UNIVERSITY OF CALIFORNIA,
IRVINE

Regulation of Macrophage Function by the Multicellular Microenvironment

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Engineering

by

Ssu-Chieh J. Hsu

Dissertation Committee:
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2021

DEDICATION

To

All my family and friends

in recognition of their love and support throughout all these years

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VITAE

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Field of Study

The regulation of macrophages by the surrounding environment, specifically those mediated by the cell population

Presentation

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Raub CB, **Hsu SC**, Chan EF, Shirazi R, Chen AC, Chnari E, Semler EJ, Sah RL: Microstructural remodeling of articular cartilage following defect repair by osteochondral autograft transfer. Osteoarthritis Cartilage 21:860-8, 2013.

ABSTRACT OF THE DISSERTATION

Regulation of Macrophage Function by the Multicellular Microenvironment

by

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Doctor of Philosophy in Biomedical Engineering

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Professor Wendy Liu, Chair

Macrophages play a key role in the innate immune system, and their activation is tightly regulated to avoid excess and harmful inflammation. Studies have revealed the roles of soluble and adhesive cues in regulating macrophage polarization. The importance of intercellular communication within a macrophage population has also been demonstrated. However, as macrophages become crowded, the increasing cell density could alter the surrounding soluble and adhesive environments, as well as the coordination within a population. How these changes in the multicellular microenvironment affect macrophage functions is not well understood.

In my dissertation, I investigated the effects of the multicellular environment and its alteration on macrophage functions, characterizing how these factors affect paracrine signaling at macroscopic and microscopic levels, as well as how it could affect the physical interaction of cells. We characterized the regulatory effects of density-dependent intercellular communication on macrophage populations by stimulating cells in bulk, in small groups, and in isolation. We also assessed how cell crowding affects the function of a mechanosensitive ion channel, Piezo1,

as this channel is important for the physical sensing of macrophages. We found that macrophage activation depends on the communication among the cell population, and both seeding density and absolute cell number contribute to the resultant responses. In addition, Piezo1 is found to contribute to the crowding-dependent regulation of macrophage activation. These works demonstrated that altering cell density could have broad-spectrum effects on macrophage functions. As a result, the impact of seeding density and its impacts on the multicellular environment should not be overlooked when designing an experiment that uses macrophages.

Overall, this study expanded our knowledge on how the macrophage population could regulate cellular functions by altering their surrounding soluble and adhesive environment in a density-dependent manner. This work could help explain the discrepancy in experimental results that can occur between different studies, as well as among different culture systems. Furthermore, this density-dependent regulation of macrophage activation may represent a novel regulatory mechanism with meaningful impacts toward cellular functions *in vivo*. Future work would focus on understanding how cell crowding could affect other aspects of cellular processes, and how these effects could further modulate macrophage functions in both *in vitro* and *in vivo* settings.

CHAPTER 1: Macrophage Biology, Heterogeneity, and Coordinated Responses

1.1 Macrophage plasticity

1.1.1 Macrophages and their functions

Macrophage is a versatile immune cell that performs a myriad of supportive functions. This cell type is best known as an integral part of the innate immune system, surveying for invading pathogens and exerting defensive responses when threats are detected. To perform their duties, macrophages physically phagocytose pathogens such as bacteria, secrete cytokines to coordinate immune functions, and in the later phase, serve as antigen-presenting cells to aid in the adaptive immune response(1). On the other hand, macrophages are integral in the process of wound healing, collaborating with other cell types such as fibroblast to promote tissue regeneration(2). Important homeostatic functions, such as clearing of dead cells and debris, also rely on macrophages to perform these tasks(3). The diverse functions of macrophages are continuing to be discovered. In a recent study, macrophages were shown to facilitate the conduction of electrical signals in the heart(4). These properties make macrophages an important regulator of biological functions within an organism. Therefore, functional abnormality in these cells can contribute to diverse pathological conditions, including atherosclerosis, different types of autoimmune diseases, cancers, and more(5). The importance of macrophage functions, and the potential adverse outcomes of their dysregulation, warrant an in-depth understanding of the underlying principles governing macrophage biology.

1.1.2 Environmental cues that modulate macrophage responses

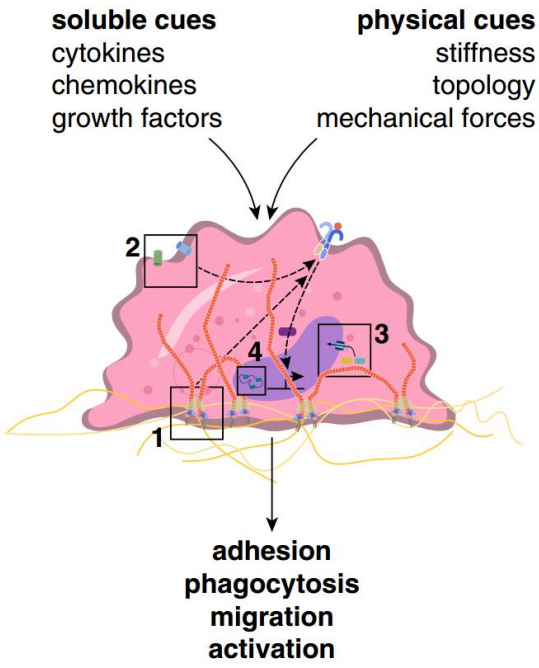

Figure 1.1: Factors that can affect modulate macrophage responses [Adapted from (9)]

