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Regulation of Macrophage Migration by the Mechanically Activated Ion Channel Piezo1

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Biomedical Engineering

by

Jiangfeng Chen

Dissertation Committee:
Associate Professor Wendy Liu, Chair
Professor Elliot Botvinick
Professor Arash Kheradvar

2020

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DEDICATION

To

my parents and friends

in recognition of their worth

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ABSTRACT OF THE THESIS

Regulation of Macrophage Migration by the Mechanically Activated Ion Channel Piezo1

by
Jiangfeng Chen
Master of SCIENCE in Biomedical Engineering
University of California, Irvine, 2020
Associate Professor Wendy Liu, Chair

Macrophage migration has been proved as an important activity involving variety of mechanosensitive molecules. The migration in vivo could lead to different physiological outcomes, it can be advantageous or detrimental. Therefore, the regulation of macrophage migration would be a novel way to cure diseases such as cancer and atherosclerosis. Macrophage behaviors could be regulated by both chemical and physical cues in extracellular matrix. However, most studies in the past focused on the chemical aspects, the physical cues are investigated until recent. As a mechanosensitive cell, macrophage activities are regulated by various mechanosensing molecules like integrins and ion channels. Piezo1, an ion channel that recently discovered in 2010, has been shown to be the most expressed ion channel in macrophage, little is known about its role in macrophage migration. In this thesis, we looked at how Piezo1 regulates macrophage migration under different conditions. First, the role of Piezo1 in macrophage random migration was discussed. Then, how the Piezo1 control macrophage migration along with adhesion was looked into. Last, the calcium cation in extracellular matrix was investigated whether it impact the motility of macrophage.

Chapter 1: Introduction

Macrophages are versatile cells that play key roles in innate immunity, adaptive immunity, metabolism and the maintenance of homeostasis [1]. These cells engulf and digest invading organisms, cellular debris, cancer cells, and microbes in a process called phagocytosis [2]. As diverse cells, macrophages can polarize to a variety of phenotypes with different functions, which is critical for many diseases (e.g. atherosclerosis [3], obesity [4], cancers [5] and wound healing [6]). Wound healing process can be divided into three phases: coagulation and inflammation, tissue proliferation, tissue remodeling [7].

In wound healing process, macrophages adopt different phenotypes that are involved in both inflammation and regeneration (**Figure 1.1**) [8]. Following tissue injury, macrophages recruited to the wound site adopt a classically activated phenotype (M1) which promote inflammation in response to foreign substances, M1 macrophages also secrete pro-inflammatory chemotactic factors that recruit other inflammatory cells. In the tissue repair stage, macrophages respond to the stimuli and other inhibitory mediators found in the local milieu, polarize toward an alternatively activated phenotype (M2), M2 macrophages can secrete pro-regenerative cytokines such as interleukin-4 (IL-4) and interleukin-13 (IL-13) to suppress

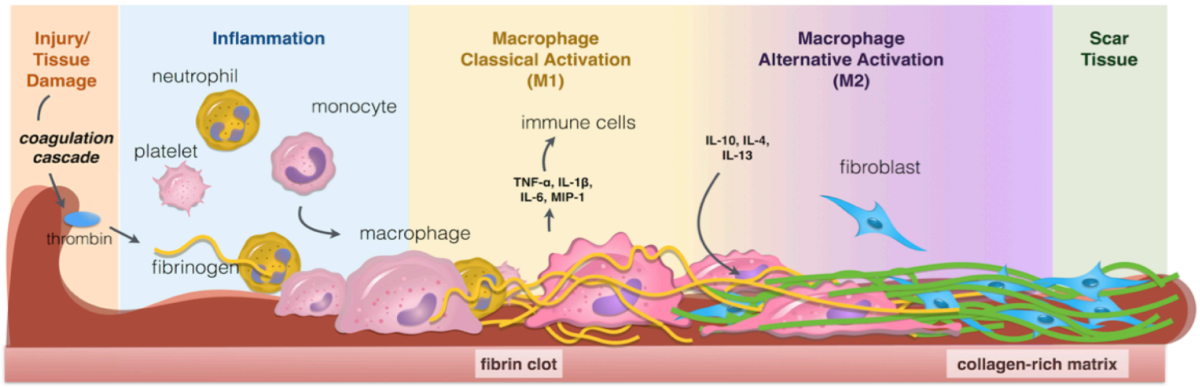


Figure 1.1. Macrophage phenotypes in wound healing. Macrophages polarize to M1 phenotype in the inflammation stage and secrete pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and macrophage inflammatory protein-1 (MIP-1, also referred as CCL4). Whereas macrophages in tissue regeneration stage adopt M2 phenotype, secreting anti-inflammatory cytokines IL-4 and IL-13. Figure courtesy of Esther Y. Chen

inflammation [9]. Though these phenotypes have different functions and were initially thought to be disparate cell states, macrophages populations in vivo do not cleanly ascribe to either the M1 or the M2 paradigm.

In many physiological processes including wound healing and diseases (such as cancers and atherosclerosis), transendothelial and interstitial motility are essential aspect of macrophages function as they are recruited to specific site upon demand [10]. Though migration of macrophages to the wound site is considered beneficial, macrophage migration facilitates formation of some chronic diseases [11]. The development of atherosclerosis is associated with accumulation of macrophages within the vascular wall, where there is enhanced oxidative stress [12]. In tumor progression, an elevated interstitial flow polarizes macrophages in tumor site towards M2 phenotype and enhances macrophages migration in three-dimensional extracellular matrix (ECM), these activations of macrophage function further improve their ability to promote cancer cell migration [13]. Therefore, regulation of macrophage migration may be a potential therapeutic method to many physiological diseases.

1.1 Role of integrins in biophysical regulation of macrophages

Macrophage can adopt various functions based on the microenvironment where it resides, through both chemical and physical stimulations. Most studies about macrophages were based on their response to soluble signals (e.g. chemokines, cytokines and damage- or pathogen-related molecular patterns), compare to other types of cues [14]. After realized how complex the microenvironment is, the biophysical cues these elastic cells are exposed to are currently considered as important as soluble cues. Macrophages in vivo undergo numerous biophysical cues, including mechanical forces and tissue physical properties. External mechanical forces like stretch and shear stress, are able to alter the morphology of cells, the functions of macrophages may also change during this alteration [15]. In addition to external mechanical forces apply on cells, extracellular matrix components (stiffness and topography)

also play a role in regulating cell function. The interaction between soluble, diffusible signals and macrophage migration is more straightforward, whereas the correlation among biophysical signals and cell migration remains unclear.

As a cell type that is able to sense mechanical forces in the environment, macrophage possesses mechanosensitive molecules, such as integrins and ion channels. Integrins are transmembrane receptors that facilitate extracellular matrix adhesion, they detect the mechanical cues in the microenvironment and transduce those external signals into biological signals that regulate cell function [16]. Through ligand binding to the extracellular matrix, integrins activate signal transduction pathways that mediate cellular activities such as regulation of the cell cycle, cell motility, organization of the intracellular cytoskeleton, morphological changes, and recruitment of other receptors to the cell membrane [17]. In immune cells, integrins are expressed in associations of α families and β families (**Table 1**). These combinations of integrins play an integral role in macrophage migration. Recent study showed that $\alpha 5\beta 1$ and $\alpha 4\beta 1$ facilitate the migration of macrophages to the lymph nodes, removal of these integrins leads to inhibition of cell migration [52]. The integrin pair $\alpha M\beta 2$ involves in migration of a variety of cell as well, the inactivation of $\alpha M\beta 2$ contributes to inhibition of macrophage efflux from the peritoneal mesothelium to the lymphatics, impaired motility of human monocytes to penetrate endothelium and connective tissues [53,54]. Moreover, $\alpha M\beta 2$ mediates the passage of neutrophils in synovial and dermal fibroblast barriers, while facilitate T cells adhere and perform transendothelial migration [55,56].

Four types of integrin-mediated cell-matrix adhesion structures have been discovered in cultured cells: focal complexes (also referred as nascent adhesions), focal adhesions (FA), fibrillar adhesions, and three-dimensional (3D) matrix adhesions, the former adhesion structure can further develop into the later ones [18,19]. The formation of focal complexes happens at the edge of migrating cells when the cells attach the ECM, and they can develop into focal adhesions when proper linkages are established with the actin cytoskeleton through external

forces [20]. External forces alter the patterns of cytoskeleton directly through integrins on the cell surface, and focal adhesions are formed due to cytoskeleton cluster. In addition to those four different adhesion structures, there are other types of integrin-mediated adhesion structures: podosome and invadopodia, they are actin-rich protrusions at the edge of plasma membrane and have been found in many cell types such as cancer cells, endothelial cells and immune cells. Podosomes are mechanosensitive organelles that are more active and unstable compare to focal adhesions [21], they are multi-molecular structures that include integrins and integrin-associate proteins such as talin, paxillin, and vinculin (**Figure 1.2.1**) [22]. In macrophages, podosomes play a key role in migration, invasion, adhesion and degradation of surrounding matrices (phagocytosis), their inhibition contributes to impaired adhesion and migration [23].

