane patch are recorded. If the pipette is pulled away, the membrane patch detaches from the rest of the cell resulting in the *inside-out* configuration. Here the intracellular side of the ion channel is in contact with the bath solution. If suction is applied whilst in the *cell-attached* configuration then *whole-cell* can be achieved. Here the combined activity of all ion channels within the cell are recorded and the intracellular solution is replaced by the pipette solution. If the pipette is pulled away, small regions of membrane detach on either side of the pipette and can re-join to form the *outside-out* configuration. Here the extracellular side of the ion channel is in contact with the bath solution.

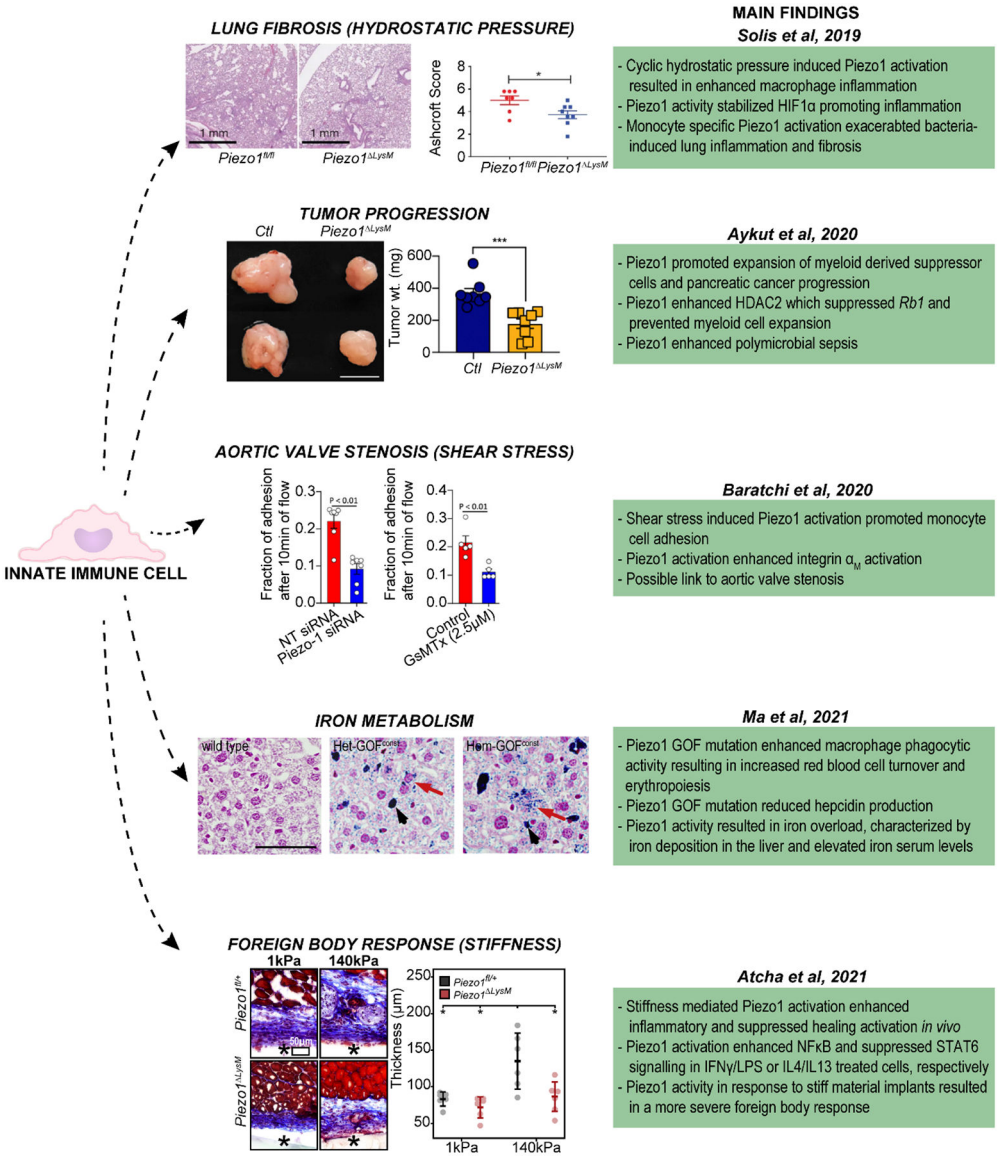

Figure 4: Piezo1-mediated regulation of innate immune cell function and disease.