Macrophages are exquisitely sensitive to their surrounding environments, which aid them in performing tasks that necessitate different functions in different contexts. One major category of cues that macrophage sense is biochemical stimuli, which are often presented as soluble factors in the surrounding environment (Figure 1.1). To sense these cues, macrophages are equipped with a diverse mix of receptors capable of detecting different biochemical stimuli, from danger signals to those discriminating friends from foes. One set of tools is pattern recognition receptors, which include various Toll-Like Receptors (TLRs), C-type lectin receptors (CLR), and Scavenger receptors(6). These receptors are used to survey surrounding extracellular environments for signs of foreign invaders, which would then activate macrophages to initiate immune responses tailored to the types of pathogen detected(6). In addition to receptors targeting pathogens, other mechanisms, including those targeting different types of cytokines and chemokines, also instruct macrophages to migrate to areas needing immune defense, exert defense mechanisms toward the outside dangers, or provide healing to the surrounding tissues(1,7). One of the cytokines being targeted is interferon-gamma (IFNγ), which primes macrophage to activate in response to pathological ligands(7). These signals, which are often presented in soluble formats, help macrophages to

respond to some of the environmental cues and exert necessary action to protect the organism and perform other vital functions.

In addition to biochemical factors, macrophages also respond to nearby physical cues (Figure 1.1). Increasing works have highlighted how factors including substrate topography, cell shape, and substrate stiffness could modulate macrophage functions(8,9). Many of the findings on topological cues originate from works that probe the effect of implanted materials on the host immune responses. In one study, macrophages cultured on rough surfaces exhibit a stronger pro-inflammatory response following activation with a bacterial ligand, lipopolysaccharide (LPS)(10). Other works have also demonstrated that in a 3D setting, porous materials induced less foreign body responses when implanted subcutaneously in a mouse model(11). To further study the role of topological cues, several engineering systems have been developed to better control the substrate geometry. One category of systems involved patterning the surface with different geometries, such as grooves with different widths. These systems revealed that on grooved surfaces, macrophages exhibited reduced inflammatory responses and promoted the expression of pro-healing markers including Arg1 and IL10(12). Similar responses were also observed in a different engineering platform, in which electrospun microfibers of different diameters were used to better mimic the physiological settings(13). These studies demonstrated that compared with planar materials, fibrous substrates again promote better healing responses.

Interestingly, macrophages exerting pro-healing phenotypes often assume a more elongated shape when compared to cells with more pro-inflammatory phenotypes(14). To address the relationship between cell shape and macrophage polarization, our lab modulated macrophages shapes by imprinting stripes of adhesive proteins onto the material surface, which causes cells to spread into the conformed geometry(14). The study found that elongated

macrophage expresses more of the pro-healing markers, Arg1, and exhibited attenuated inflammatory activation when stimulated with LPS and IFNγ. The shape-induced effect appeared to involve cytoskeletal contractility, as various cytoskeletal inhibitors abrogated the increased expression of Arg1 observed for cells on patterned surfaces.

Besides topography, mechanical properties, including substrate stiffness, mechanical stretches, and applied pressures, also modulate macrophage activations. Depending on the tissue types, macrophages can experience diverse mechanical environments, including tissues with different stiffness, as well as the presence of fluid shear, cyclic stretches, and pressure as in blood vessels and lungs(15). Recent studies have suggested that substrate stiffness can regulate different macrophage functions. Cells seeded on stiffer surfaces assumed more rounded shapes at lower stiffness and spread out more with flatter shapes at higher stiffness(16). Cells also exhibit increased adhesion(16), migration(16), and phagocytosis(17). In addition, several studies found that stiffness modulated macrophage activation, with higher stiffness contributing to stronger activation following pro-inflammatory stimulations *in vitro*(18–21). In the *in vivo* setting, implantation of stiffer hydrogel in mouse models led to more recruitments of macrophages and more prominent foreign body responses(20). Yes-associated protein (YAP), a mechanosensitive transcriptional factor, contributes to the modulation of stiffness on macrophage activation(19). However, there are also conflicting results on the effect of stiffness(22) and more work is needed to clarify the discrepancy between them.

Other mechanical properties, including mechanical stretch and pressures, also influence macrophage functions. Mechanical stretches promote cells to adopt an elongated shape, as well as an increase in the expression or secretion of certain inflammatory markers(23,24). However, there are also studies reporting conflicting effects of stretches on macrophage activations(25).

Recently, a report found that cyclical hydrostatic pressure increases the inflammatory responses of macrophages via a Piezo1 and EDN-1 mediated mechanism(15). In this case, cyclic hydrostatic pressure mediates a Piezo1-mediated calcium signaling that drives the transcription and secretion of endothelin-1, which in-term promote stabilization of hypoxia-inducible factor 1a (HIF-1a) through paracrine signaling to exert its pro-inflammatory effects. Although recent studies have helped clarify the roles of mechanical properties in modulating macrophages behaviors, more studies are needed to better elucidate the underlying governing mechanism and clarify the conflicting results observed in some studies.