Table 1. Monocyte and macrophage integrins [41]

β chain family	Associated α	Ligands
β1	α1	Collagen
	α2	Collagen, Laminin
	α3	Laminin
	α4 (CD49d/CD29, VLA-4)	Fibronectin, VCAM-1 (CD106)
	α5 (CD49e/CD29, VLA-5)	Fibronectin
	α6 (CD49f/CD29, VLA-6)	Laminin
β2	αM (CD11b/CD18, CR3, Mac-1)	ICAM-1, ICAM-2, C3bi, fibrinogen, Factor X
	αL (CD11a/CD18, LFA-1)	ICAM-1, ICAM-2, ICAM-3
	αx (CD11c/CD18, p150/95)	RGD-containing proteins, Fibrinogen, Lipopolysaccharide, C3bi
β5	αV (CD51/CD61)	Vitronectin, Entactin, RGD- containing proteins

1.2 Role of ion channels in biophysical regulation of macrophages

In addition to integrins, ion channels are also capable of transducing mechanical signals into electrical signals. Ion channels are porous membrane proteins that establish a membrane

potential, tune action potentials and other electrical signals by controlling the passage of ions [24]. Impaired ion channel functions lead to neuronal and muscular degeneration [25,26], cardiac arrhythmias [27], hypertension [28], and polycystic kidney disease [29]. Two ways of force transmission and gating methods have been presented: 1. Ion channels are activated by the forces applied to the lipid bilayer on the cell membrane, the membrane tension generated through the force and gate the channels. 2. Forces applied to the cell or ECM are transduced through a tether connection between ion channels and ectomembrane components.

1.2.1 Transient receptor potential (TRP) channels: TRP channels are a family of ion channels reside on the plasma membrane, there are about 30 TRP channels that share similar structures [30]. These 30 TRP channels can be classified into 6 subgroups according to their structural similarity and domain architecture: TRPC ("C" for canonical), TRPV ("V" for vanilloid), TRPM ("M" for melastatin), TRPN ("N" for no mechanoreceptor potential C) , TRPA ("A" for ankyrin, TRPP ("P" for polycystic) and TRPML ("ML" for mucolipin) [31]. TRP channels respond

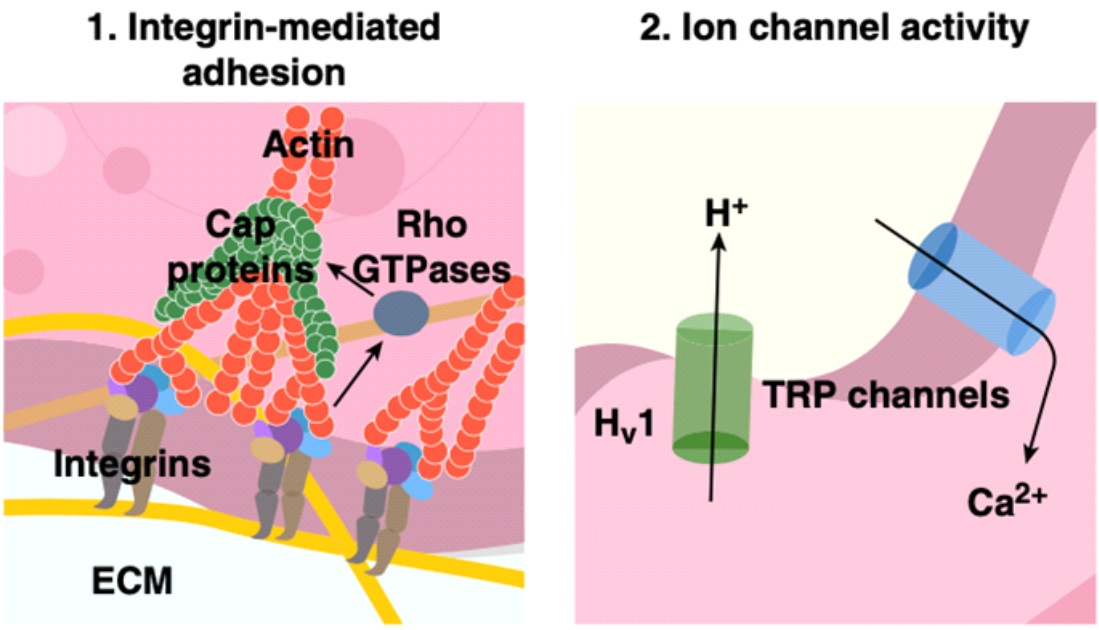


Figure 1.2 Representative ion channels. (1). Podosomes that include integrins and other integrin-associate proteins, they sense the changes in ECM by binding to it, transduce the physical signals to biological signals through the binding with actin cytoskeleton. (2). Ion channels on the membrane are activated due to forces or changes in membrane tension, allowing the passage of cations [16].

to various stimulations in the environment including temperature, pH, and osmolality, making them also function as environmental sensors of pain, tastes, pressure, and temperature [32].

TRP channels conduct cations, are weakly voltage-sensitive and non-selective for calcium, sodium and other cations, contributing to immune and inflammatory responses, these channels play major roles in mediating several macrophage functions such as cell survival, proliferation, polarization, migration and phagocytosis. TRPM4 appears to control macrophage survival in sepsis, the lack of TRPM4 affects macrophage infiltration and increases the monocyte number. The knockout of TRPM4 in macrophage inhibits the Akt pathway due to reduced calcium mobilization, and consequently macrophage survival, phagocytosis of bacteria [33]. The ion channels in a cell may have opposite functions, TRPV4 downregulates inflammation induced by Lipopolysaccharides (LPS), but upregulate LPS-mediated phagocytosis and ox-LDL uptake [34]. On the contrary, TRPM7 enhances endocytosis of the LPS-TLR4 complex, which positively regulates inflammation induced by LPS, the knockout of TRPM7 in mice protect them from LPS-induced peritonitis [35]. In addition to their role in manipulating inflammation, TRPM7 also controls the formation of focal adhesions into podosomes by a kinase-dependent TRPM7-mediated activation [36]. Similar to TRPM7, TRPM4 channels are localized in the focal adhesions, they modulate cellular contractility and migration in macrophages by regulating FAK and Rac GTPase activities [37]. The blockage of TRPC6 or TRPV1 channels is reported to partially impair monocyte chemotaxis, when TRPC6 and TRPV1 are blocked at the same time, the migration in monocytes induced by chemoattractant is abolished [38].

1.2.2 Hv1 channels: In macrophages, there are ion channels that gate other cations, such as the voltage-gated proton channel Hv1 (**Figure 1.2.2**). Hv1 channels regulate the protons into cell, are responsible for the NADPH oxidase complex, they contribute a majority of charge compensation that is critical for optimal NADPH oxidase activity. The absence of Hv1 in cells attributes to substantially reduced NADPH oxidase-dependent superoxide anion

production, this superoxide anion production is required for efficient phagocytosis of bacterial pathogens [39].

1.2.3. Piezo channels: Piezo family of genes were discovered in 2010 as essential components of mammalian mechanosensitive ion channels, including Piezo1 and Piezo2. Piezo1 was first considered as a mechanosensitive channel responsible for the mechanically activated in Neuro2A mouse neuroblastoma cells, and Piezo2 was required for a subset of mechanically activated current in dorsal root ganglia sensory neurons. Piezo is the largest ion channel subunit that contains large proteins with numerous predicted transmembrane domains across species, 2521 amino acids for human Piezo1 and 2752 amino acids for human Piezo2, share no similarity to other proteins. Piezo proteins are pore-forming subunits of ion channels, they are mediated by mechanical forces, allow the entrance of ions such as calcium and sodium [40]. The structures of Piezo1 and Piezo2 are analogical, though they only share 42% sequence homology, both of them are homotrimeric channels [42]. This trimeric complex looks like a propeller from the top side, with three blades surrounding the central pore beneath a cap region referred to as the C-terminal extracellular domain **(Figure 1.3**).

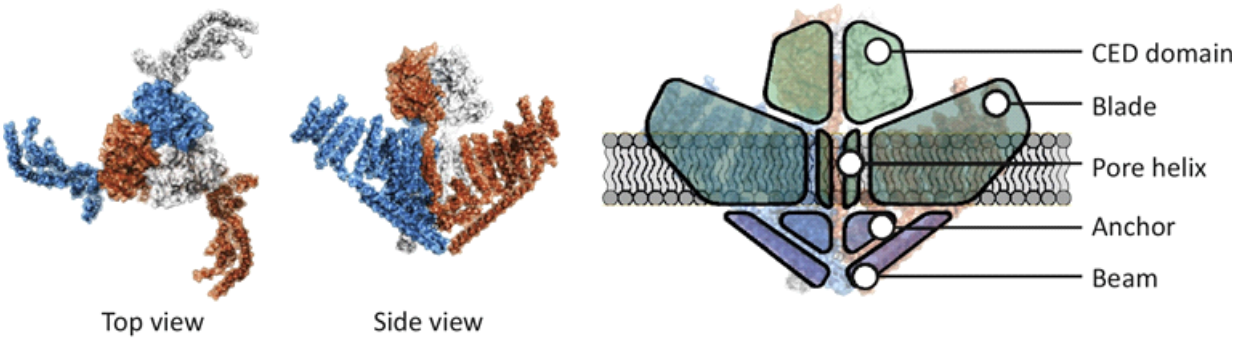


Figure 1.3 The structure of Piezo1 under cryo-electron microscopy (left) and other structure domains (right) [43].