Summary of recent studies that identify roles for myeloid cell specific Piezo1 in regulating lung fibrosis [95], tumor growth [100], aortic valve stenosis [104], iron metabolism [106], and the foreign body response [83]. Mechanical stimuli identified as key regulators of channel activity are indicated in parentheses. Data shown indicates that myeloid cell specific Piezo1 deletion (*Piezo1*$^{\Delta LysM}$) reduced bacteria-induced lung fibrosis, tumor growth, and fibrous encapsulation of stiff implanted materials as is indicated by a reduction in the Ashcroft clinical score, tumor weight, and fibrous capsule thickness, respectively. Moreover, siRNA mediated Piezo1 knockdown or pharmacological inhibition of mechanically-gated ion channels revealed a reduction in monocyte cell adhesion, whereas myeloid cell specific heterozygous (Het-GOF$^{const.}$) of homozygous (Hom-GOF$^{const.}$) Piezo1 GOF mutation resulted in enhanced iron deposition in the liver. Arrows indicate presence of iron in hepatocytes.

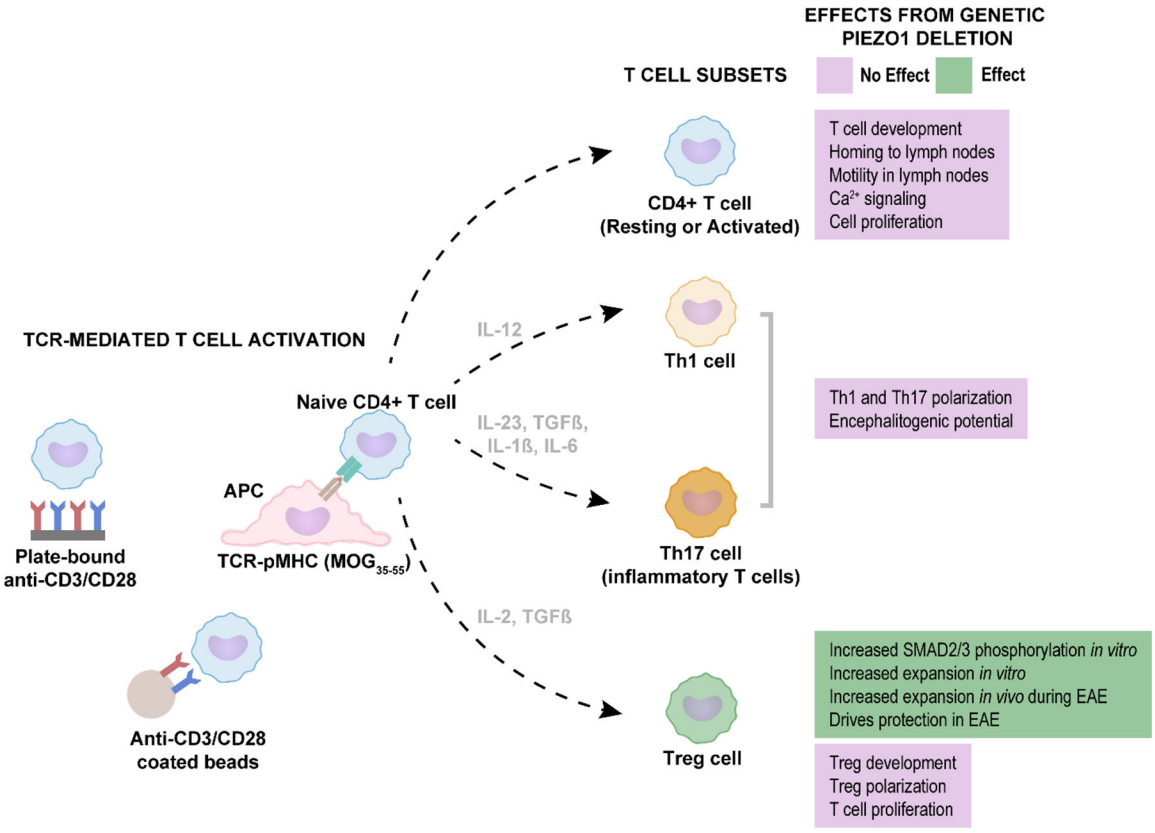

Figure 5: Effects of Piezo1 deletion in CD4$^+$ T cell subset functions.

CD4$^+$ T cells were activated either by peptide recognition on antigen presenting cell (APC) or by TCR-crosslinking antibodies immobilized on beads or a flat-substrate (left). T cell subsets generated by TCR activation in association with specific cytokine cocktails as indicated. Panel on the right shows the effects of Piezo1 deletion in various subsets (see text for details).