1.1.3 Macrophage polarization

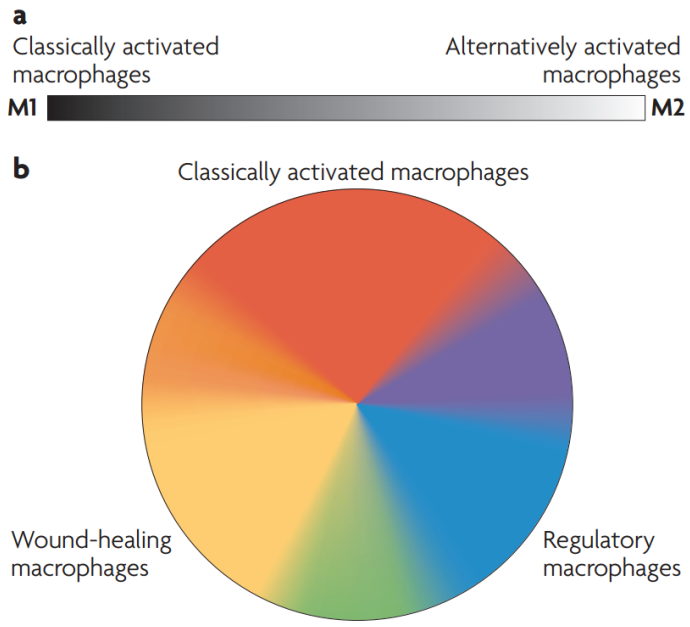

Figure 1.2: Macrophage can exert significant variations of phenotypes, in addition to the traditional M1 M2 dichotomy [Adapted from (7)]

While macrophages can sense different chemical and physical stimuli, they are also known to exert diverse functions in response to different needs. In *in vitro* conditions, macrophages are known to polarize into two opposite phenotypes. These phenotypes at the extreme ends are usually referred to as M1 and M2, mirroring toward the TH1 and TH2 paradigm used to describe T-cell activation(26). Under signals including lipopolysaccharide (LPS) and interferon-gamma (IFNγ), cells can activate into a pro-inflammatory M1 phenotype. This is characterized by secretion of pro-inflammatory cytokines such as TNFα and IL6, as well as expression of markers related to

inflammation and immune responses, including iNOS and CD86(27). On the other hand, following with stimulation with interleukin 4 (IL4) and interleukin 13 (IL13), which leads to TH2 responses for T-cells, macrophages can exhibit pro-healing M2 phenotypes that suppress inflammation and promote repairing of the tissues(28). These M2 macrophages are often characterized by high expression of arginase 1 and mannose receptor CD206, as well as upregulation of certain cytokines such as IL10 and TGFβ(28,29). Additional studies have found that under specific stimulations, the M2 phenotype of macrophage can be further separated into different subtypes, M2a, M2b, M2c(27,29). These subtypes facilitate different immunoregulation and pro-healing functions.

On the other hand, macrophage polarization *in vivo* is often more complex than the simple M1-M2 dichotomy. The current consensus agrees that macrophage polarization is viewed as a spectrum, in which cells can exhibit both pro-inflammatory and pro-healing markers(7). Indeed, subjecting macrophages to both pro-inflammatory and pro-healing stimuli can produce cells with mixed activation states(30,31). There are also reports on the existence of macrophages expressing both M1 and M2 markers in pathological conditions such as cancer(32). Finally, macrophage polarization can be a dynamic process. Studies on macrophages subjected to mixed M1 and M2 stimulation observed that M1 makers gradually decreased with time, while M2 markers exhibited an opposite trend(31). This result seems to agree with the trend in wound healing, in which pro-inflammatory macrophages important for host defense are gradually replaced with pro-healing macrophages over time(2). Overall, the diverse phenotypes that macrophages can exert in response to different combinations of stimulations exemplify their ability to perform many different functions. Further works on characterizing and modulating

macrophage polarization could yield more insights on not only the innate immune systems but also clues to address multiple pathological conditions.

1.2 Macrophage Heterogeneity and Coordinated Control of Macrophage Population

1.2.1 Heterogeneity within a macrophage population and its implication

As macrophages are essential in the host defense and their dysregulation has been implicated in many diseases, better understandings of the macrophages population and their functions could help identify novel therapeutic targets. Advancement in single-cell technology has enabled researchers to explore the heterogeneity within macrophage populations, providing new insights on macrophage functions in both healthy and diseased states. In one study, a study explored macrophage phenotypes in two mouse models of atherosclerosis and identified three macrophage subsets in the atherosclerotic arteries, including a previously unidentified TREM2^{hi} (triggered receptor expressed on myeloid cells 2) population(33). This subtype exhibited features associated with lipid handling and catabolic process, and the subpopulation is also speculated to have specialized functions in atherosclerotic lesion calcification. Similar studies on different physiological or pathophysiological conditions could help clarify how different subpopulations of macrophages contribute to these processes, which could potentially help identify novel targets for treatments.

On the other hand, single-cell analysis on a relatively homogeneous population of macrophages also yields strikingly variations across all aspects of biological processes. Transcriptomic and proteomic analysis on U937 monocytic cells differentiated in identical conditions revealed that these macrophage-like cells exhibit a continuum of phenotypes(34).

Interestingly, within this continuum of cells, genes associated with M1 and M2 polarization are reciprocally expressed. The heterogeneity of differentiated U937 cells was also evident in their cytokine secretion. Using a single-cell barcode chip, Xue et. al. unveiled that following stimulating with LPS, a small subset of high-secreting cells exists and is responsible for producing significant amounts of certain cytokines such as TNFα(35). This trend was also observed in primary monocyte-derived macrophages(35). Another study examined single-cell responses toward mixed M1/M2 polarization signals and found that cells in general exhibit distinct global transcriptional programs(30). However, for certain sets of genes belonging to the core gene programs specific to LPS and IFNγ (*Il6* and *Il12b*) or IL4 (*Arg1* and *Chil3*) activation, many of the cells would only express either one set or the other. This results in increased heterogeneity of the cell population. The sources of such heterogeneity remain to be investigated, and future work would aim to understand how stochastic or deterministic processes could contribute, as well as the significance of cellular heterogeneity in macrophage functions.