Physiologically, Piezo1 functions as a mechanosensitive ion channel required for vascular development, remodeling, and blood pressure regulation [44]. Expression of Piezo

channels is critical for the survival of vertebrates, the fully knockout of Piezo1 channels during midgestation in mouse individual is fatal, by disrupting the formation of vasculature system [45]. The absence of Piezo1 in smooth muscle cells leads to mice have deficits in arterial remodeling upon hypertension [46]. Besides, Piezo1 also plays key role in a variety of cell activities, such as homeostasis of epithelial cell number [47], red blood cell regulation [48], neural stem cell fate determination [49].

As the most expressed mechanosensitive ion channel in macrophages, our laboratory has recently found that Piezo1 is an important regulator in macrophage behaviors (**Figure 1.4**) [50]. Cyclical hydrostatic pressure triggers upregulation of proinflammatory genes such as Il1b, Cxcl10 and Ptgs2 in macrophages with the presence of Piezo1, however, this transcriptional reprogramming is abolished when Piezo1 is removed [50]. Besides the fact that Piezo1 promote inflammation in macrophages, its roles in other macrophage activities like migration and adhesion have not been well-studied. Therefore, some fundamental experiments had been conducted to have the basic understanding of how Piezo1 regulate macrophage migration.

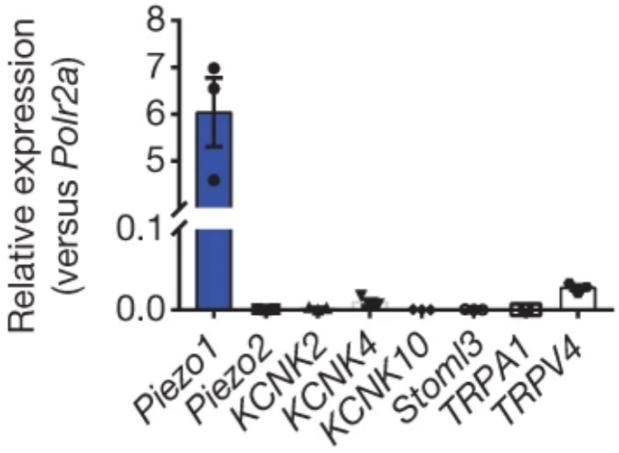


Figure 1.4 RT–qPCR analysis of known mammalian mechanosensory ion channels from unstimulated BMDMs. Data are presented as three biological replicates from two independent experiments [50].

1.3 Summary

In this chapter, macrophage phenotypes and their role in wound healing are discussed. To figure out how macrophage activities are regulated, the chemical and mechanical cues in the extracellular matrix are investigated. Specifically, we discuss the mechanism of mechanosensing in macrophages, involving various mechanosensory molecules such as integrins and ion channels. Piezo1 is a recently discovered ion channel, and is the most highly expressed ion channel in macrophages, but its role in regulating macrophage behaviors has not been well understood, especially in macrophage migration. Macrophage migration involves in numerous diseases besides and during wound healing, the migratory macrophages could lead to beneficial or harmful pathological results. In this thesis, the role of ion channel Piezo1 in macrophage motility has been investigated. In chapter 2, study involving the role of Piezo1 in macrophages random migration on the glass will be elaborated upon. Further, in chapter 3, the migration is observed in different stiffness gels, the correlation of adhesion and migration will be discussed. Last, in chapter 4, we investigate the role of Ca^{2+} signals in regulating macrophage motility. As an ion channel that allows passage of calcium cation, Piezo1 also control macrophage motility in a way involving Ca^{2+} events.

Chapter 2: The role of Piezo1 in macrophage migration

2.1 Background

Macrophages polarize toward M1 phenotype when stimulated by LPS and IFNγ, when macrophages exposed to IL-4 and IL-13 adopt M2 phenotype. Despite the difference in functions, they are morphologically diverse, M1 macrophages are considered as more rounded, whereas M2 macrophages are more elongated. Recent study showed that M2 macrophages activation can be induced by morphological alternation, the shear stress caused by elevated interstitial flow polarize the local macrophages to M2 macrophages [13]. Therefore, the expression levels of mechanical molecules may differ in macrophage phenotypes. Previous study has shown that expression level of integrin αD in M1 macrophage is upregulated compare to M2 macrophage [51].

2.2 Methods

2.2.1 Cell culture conditions: To investigate the how Piezo1 affect macrophage migration on glass, a simple experiment was designed. Since the deficiency of Piezo1 in embryo leads to death, to obtain bone marrow derived macrophages that Piezo1 is knocked out, Piezo1-floxed mice was crossed to LysM-Cre mice to delete PIEZO1 in myeloid cells. $Piezo1^{fl/fl}$ (Het) and $Piezo1^{\Delta LysM}$ (cKO) cells were harvested from bone marrow in mice, and cultured for 7 days for further usage. On day 7, these cells were seeded in 8-well chamber slides (Thermo Scientific Lab-Tek®), at a cell density of 40,000 cells/mL (20,000 cells per well) in 500 μL D10 media. This seeding density was selected to prevent overcrowding and to have sufficient cells in the analysis frame.

2.2.2. Image acquisition and analysis: The chamber slides were placed in incubator for 6 hours after seeding, allowing the cells to fully adhere to the wells. Half of the D10 media was taken out from each well (250 μL) after 6 hours incubation, new media (250 μL) with stimulation

was added to each well. Macrophages were stimulated with 1ng/mL LPS+IFNγ or 1ng/mL IL-4+IL-13, after adding the media with stimuli, slides were placed on a stage incubator under microscope. The temperature in the stage incubator was maintained at 37°C, with a constant 5% CO2 atmosphere, temperatures were periodically measured with a thermosensitive probe. Chambers were observed with an Olympus IX-83 inverted microscope equipped with a Tokai Hit stage incubator and controlled by Micro-Manager. Cells in chambers were imaged at 2 minutes intervals for a total of 6 hours using the built-in Multi-Dimensional Acquisition function in Micro-Manager. Two views were selected in each condition, 30 cells were tracked using MTrackJ in ImageJ in each duplicate, cells that went out of view during the video were not tracked. The tracks were further quantified by using an in-house developed Python script by Dr. Tim Smith.

2.2.3 Flow cytometry: Het and cKO cells were seeded according to 2.2.1 with no stimulation added, then were retrieved using cell dissociation buffer and cell scrapers. Antibodies and their corresponding isotype antibodies were used, to look at the expression levels of integrin αm, αv, αx, β1, β2 and β3. Flow cytometry was performed on a BD LSRII flow cytometer using BD FACSDiva software (BD Biosciences). Post-processing was performed in FlowJo (Treestar) and further data analysis and quantification was done in Excel. Cell populations were gated on forward and side scatter to select for intact, single cells. Acquisition was performed until at least 10,000 events were collected using a preliminary analysis gate or until the sample was exhausted.

2.3 Results

2.3.1 *Piezo1 knockout increases migratory capacity in unstimulated macrophages*

The trajectories of macrophages on the video stacks that compose of 180 frames show that unstimulated macrophages with deletion of Piezo1 seems to have the most spreading tracks (**Figure 2.1. A**). To further confirm this hypothesis, displacement plots were generated by R (**Figure 2.1. B**). These plots also display the same result, as the displacement of unstimulated

macrophages without Piezo1 reaches over 200 in x axis and y axis, whereas the displacement plots of cells in other conditions stay [-200,200] in both directions of axis. The scatterplots were made using R (**Figure 2.2.**), asterisks denote $p < 0.05$ in student's t test, which means the difference between two group is significant. The rms displacement refers to the root mean square displacement, is defined as the square root of the mean square (the arithmetic mean of the squares of a set of numbers). In the case of a set of n values $\{x_1, x_2, x_3, \dots, x_n\}$, the rms is:

$$x_{rms} = \sqrt{\frac{1}{n}(x_1^2 + x_2^2 + \cdots + x_n^2)}$$

In the bar of unstimulated cells, the macrophages with Piezo1 removed have significantly greater mobility since they have more path length, max displacement, rms displacement and velocity.

2.3.2 *Motility of activated macrophages is independent of Piezo1*

Though there is a remarkable difference between unstimulated Het and cKO macrophages, no significance difference is shown in the Het and cKO macrophages stimulated with LPS and IFNγ, or cells stimulated with IL-4 and IL-13. Macrophages behave similarly in migration when they are polarized to M1 phenotype or M2 phenotype, regardless the presence of Piezo1. Unstimulated Het macrophages share similarity in path length and velocity with macrophages stimulated with IL-4 and IL-13. Though unstimulated cells have less maximum displacement and rms displacement compare to cells stimulated with IL-4 andIL-13, the difference is not statistically significant. However, the differences are obvious in path length, velocity and displacement between macrophages stimulated with LPS and IFNγ and unstimulated macrophages or macrophages stimulated with IL-4 and IL-13. Macrophages stimulated with LPS and IFNγ migrate the slowest and stick closely to their original positions.