Recent works revealed that different signal transduction dynamics contribute to the phenotypic and functional heterogeneity in a population of macrophages. One of the master immune regulators is the NFκB pathway, which itself exhibits considerable heterogeneity in its signal dynamics. One study explored the heterogeneity in the NFκB signaling following LPS activation, as well as the subsequent effects on cellular phenotype and function. The result categorized cells into three subpopulations with distinct types of NFκB signaling dynamics, and it suggested that each type of signaling dynamic is associated with distinct profiles of gene expression patterns(36). Signaling pathways upstream of NFκB also contribute to cellular heterogeneity. Cheng and colleagues explored the TLR4 pathway upstream of the NFκB transduction, whose activation depends on two adaptor proteins, Myd88 and TRIF. They found

that while Myd88 functions as a digital switch and activates consistently across the population, the TRIF adaptor exhibits more variability in its signaling dynamics(37). The results suggest a model in which Myd88 activation leads to a reliable, digital initial response for NFκB while TRIF contributes to extended activation of NFκB in a subset of cells with variable levels to adapt to the magnitude of the threat. Although the effect of TRIF on NFκB signaling dynamics is challenged in a subsequent study, TRIF nevertheless does help control some inflammatory processes, such as the production and secretion of TNFα(38). Thus, the variabilities in the signal transduction dynamics contribute to cellular heterogeneity, and such variations may help tune the overall responses.

Besides signal transduction dynamics, different baselines states of macrophages also contribute to heterogeneity in macrophage functions. In one study, Chen and colleagues used single-cell RNA-seq and a single cell barcode chip to examine transcriptional and secretion variations within a macrophage population following the TLR4 pathway via lipopolysaccharide (LPS) at different time points(39). They found that there existed two major activation states of cells, and the protein secretion data suggested that the activation states are dependent on the respective baseline status. This finding was supported by another study that investigated the NFκB dynamics and TNFα transcription before and after LPS stimulation using two reporter systems, an EGFP-RelA fusion protein to track NFκB dynamics and a mCherry based reporter to monitor TNFα transcription (40). The result also indicated that two activation states existed following pro-inflammatory stimulations, and it suggested that basal NFκB level likely contributed to the resultant differences in activation states. With increasing knowledge on macrophage heterogeneity in both basal and activated states, understanding how these variations

contribute and coordinate the overall cell population response may yield more insights on the regulation of macrophage functions.

1.2.2 Roles of cellular coordination in macrophage functions

Macrophages exhibit tightly controlled functions during inflammatory activation, as excessive activation can damage the host tissue. Thus, it is remarkable that a highly heterogeneous macrophage population can generate such coordinated actions. Recently, studies adopting the use of single-cell analysis increasingly suggests that coordination within a group of immune cells contributes to the overall responses of the whole population(41). In one study utilizing U937-derived macrophage-like cells, Xue and colleagues found that following LPS polarization, cells in isolation secreted significantly less IL6, IL8, and IL10 when compared with cells in the population(35). Our group also performed similar studies on bone marrow-derived macrophages and found that MCP1 secretion was significantly less for cells in isolation than in a population(42). As isolated cells lack communication with neighboring cells, these results suggest that homotypic interaction, whether through shared secreted factors or physical interactions, is necessary for the full activation of macrophages.

Emerging evidence supports the role of secreted factors in coordinating the collective actions of an immune cell population. A study on dendritic cells, a type of cells important for the innate immune system, demonstrated that following activation with LPS, a subset of precocious cells is responsible for coordinating the expression of antiviral-related genes within the population(41) (Figure 1.3). Similarly in macrophages, TNFα secreted from a small percentage of high-responder cells mediate the larger secretion of IL6 and IL10 in the cell population(35). These paracrine signals not only contribute to the activation of the population but also regulate

the magnitude of the response. For example, IL10 has been found to restrain the subpopulation of the low-responding macrophage from activating, as well as to resolve the inflammatory responses from the highly activated cells(43). As these paracrine interactions hold importance in modulating macrophage functions, the consequences of their dysregulation have also been implicated. In a study that evaluated the potential of IL23 targeting therapies in treating inflammatory bowel diseases, the researchers found that autocrine/paracrine signaling of IL10 and IL1 exerted significant controls in modulating IL23 production(44). Based on this finding, they suggested that IL1-targeting therapies could potentially benefit patients with defects in IL10 signaling. Overall, these soluble factors-based intercellular communication may present a strategy to enable reliable responses out from heterogeneous populations, and even present benefits for their functions(45). Likewise, a better understanding of these paracrine mechanisms and how their dysregulation contributed to pathogenic outcomes will help guide the applications of relevant treatment plans.