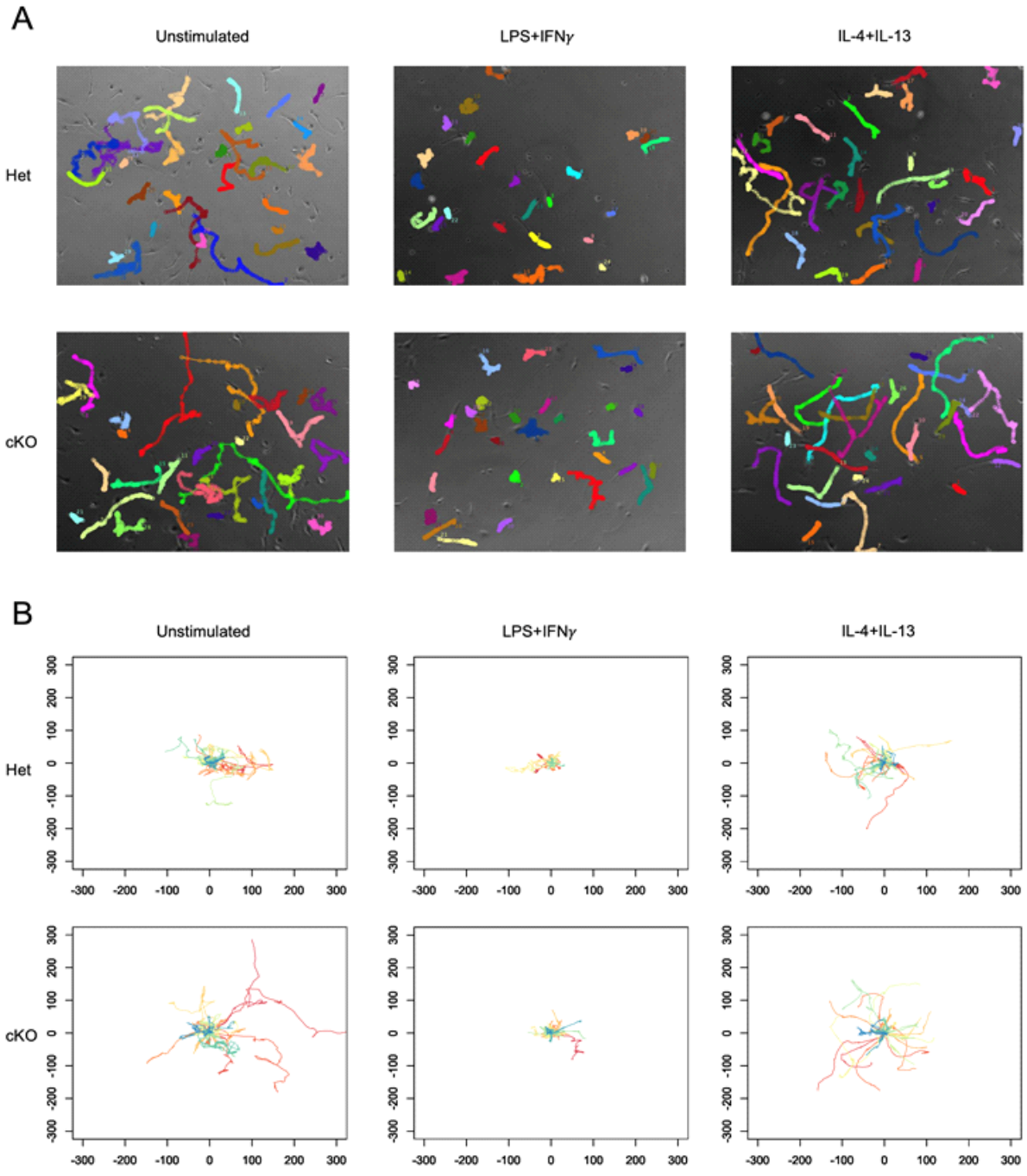


Figure 2.1. Macrophages exhibit different motility patterns dependent on Piezo1 and activation. (A) Representative images acquired from DREAM scope under 10X of different macrophage phenotypes with or without Piezo1, tracks of 30 cells are shown in each condition. Scale bar: 100 μm. (B) Representative displacement plots of different macrophage phenotypes with or without Piezo1, unit: μm.

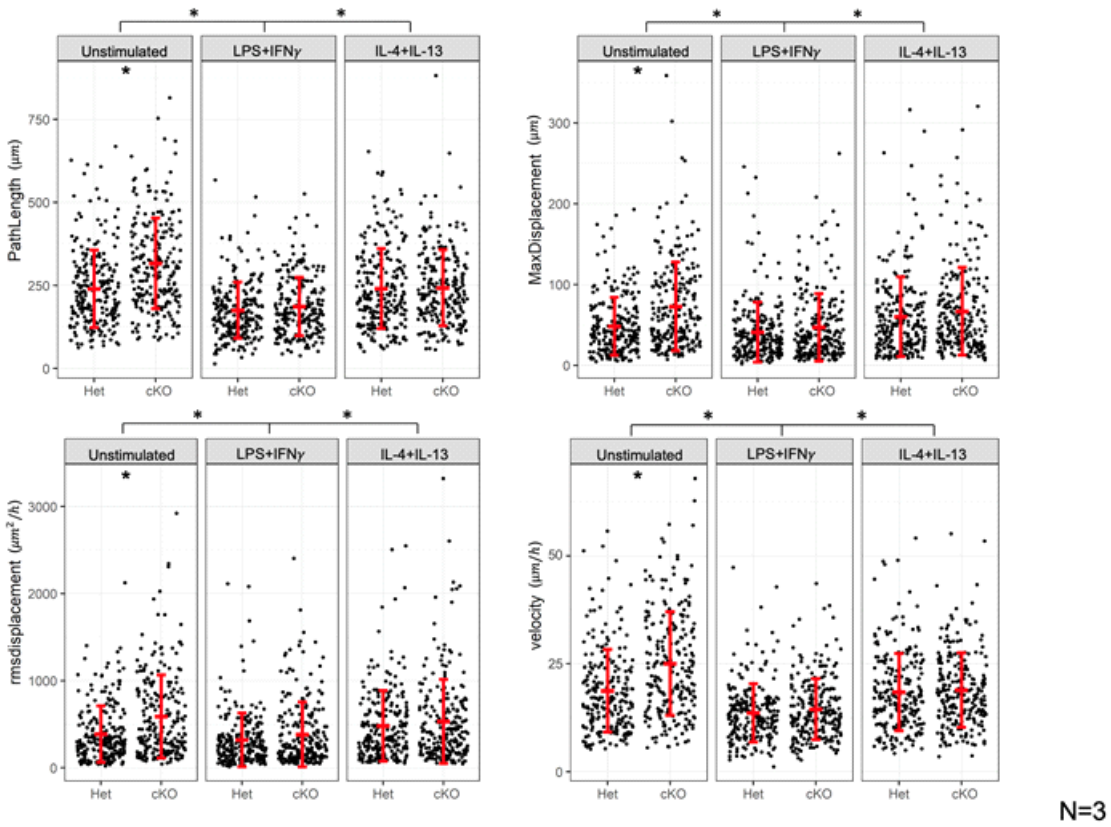


Figure 2.2. Macrophages exhibit different motility patterns dependent on Piezo1 and activation. Scatterplots of path length, max displacement, rms displacement and velocity are shown. For all the plots, bar and plot values are mean ± SEM of n = 3 biological replicates.

2.3.3 *Knockout of Piezo1 leads to differences in integrins expression*

We performed flow cytometry to examine αm, αv, αx, β1, β2 and β3 integrin expression levels in unstimulated Het and cKO cells. We observed some differences in integrins expression in Het and cKO cells, there was an increase in αm integrin when Piezo1 was knocked out, whereas αv, β1 and β2 integrins expression decreased in cKO cells (**Figure 2.3**). However, this experiment was only performed twice, and further studies will be needed to confirm results.

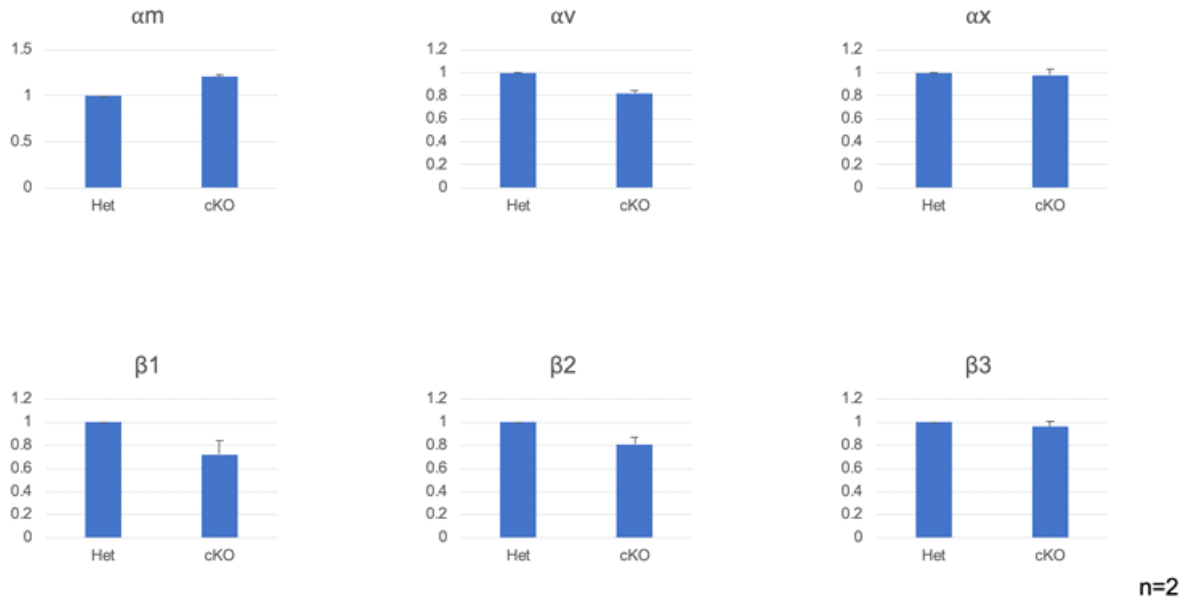


Figure 2.3. Expression levels of αm, αv, αx, β1, β2 and β3 integrins in unstimulated Het and cKO macrophages. The value on y axis refers to relative expression level of integrins in cKO macrophages compares to Het macrophages. The expression levels of Het macrophages have been set to 1, bar values are mean ± STD of n = 2 biological replicates.

2.4 Discussion

Similar to the finding above, several studies have shown the knock down of Piezo1 in other cell type leads to enhanced migration. The loss of Piezo1 in lung endothelial cells causes anchorage independence and increased cell migration, through inactivation of beta1 integrin and reduction in cell detachment [57], which was consistent with our flow cytometry data (**Figure 2.3**). Integrins respond to cytoplasmic signals through changes in integrin-ligand affinity, and integrins functions are modulated by the Ras GTPase family of cytoplasmic signaling proteins, whereas the R-Ras activates integrins affinity and increases cell adhesion [58]. Therefore, integrins and cytoskeleton may play a role in the enhanced migration in macrophages. Although there is an inactivation of beta1 integrin in macrophages and endothelial cells, it does not necessary mean they share the same signal pathway transduced by Piezo1.

Although the absence of Piezo1 increases macrophage migration in unstimulated cells, it does not impact the migration of cytokine stimulated macrophages. This suggests that the enhanced migration due to knock down of Piezo1, which is considered to be mediated by the mechanosensing mechanism of integrins and cytoskeleton, is eliminated due to the polarization induced by the cytokines.

In addition, the unstimulated macrophages have similar motility when compared to macrophages stimulated with IL-4 and IL-13, which are more motile compare to the macrophages stimulated with LPS and IFNγ. This variation in motility may reflect the behaviors of these cells in vivo, macrophages induced by LPS and IFNγ polarize toward a pro-inflammatory M1 phenotype that present in the early stage of immune response, and remain stable at the site to clear pathogens. Also, it has been shown that in disease pathogenesis, M1 macrophages tend to accumulate in the diseased site, such as obese adipose tissue or atherosclerosis plaques [59,60]. In contrast macrophages induce by IL-4 and IL-13 adopt an anti-inflammatory M2 phenotype that may require motility as the tissue remodels. Thus, to maintain the homeostasis, M2 macrophages may require enhanced migratory capacity than M1 macrophages.

To conclude, the mechanisms of Piezo1 in mediating the macrophages motility remains unknown. The signal pathway involving Piezo1, integrins and cytoskeleton in macrophages need to be further studied. The interaction of chemoattractant and mechanosensation is also an interesting aspect to look at.

2.5 Future work

To begin with, flow cytometry that look at integrins expression level in Het and cKO unstimulated macrophages should be conducted, and more integrins can be involved. This will help obtaining a better understanding of how the mechanosensitive molecules mediate the

migration of macrophages, a signal pathway containing Piezo1, integrins and other molecules can be discovered in the future.

Also, integrins expression level in stimulated Het and cKO macrophages is another aspect worth studying further. Het and cKO macrophages will be seeded and stimulated with LPS and IFNγ or IL-4 and IL-13, and let them migrate for 6 hours under microscope, then collect them for flow cytometry. If the velocity of Het and cKO macrophages with same stimuli are similar, like the data showed above, the difference in integrins expression would illustrate that the impact of cytokines on macrophages migration overwhelms the impact of Piezo1 knock out.

Additionally, chemoattractant can be placed inside the chamber slide before the imaging, a chemoattractant dissolved in agarose that can degrade slowly and release it. This would help to study if there is a pattern in macrophages migration. During the migration towards chemoattractant, macrophages may start to polarize, which mimic the real migration in vivo.

To further simulate macrophages migration in vivo, three-dimensional experimental setup is required. A penetrable material like hydrogel can be used to conduct the experiment, Het or cKO macrophages are seeded below the hydrogel, and a chemoattractant is placed on the top of the hydrogel. The cells will be attracted to move upwards due to the chemoattractant.

Chapter 3 The effect of stiffness on Piezo1-dependent macrophage migration

3.1 Background

As cells that respond to mechanical cues in the extracellular matrix, macrophage activities such as adhesion, migration and differentiation, can be modulated by substrate stiffness [61]. The stiffness of tissue varies from 1kPa in brain to 10 kPa in muscle, and ~ GPa for bone [62]. In many physiological diseases, the local substrate stiffness has been shown to be greater than the native healthy tissue. Tissues in many tumors and fibrous scar are 10 times stiffer than healthy tissue, and the presence of calcium deposits in atherosclerotic plaques leads to greater stiffness [63,64]. As a method to mimic the tissue stiffness in vivo, crosslinking the extracellular matrix-based materials can be used to control the stiffness of the substrate. Studies have shown that macrophages generally have enhanced adhesion and migration cultured on stiffer substrates (hundreds of kPa) compared to soft substrates (1-10 kPa) [65], these changes in adhesion that depend on substrate stiffness are generally associated with increased actin and cytoskeleton stiffness. Also, stiffer substrates facilitate polarization of macrophages toward M1 phenotype when stimulated with LPS [66]. Meanwhile, bone marrow derived macrophages that cultured on soft polyacrylamide substrates produce less pro-inflammatory cytokines, which is associated with TLR4 signaling pathway [67]. In addition to macrophages activation, stiffness plays a role in phagocytosis as well, macrophages tend to uptake significantly more hard nanoparticles (3000kPa) than soft nanoparticles (10kPa) [68]. The increase of substrate stiffness also leads to enhanced proliferation and migration [69].

3.2 Methods

3.2.1 Polyacrylamide gel fabrication: To fabricate polyacrylamide gel that can fit into the 8-well chamber slides, coverslips were cut to the size as the chamber. After the gels that

have different stiffness of 1 kPa, 20 kPa, 40 kPa and 280 kPa were made (**Table 2**), gels were placed into the chamber and coated with fibronectin (0.1mg/mL) under 4 degree overnight.

Table 2. Volume needed to make 1 ml of polyacrylamide gel

Stiffness	Acrylamide	Bis-acrylamide	PBS	TEMED	APS
1 kPa	125µl	15µl	860µl	1µl	10µl
20 kPa	200µl	132µl	668µl	1µl	10µl
40 kPa	200µl	240µl	560µl	1µl	10µl
280 kPa	375µl	600µl	25µl	1µl	10µl

3.2.2 Cell culture conditions: Het and cKO cells were seeded into sperate chambers with different stiffness on the next day, with cell density of 40,000 cells/mL (20,000 cells per well) in 500 µL D10 media. The chamber slides were placed into incubator for 6 hours of adhere, followed by 6 hours of live imaging of macrophages migration.

3.2.3 Image acquisition and analysis: Cells in chambers were imaged at 2 minutes intervals for a total of 6 hours using the built-in Multi-Dimensional Acquisition function in Micro-Manager. Two views were selected in each condition, 30 cells were tracked using MTrackJ in ImageJ in each duplicate, cells that went out of view during the video were not tracked. The tracks were further quantified by using an in-house developed Python script by Dr. Tim Smith.

3.3 Results

3.3.1 Macrophages on 40 kPa gels exhibit enhanced migration

Although the displacement plots from **Figure 3.1.B** showed no significance, there are some difference when analyzed in a single cell way. The scatterplots show that cells seeded on 40 kPa gels exhibited greater displacement and velocity compared to cells on other stiffness. Meanwhile, migration of macrophages seeded on 280 kPa gels was not significant comparing to cells on 1 kPa and 20 kPa. Although the representative tracks and displacement plots of cKO

cells on 280 kPa showed significant differences (**Figure 3.1**), the scatterplots (**Figure 3.2**) of n=4 biological replicates exhibited less migration compared to Het cells on 280 kPa.