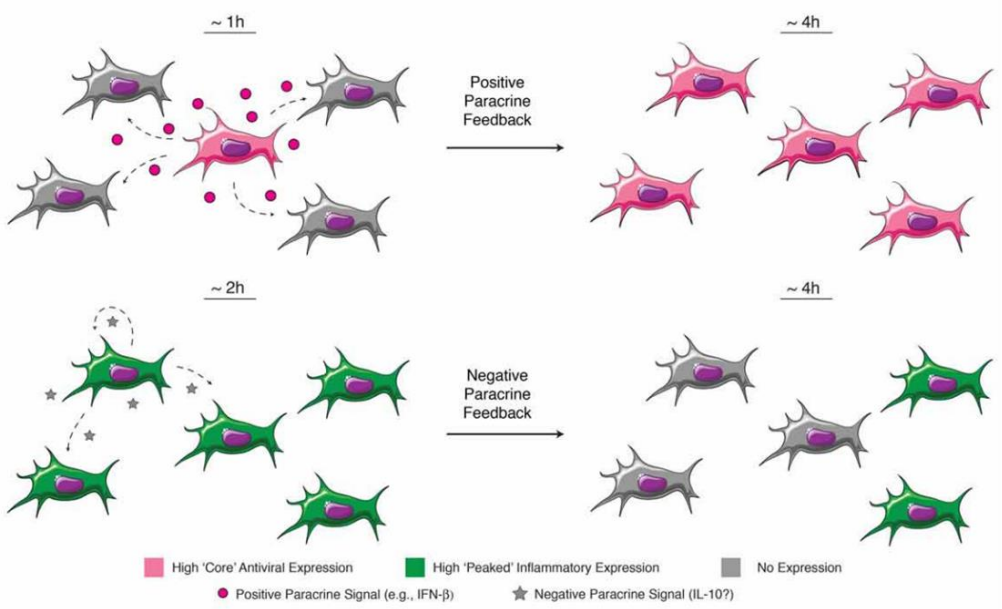

Figure 1.3: a schematic describing how small subpopulations of dendritic cells can exert important effects in regulating overall activation of the whole population. [Adapted from (41)]

On the other hand, more efforts are focusing on identifying the secreted products contributing to the collective responses of a heterogeneous population. Some of the cytokines with well-known autocrine and paracrine effects are TNFα, IL10, and interferon-beta (IFNβ). As mentioned earlier, paracrine signaling of TNFα contributes to the inflammatory responses, while IL10 dampens it(35). TNFα is one of the major pleiotropic cytokines produced by macrophages during pro-inflammatory activation, and it is also responsible for inductions of several pro-inflammatory cytokine cascades(46). Interestingly, the autocrine or paracrine function of TNFα may depend on the dynamics of the signaling pathway, suggesting a context-dependent role of TNFα in coordinating the population-level response(47). On the other hand, IL10 is the major immunosuppressive cytokine that imposes potent negative feedback during inflammation. As mentioned previously, it appeared to exert different controls to different subsets of macrophages with varying levels of activation(43). Interferon beta is a cytokine that could both promote and inhibit inflammation. On one end, IFNβ could work synergistically with TNF to promote inflammation(48). On the other end, it also augments IL10 secretion and suppresses macrophage activation above a threshold of LPS stimulation(49,50). Thus, the examples of IFNβ highlight how crosstalk among different secreted factors could produce complex regulatory mechanisms. Besides these cytokines, more secreted factors with autocrine/paracrine roles are being discovered. Some examples include ApoE, PGE2, and even small molecules like ATP(51–54), which interestingly facilitate intercellular calcium propagation. Future discoveries on more paracrine factors, as well as their potential crosstalk, would help uncover the diverse processes that modulate macrophage functions at the population level and possibly identify potential targets for new therapies.

1.2.3 Recent development in tools for investigating macrophage heterogeneity

Many of our understandings on macrophage plasticity and polarization relied on experiments conducted using bulk cell culture and assessed with techniques that give an average measurement for the desired parameters. These approaches have been useful, contributing invaluable insights into the phenotypes and functions of macrophages. However, bulk measurements have limitations. Average measurements are unable to reflect the variations in individual cellular states, which could provide greater mechanistic insights underlying a given process. Until recently, there were only a limited number of available single-cell techniques, including patch-clamp electrophysiological techniques, immunofluorescence microscopy, ELISpot, and flow cytometry(55). These techniques provide great tools in understanding macrophage electrophysiology, signaling dynamics important for macrophage activation, cytokine secretion in response to stimulation, and characterization of macrophage phenotypes at the single-cell level. Nevertheless, shortcomings such as lack of multiplexing capability limited their ability in elucidating broader variations within the cell population(55). In recent years, there are major progress in the tool developments to perform single-cell analysis at both genetic, proteomic, and functional levels, and approaches to enable multi-omics analysis are also emerging. These innovations will likely enable us to understand macrophage heterogeneity and its impacts in details that have never been seen before.

One major function of macrophages is secreting cytokines that perform diverse jobs such as immune and inflammation responses, as well as promotion of wound healing(27,29). Traditionally, cytokine level has been measured with enzyme-linked immunosorbent assay (ELISA), which only provides average values of secreted factor concentrations. Techniques such as ELISpot and intracellular flow cytometry coupled with the use of brefeldin A enable assessing cytokine production at the single-cell level. However, ELISpot suffers from the lack of capability

for quantitative measurement, and the use of brefeldin A in intracellular flow cytometry could alter cellular responses.