3.3.2 Improved adhesion is required to observe macrophage migration

From the images acquired from the microscope, conclusions can be made that the cells were not fully adhere to the gels, since the shape of macrophages tends to be similar on these different stiffness gels, which appears to be rounded (**Figure 3.1. A**). Whereas studies investigating the effect of substrate stiffness have shown that the morphology of macrophages are more spread and flattened on rigid substrate, and more rounded on soft substrate [70]. However, the cells seeded on 280 kPa gels were rounded whether Piezo1 was knock out or not.

Thus, to have better conclusions about how substrate stiffness and Piezo1 play roles in macrophages migration, the cells should have better adhesion status. The displacement plots show that the cells were barely moving among the 6 hours of imaging, whereas the macrophages that seeded on glass have significant movement, with displacement up to 300 μm in one direction (**Figure 3.1. B**). Since this process was considered as a status cells trying to adhere, it might explain why there is no significant difference in migratory capacity between Het cells and cKO cells, Piezo1 was regulating the adhesion of macrophages instead of mediating their migration.

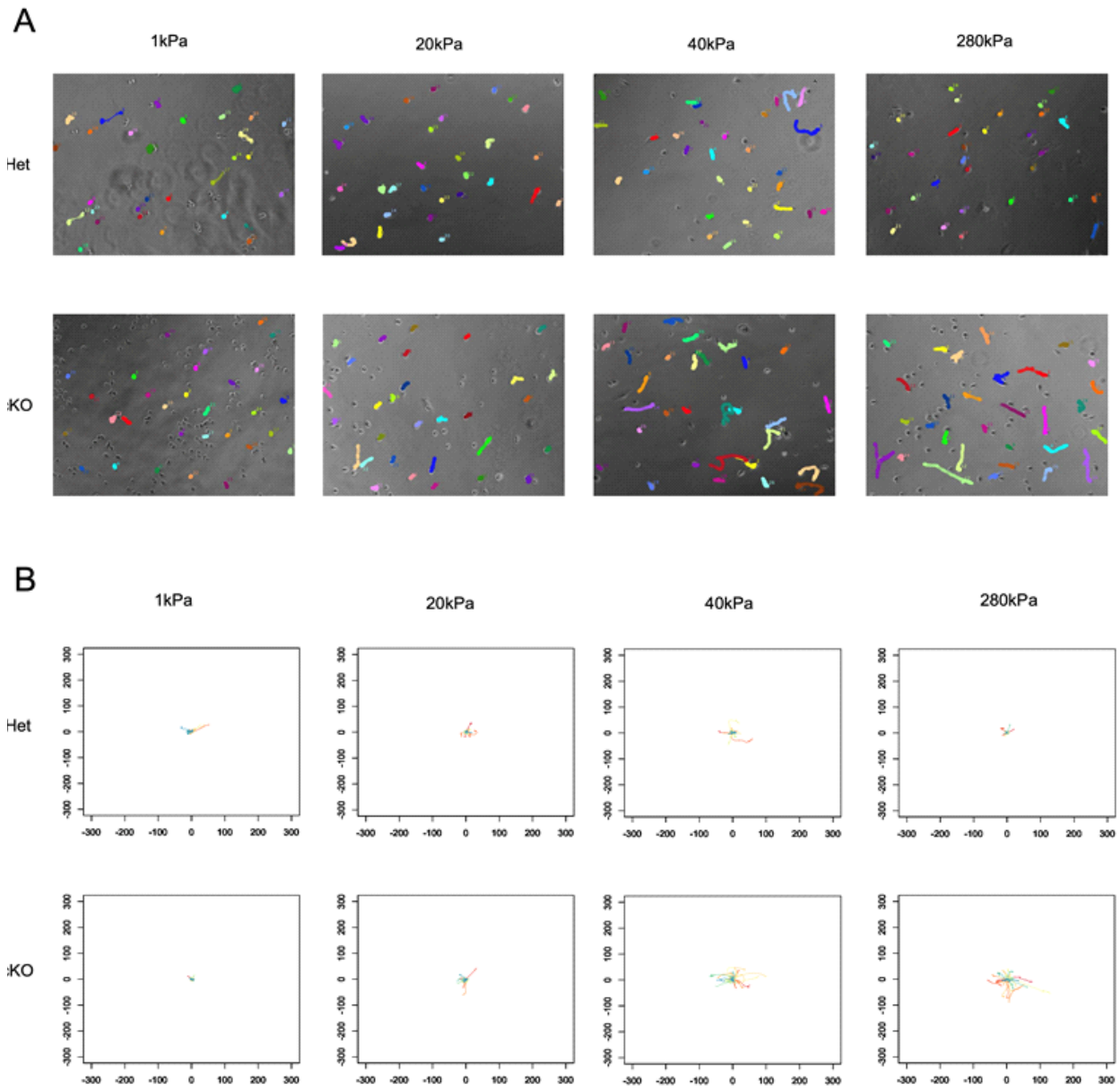


Figure 3.1. Macrophages with or without the presence of Piezo1 exhibit similar motility patterns on different stiffness gels. (A) Representative images acquired from DREAM scope under 10X of macrophages migration on different stiffness gels with or without Piezo1, tracks of 30 cells are shown in each condition. Scale bar: 100 μm. (B) Representative displacement plots of macrophages migration on different stiffness gels with or without Piezo1, unit: μm.

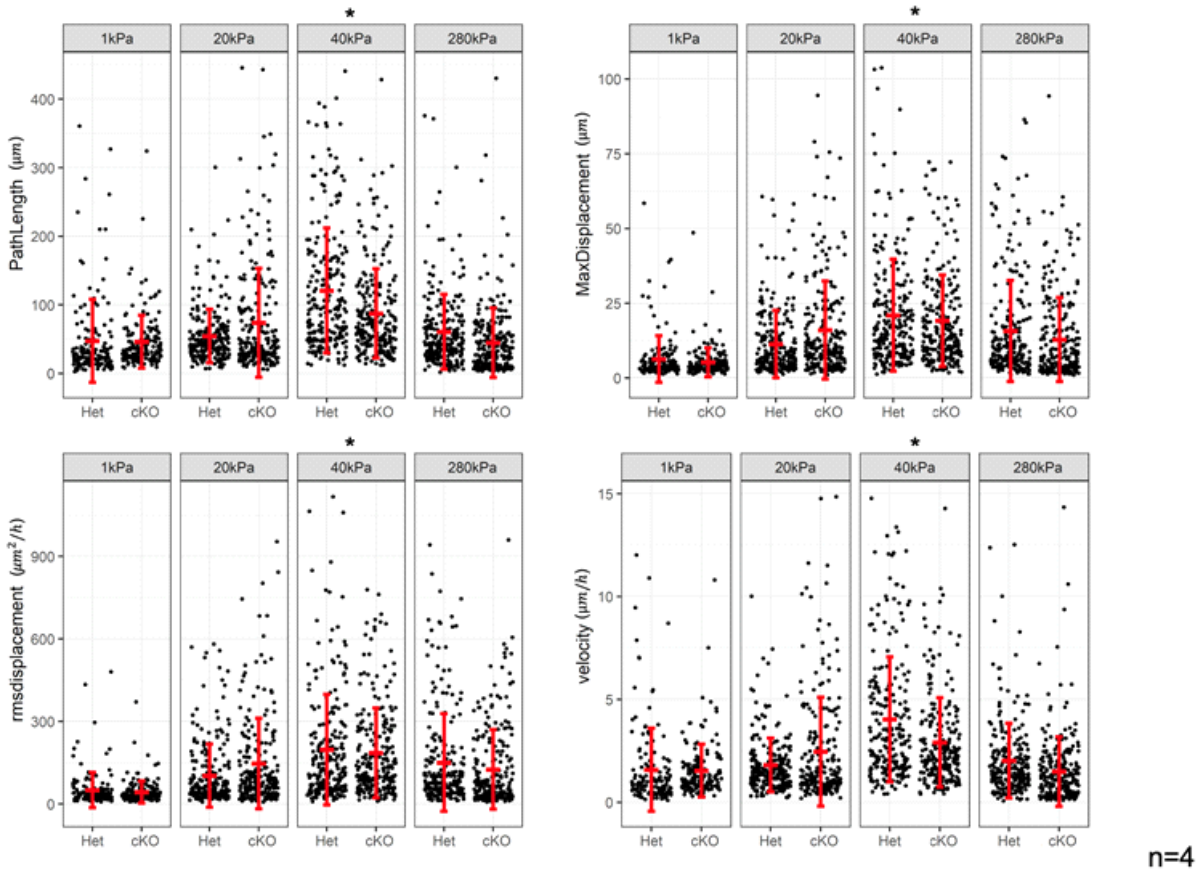


Figure 3.2. Macrophages with or without the presence of Piezo1 exhibit similar motility patterns on different stiffness gels. Scatterplots of path length, max displacement, rms displacement and velocity are shown. For all the plots, bar and plot values are mean ± SEM of n = 4 biological replicates.