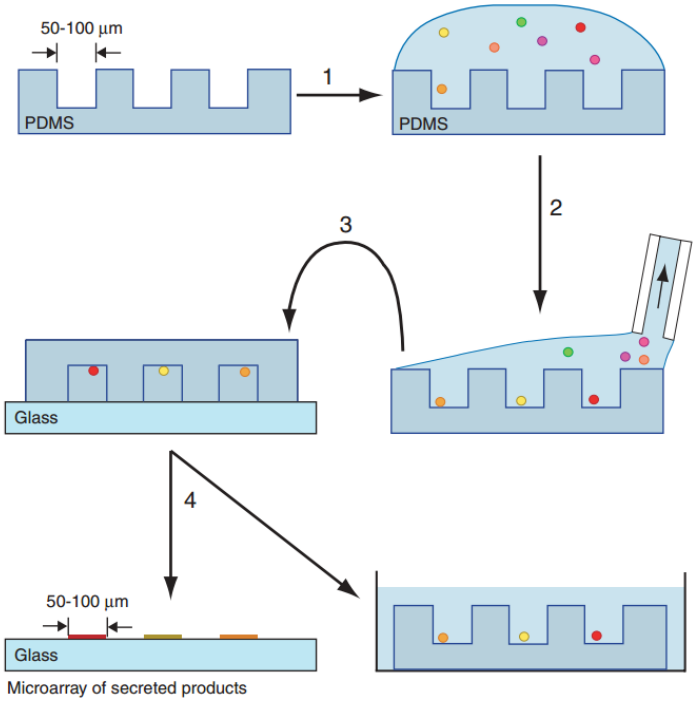

Figure 1.4: Microengraving technique developed to probe cytokine secretion at single cell level [Adapted from (56)]

In response to these shortcomings, Love et al. developed a microengraving technique (Figure 1.4), which utilized a microfabrication-based approach to enable quantitative measurement cytokine secretion without the need for brefeldin A(56). In this process, cells were seeded into arrays of nanoliter- to picoliter-sized microwells, treated with stimulants if needed, and then sealed with a glass slide coated with antibodies to detect solutes of interest. At the end of the experiment, the detection glass slide was separated from the cell-containing microwells, probed with fluorescently tagged antibodies against the captured solute, and scanned under a microscope. The final image would consist of arrays of fluorescent spots representing the concentration of the targeted solute in each well. With modifications, this technique enables measurements of two or more cytokines in an experiment(57). The adaptation of a quantum dot-based detection mechanism can significantly increase the sensitivity in protein detection(58). On the other hand, by modifying the geometry of the microwell, our lab developed a version of the system that enables the study of cell shapes on macrophage activation at the single-cell level(42).

The original microengraving technique suffers from the limited capability to multiplex. This shortcoming was addressed with the introduction of an antibody barcode array(59,60) (Figure 1.5). Instead of using different colors to differentiate distinct cytokines, the position of the detection antibody on the barcode was used. Combining this approach with microwell arrays, this new system can detect up to 15 proteins using one color channel and up to 45 proteins using three channels(61). The system was capable of measuring thousands of single cells in a single experiment, and the throughput could be increased by decreasing the numbers of cytokines for detection(39). These advancements in measurements of single-cell cytokine secretion enable better profiling of immune cells and allow us to better understand macrophage heterogeneity and its importance in cellular functions.

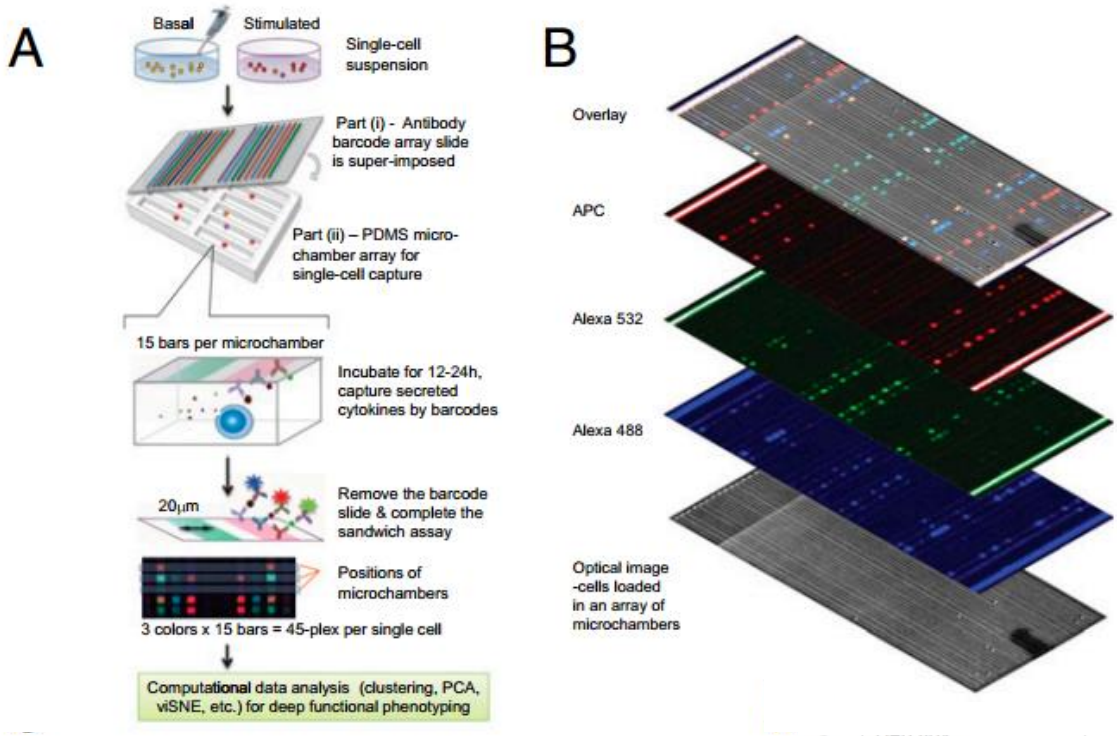

Figure 1.5: Adaptation of the antibody barcode array techniques to enable highly multiplexed measurement of cytokine secretion [Adapted from (61)]