3.4 Discussion

Previous study that also looks at how substrate stiffness regulates macrophages behaviors, did 18 hours of live imaging right after seeding, which has longer timeline. However, they observed significant morphological changes in macrophages plated in 280 kPa substrate less than 1 hour, whereas most of the cells in our experiment stayed rounded after 6 hours. Then concluded that cells moved fastest on 280 kPa substrate at a velocity of 12.0 ± 0.5 μm/h, moved slowest on 3 kPa substrate at a velocity of 5.0 ± 0.4 μm/h, and have the velocity of 7.4 ± 0.6 μm/h on 1 kPa, 7.3 ± 0.6 μm/h on 5 kPa and 7.5 ± 0.2 μm/h on glass [71]. Evidently, the migratory capacity does not increase as the substrate stiffness increases, which means cell

behaviors vary from stiffness. Thus, the increased velocity in 40 kPa in our experiment could be explained.

Meanwhile, in a separate study, they found no significant difference in adhesion of RAW264.7 cells seeded onto PEG hydrogel substrate stiffness ranging from 130 to 840 kPa. Then they used bone marrow derived macrophages, and still no significant differences were found. However, with the presence of LPS, the expression of TNF-α, IL-6, IL-1β and IL-10 all increased with increasing rigidity [72].

These differences in observations may be caused by diverse experimental setups such as the source of macrophages, adhesion time for macrophages, the presence of chemokines and the selection of adhesive proteins. To have better understanding of substrate stiffness and migration, the experimental design parameters must be well selected.

3.5 Future work

To begin with, live imaging of Het and cKO macrophages activities on different substrate stiffness after seeding is essential. This would give a general idea of how much time macrophages needed to fully adhere to the different stiffness gels. Also, how these cells respond to the gels would show the role of Piezo1 in regulating macrophage adhesion.

Then, this experiment of Het and cKO cells migration on different stiffness gels can be repeated again. But the adhesion time will be extended based on how much time they need to adhere, before the cells are being live imaging. And the media should be renewed after 24 hours of adhesion, in case of the depletion of media.

What's more, adding stimulations to polarize the cells is worth trying. Base on the previous studies, stimulations may contribute to significant changes where there were no differences before. There could be some differences in the pattern of macrophages migration after the addition of cytokines.

Chapter 4 Ca^{2+} signals regulate macrophage motility

4.1 Background

Ca^{2+} signals have been shown to play an essential role in cell migration ever since 1970s [73]. In leukocyte cells, migration ended due to the depletion of Ca^{2+} in extracellular medium [74]. Also, adding La^{3+} that blocks the calcium channels, would lead to termination of migration [75]. In addition, the intracellular Ca^{2+} signals regulate cell migration as well, movements of cells slowed down as the concentration of intracellular Ca^{2+} went down [76]. While in T cells, there is an elevated cytosolic Ca^{2+} concentration that decreases T cells mobility and anchor the cells to the site of antigen presentation, when T cell receptors engage in discerning cognate antigen [77]. In macrophages, Ca^{2+} signals are shown to enhance macrophages phagocytosis, which is induced by the releasing of ATP [78]. However, the function of Ca^{2+} signals in macrophage migration has not yet been determined. Piezo1, an ion channel that allow the passage of calcium cations, is the most expressed ion channel in macrophage, no role has been identified for Piezo1 channels in macrophage motility involving Ca^{2+} signals.

It has been a technical challenge to have the clear measurement of the amount of calcium in moving cells, however, Dong, Othy et al. genetically engineered mice to produce a new calcium-sensitive reporter protein in cells to overcome this problem. This novel protein, named as Salsa6f, is a combination of a red fluorescent with another protein that glows green when it binds to calcium ions. A measurement of the iii concentration of calcium ions inside the cell can be obtained from the ratio of the red and green fluorescent. Salsa6f is localized to the cytosol, with red fluorescence from tdTomato that reflects fluctuations in cell movement and very low baseline green fluorescence from GCaMP6f that rises sharply during Ca^{2+} signals [79].

4.2 Methods

4.2.1 Cell culture conditions: Macrophages transfected with Salsa6f were seeded into the center well of petri dish (Nunc™ Center Well Dish for IVF, Center well, Thermo Scientific™), at a cell density of 100,000 cells/mL (25,000 cells in 0.4 mL medium). The cells were incubated for 6 hours after seeding. The media was taken out and replaced with 1 mL of calcium solution (2mM), then the petri dish was placed for confocal imaging.

4.2.2 Confocal imaging: For Ca2+ imaging of Cd4+ macrophage cells from Cd4-Salsa6f mice, we used an Olympus Fluoview FV3000RS confocal laser scanning microscope, equipped with high-speed resonance scanner and the IX3-ZDC2 Z-drift compensator (Olympus Corp., Waltham, MA). Diode lasers (488 and 561 nm) were used for excitation, and two high-sensitivity cooled GaAsP PMTs were used for detection. Cells were imaged using the Olympus 40x silicone oil objective (NA 1.25), by taking five slice z-stacks at 2 μm/step, at 2 seconds intervals, for up to 60 min. Temperature, humidity, and CO2 were maintained using a Tokai-Hit WSKM-F1 stagetop incubator.

4.2.3 Image analysis: The migration of macrophages was analyzed in ImageJ using MTrackJ, the tracks were further quantified by using an in-house developed Python script by Dr. Tim Smith. To calculate the amount of calcium events, an in-house developed Matlab script by Hamza Atcha, Andrew Flach and Huy Eng Lim was used. This general logic of this script is to input matrix of green/red mean intensity values, subtract baseline values, use gaussian average to smooth signal, calculate threshold for each cell, use builtin peak detection algorithm to determine if event has occurred.

4.3 Result

4.3.1 *Instantaneous calcium events are negatively correlated with macrophage motility*

All the macrophages in the view of image were tracked, except the ones went out of view while tracking, the trajectories of those cells are random and similar (**Figure 4.1.**). To

amount the numbers of calcium events, two methods were used. Firstly, the instantaneous calcium signals were counted manually on every cell throughout 1 hour of imaging. To make this process easier, each video that contains 1800 frames was separated into 6 substacks. The calcium transients were identified into two types: numerous small and brief spots (referred as sparkles); and less-frequent large, cell-wide transients. After manually counted the calcium events, a Matlab script was generated to calculate the green to red ratio at a specific area in the image, and a filter made by the script based on ration baseline of that individual cell, then some peaks were shown after applying the filter. The analysis was conducted in 10 minutes interval as well, since the cells were migrating, cells might migrate out of the area that circled to calculate green to red ratio. Those peaks were considered spontaneous calcium signals, then were compared to the numbers that obtained from manual counting, the summation of calcium events was further optimized. The calcium events analysis showed that, most of the cells were not considered to have calcium events (55 cells out of 96 cells), only 41 cells had calcium signals and most of them have only one event throughout one hour's live imaging.

The displacement plots are uniform, all of them have the max displacement in each direction up to ~100 μm in one-hour migration (Figure 4.2.A). After acquired the migration parameters, they were correlated to number of calcium events. At first, we analyzed it using all the cells, sample size: 96. However, the trend line was flat and the R-squared value was very low, which made this analysis not reliable. Therefore, we tried to analyze only the cells with calcium events. This would give us a general idea of how calcium signals regulate macrophages motility. After the depletion of cells without calcium events, the trend line began to skew, suggesting that more calcium events would lead to less displacement, rms displacement and decreased velocity (Figure 4.2.B). It shows that the calcium events are negatively correlated to macrophage motility. Nevertheless, this analysis discarded a mass of samples (more than 50%).

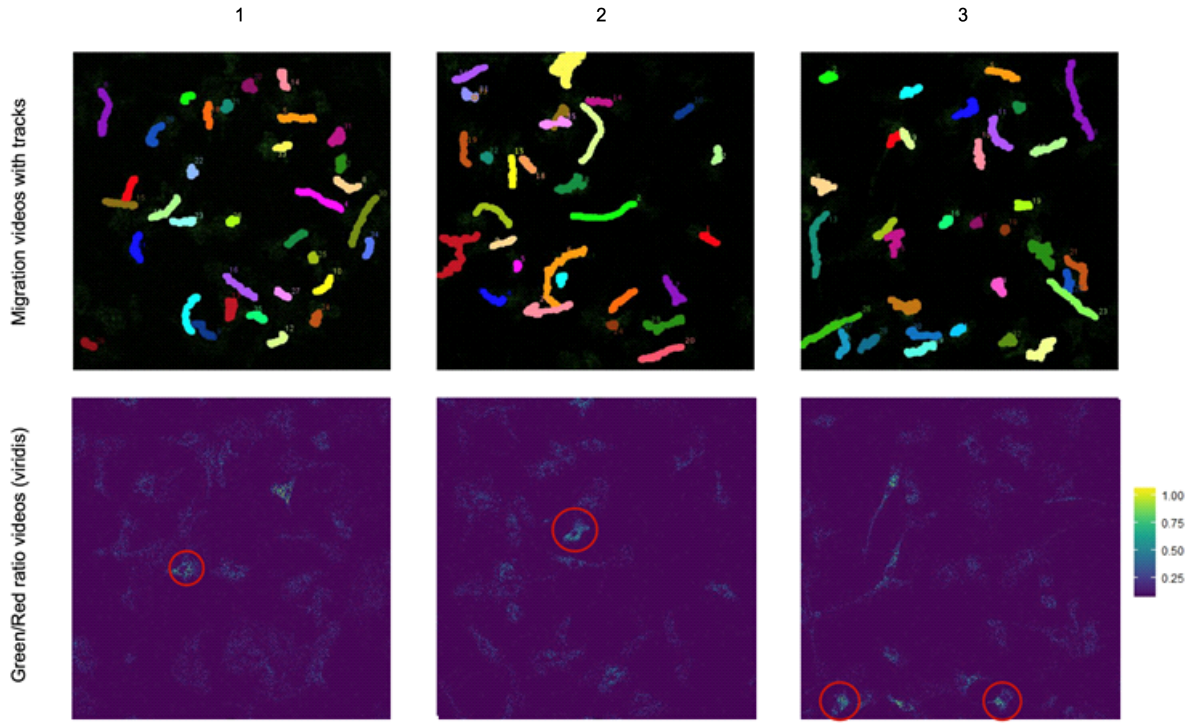


Figure 4.1. Migration trajectories of macrophages and representative instant calcium events. Each column represents an experiment, this experiment was repeated three times. Above is the tracks of 96 cells in three images, these images were at the same condition, no differences in setup. Below are images that have been disposed to have better visualization of spontaneous calcium events. The cells that are circled showed calcium transient at the moment of this frame. Some cells that have higher ratio in these images are not considered having calcium events, because these cells have relatively higher ratio baseline, the baseline varies in every cell.

To obtain a more accurate result with all the samples, the migratory parameters of one-hour were separated equally into 6 substacks, the interval was 10 minutes. The separation gave us a sample size of 576, which was considered sufficient. The analysis of these substacks are similar to the former analysis, the macrophage motility is negatively correlated with calcium signals (Figure 4.2.C). Although the R-squared values of all these trend lines are not significant, conclusion can still be made that calcium signals are correlated with reduced macrophage motility.

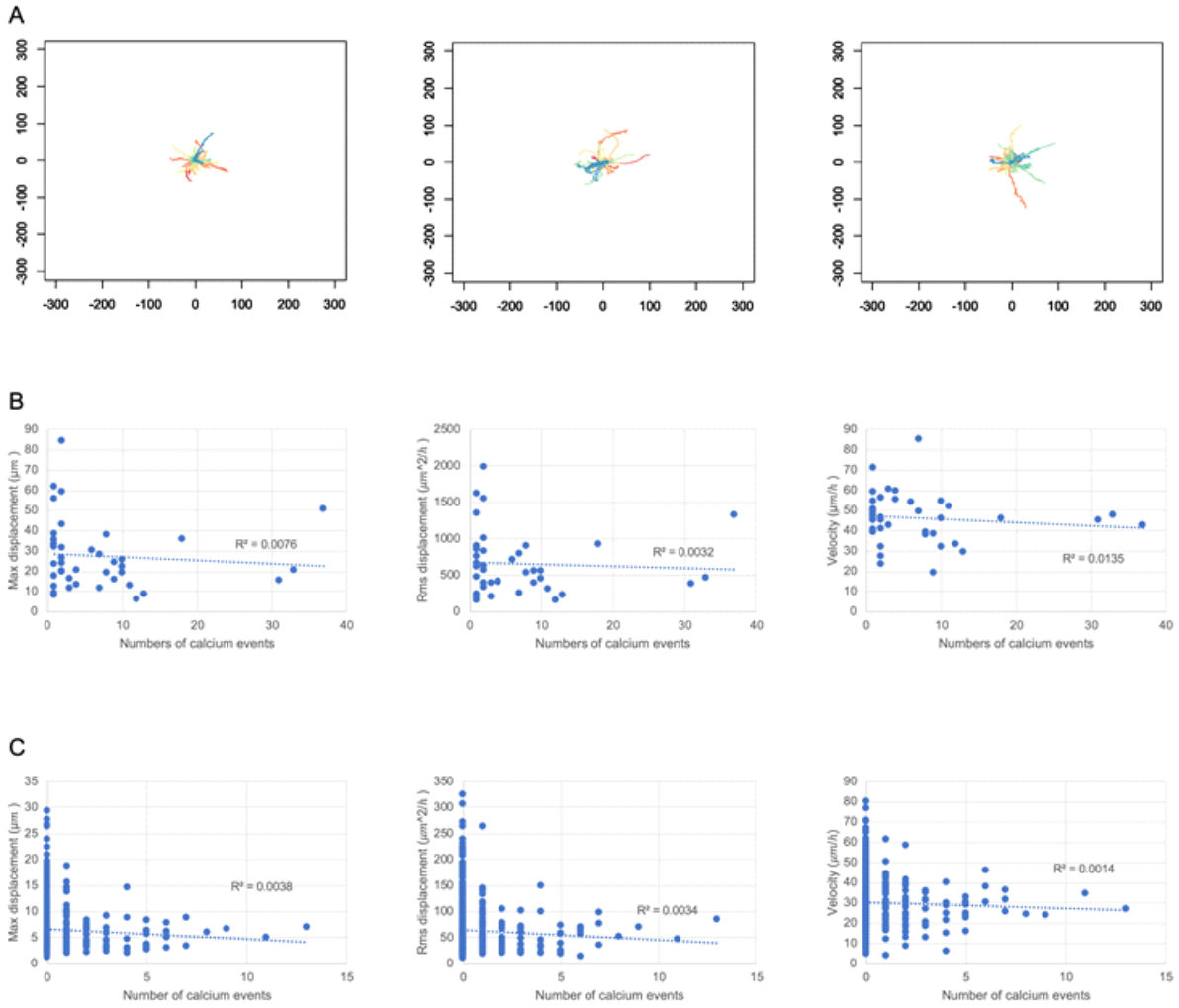


Figure 4.2. Displacement plots and scatterplots corelate migration parameters with numbers of calcium events. (A). The displacement plots for 96 cells in three views in one hour, unit: μm. (B). Scatterplots of max displacement, rms displacement and velocity relate to number of calcium events. Sample size: 41. (C). Scatterplots of max displacement, rms displacement and velocity relate to number of calcium events. Sample size: 576.

4.4 Discussion

Our study showed that Instantaneous calcium events are negatively correlated with macrophage motility, by correlating migratory parameters acquired from ImageJ and the summation of calcium events. However, the definition of calcium signals is considered as spontaneous, whereas these migratory parameters are average or summational. Therefore, the linkage of these numbers may not be accurate. The correlation could be precisely proved if we can acquire the instantaneous velocity, and relate it to the transient calcium signals.

In addition, we observed 41 cells out of 96 cells were exhibiting calcium signals. This suggests that the concentration of calcium solution we used maybe insufficient, less than half of the cells were activated.

In a study investigating the relation between intermittent calcium signals and basal T cell motility, they showed that spontaneous calcium signals during confined motility in vitro are correlated with reduced T cell velocity. The mechanosensitive molecule they investigated was Orai1, an ion channel in T cells. Orai1 allows the passage of calcium cations, and its activity triggers pauses during human T cell motility in vitro in the absence of extrinsic cell contact [80]. The role of Orai1 plays in T cells seems like the role of Piezo1 plays in macrophages.

4.5 Future work

Further analysis of the data to characterize instantaneous velocity will provide more precise information to correlate calcium signals and motility (Dong, Othy et al). In addition, variations in extracellular calcium during live imaging will show how the ion channels respond to different concentration of ions, and how the changes in ion channel activities affect macrophage motility. Finally, comparing the motility of macrophages with or without Piezo1 in calcium solutions, and correlate it to calcium signals, would give us a better understanding of the role Piezo1 plays in macrophages and the downstream effects on calcium signals.

Chapter 5: Summary and conclusion

In this thesis, the role of Piezo1 in macrophage migration was investigated. In chapter 2, we discovered that the knockout of Piezo1 would facilitate the migration of unstimulated macrophages. However, this enhancement has not been shown in stimulated macrophages, which means the motility of activated macrophages is independent of Piezo1. In addition, this regulation of migration may contribute to the activation of integrins. In chapter 3, to have a more conclusive observation of macrophage migration on gels, the cells should be fully adhered to the gels. More adhesion time is required to figure out the how Piezo1 controls the migration of macrophages on different substrate stiffness. In chapter 4, we showed that calcium events in macrophages would lead to impaired motility. As the most expressed ion channel that allows passage of calcium cation, Piezo1 definitely is involved in this regulation. To conclude, Piezo1 conducts calcium cation that reduces macrophage migration, therefore, the absence of Piezo1 in unstimulated macrophages would enhance the motility of these cells. This regulation may due to the differences in integrins activation. The motility of macrophages that had been polarized is independent of Piezo1. However, whether Piezo1 mediates macrophage migratory capacity on different substrate stiffness remain unknown.

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