1.3 Cellular Density/Crowding as a Regulator for Macrophage Function

1.3.1 Regulatory roles of cell density/cell crowding in other cell types

Cell-cell interaction, whether through physical contacts or soluble factor signaling, is important in shaping macrophage responses within a population. These interactions can be altered by changes in the surrounding microenvironment, and some of these changes can be due to cell-intrinsic features. One of these features is cell density, which can vary and subsequently influence cell functions. Indeed, one study has examined how the degrees of culture confluency affect bone marrow mesenchymal stem cells and found that viability, proliferation, and osteogenic differentiation potential vary with the level of confluence(62). Seeding density can alter the behavior of the cell population by changing both the physical and soluble environments within the cell population, and emergent studies have begun to identify the associated signaling pathways and the underlying mechanisms.

Recent studies are suggesting that physical crowding can affect the behaviors of the cell population. In a dense culture, there are increased cell-cell contacts and reduced cell spread, and both factors could alter cellular interactions. One work compared cells in the spare and dense regions of the culture and found that crowding altered the endocytosis, virus infection, and lipid composition of cells(63). Subsequent studies implicated the role of focal adhesion kinase (FAK) in some of these observations(64). FAK is observed to have higher levels of activation in spare culture, where cells can spread out more and have more focal adhesion sites. The higher activation of FAK led to suppression of ABC transporter A1 (ABCA1), which can alter the physical properties of a lipid bilayer. Thus, cell crowding could reduce FAK activation and contribute to a stronger expression of ABCA1, resulting in changing membrane properties when

compared to sparse culture(64). This effect, together with other changes, could contribute to alterations in cellular properties such as intercellular trafficking, the bending rigidity of cellular membrane, and cell stiffness(65,66). In addition, physical crowding could even affect how cells sense the surrounding environment. One study found that cell density can override the mechano-sensing of substrate stiffness for mesenchymal stem cells, causing crowded cells on soft materials to exhibit properties that resemble those on stiffer surfaces(67). Other signaling pathways affected by cell density, such as mTOR pathways(68), have been identified. Further studies could help uncover novel cellular mechanisms governing these density-dependent changes, as well as enabling better understandings of how *in vitro* culture parameters could affect the experimental outcomes.

Cell crowding can also increase the local concentration of soluble factors and cause density-dependent changes in signaling (Figure 1.6). It has been observed that microbial populations could produce small signal molecules, termed autoinducers, to communicate in a density-dependent manner(69). When the bacterial population reached a certain density, autoinducer concentration would reach a threshold that induces collective responses for the whole community. This phenomenon, termed quorum sensing, has also been observed at the tissue level, in which significant hair regeneration was observed if damaged hair follicles reached a certain density(70). CCL2, which was secreted in response to the injury, contributed to this organ-level density-dependent effect. Recently, studies are suggesting that the immune system adapts a similar approach in controlling cellular functions. For T cells, autocrine/paracrine signaling of IL2 can control different behaviors of $CD4^+$ T cells in a density-dependent manner. A critical density of T_{eff} cells is required for the secreted IL2 to reach a concentration threshold that enables sustained phosphorylation of STAT5 and the resultant proliferation(71). Similar

mechanisms also govern the differentiation of naïve $CD4^+$ T cells into memory precursors via the signaling of IL2 and IL6(72). On the other hand, myeloid cells exhibit comparable mechanisms(73). For plasmacytoid dendritic cells, the secretion of interferon-alpha (IFNα) following stimulation of CpG-C, a TLR agonist, is density-dependent(74). Such stimulation results in an initial secretion of IFNα in a subset of cells. The cytokine would then act as an autoinducer to amplify the subsequent responses for the whole population in a density-dependent manner. Quorum sensing, or other similar mechanisms, enables coordinated control of the population to exert context-dependent responses. Thus, a better understanding of similar controls at the population level could help understand the complex regulatory networks inside a living system.

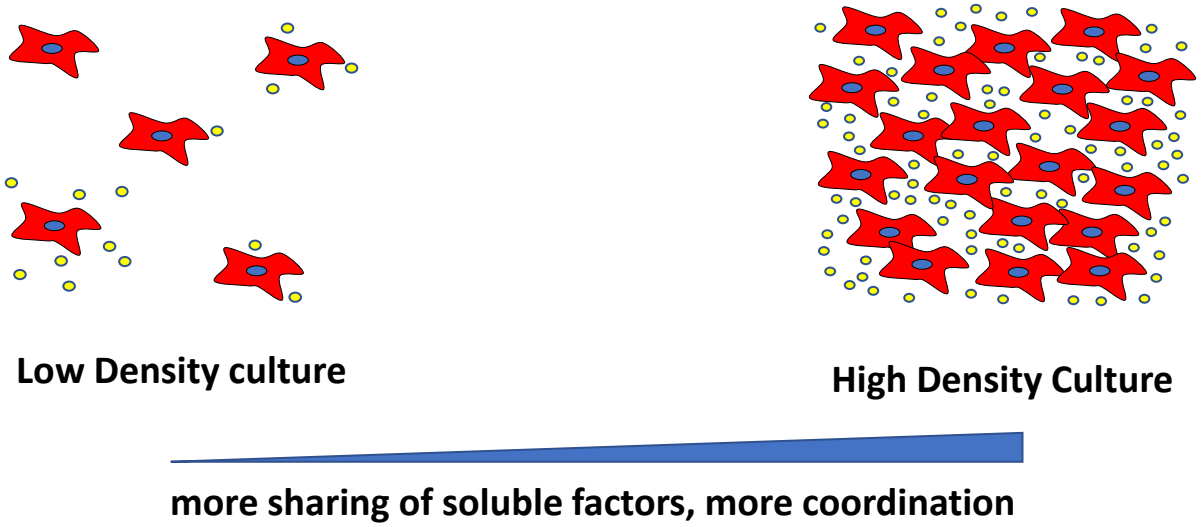

Figure 1.6 Schematics describing how seeding can affect the paracrine interactions among a population of macrophages and cause density-dependent regulation of macrophage function

1.3.2 Regulatory roles of cell density/cell crowding in macrophages

Cell density-associated changes in the microenvironment also affect different aspects of macrophage biology, including macrophage differentiation, activation, and antibacterial functions. Studies on *in vitro* differentiation of murine bone marrow-derived macrophage

demonstrated that seeding density at the beginning of the differentiation process can alter the phenotype of the differentiated cells(75). This resulted in different phagocytosis capabilities, altered expression of markers such as iNOS and mannose receptors, as well as changes in NO production and cytokine secretion under LPS stimulation, including TNFα, IL-12, and more. This density-dependent difference was also observed in the differentiation of THP-1 cells(76). In addition to differentiation, macrophage polarization was also affected by seeding density. Muldoon et. al. found that high cell density appeared to increase the proportion of cells in the high activation state(40). Further investigation suggested that secreted factors from macrophages during resting state could prime the population for subsequent activation in a density-dependent manner, which is likely due to increased accumulation of NFκB in cells before activation. The authors termed this phenomenon as "quorum licensing", as it resembles the density-dependent quorum sensing observed in bacteria but produces a graded response as opposed to a switch-like behavior in microbial populations. Finally, macrophages in high density can increase their ability to control mycobacterial growth(77). Small molecules secreted by cells appeared to contribute, as the mycobacteria inhibiting effects of the conditioned media from high-density culture were lost after dialysis. Interestingly, the study also noted that dense macrophage culture has significantly different morphology than those in sparser conditions, suggesting that membrane contact may also play a role. These studies suggest that soluble factors and physical contact could contribute to the density-dependent changes in macrophage functions, but more studies are still necessary to elucidate the underlying mechanisms.

While the mechanisms behind the density-dependent regulation of macrophages have not been elucidated yet, some contributing factors have been identified. Several soluble factors, including TNFα, IL10, and interferon-beta (INFβ), have known paracrine properties, and some

studies have investigated their ability to modulate macrophage populations. Lane et al. have investigated NFκB dynamics under different perturbations and found a role of seeding density in alternating the dynamics(36). They tried different treatments, including brefeldin A and soluble TNFα receptors to block cytokine secretion and TNFα signaling respectively, and found that only the latter resulted in a significant change in NFκB dynamics. Muldoon et. al. also investigated the role of TNFα and IL10 in modulating the density-dependent effect. Interestingly, the results suggested that both cytokines have limited density-dependent regulatory effects(40). On the other hand, INFβ appeared to exert density-dependent control in iNOS expression in RAW 264.7 cells. In the study, higher seeding density, as well as the supernatant from high-density cultures, led to increased iNOS expression in RAW 264.7 cells(78). Such increase was attenuated when the neutralizing antibody against INFβ was added, indicating the involvement of the cytokine. Other candidates for density-dependent controls, such as gelsolin, are continuing to be identified(79). Continued work on how these cytokines contribute to density-dependent regulation of macrophage activation would further uncover the complicated regulatory mechanism that enables tight control of macrophage activation.

On the other hand, physical interaction is also implicated in the density-dependent effect. One study investigated how physical confinement, including cell crowding, regulates macrophage polarization. It found that inflammatory activation was lower for bone marrow-derived macrophages in confined or crowded conditions(80). The reduced actin polymerization in crowded conditions appeared to contribute to diminished late-phase inflammatory activation, but the cause was not elucidated. A separate study ruled out the role of a transcription factor, Yap, as it did not promote density-dependent changes in macrophages(19). Interestingly, this inhibitory crowding effect was in contrast with the density effect observed in other studies, in

which increasing seeding density contributes to macrophage polarization(35,40,81). The different cell types (BMDM vs. Raw 264.3 cells) used in the studies could account for the differences, suggesting that some functions may be lost in the commonly used cell lines when compared to primary cells. This highlights the complex mechanisms employed by macrophages, as well as the shortcoming of using cell lines to study some of the immune functions.

1.4 Piezo1 and its sensing and regulation of cellular crowding

1.4.1 Structure and function of Piezo1

Cell crowding can alter cellular interactions and result in density-dependent changes in macrophage phenotype and functions. These interactions can occur either via sharing of soluble factors or through physical interactions. While the importance of soluble factors in macrophage modulation has been well appreciated, the roles of physical interactions are gaining more attention now. One important class of physical sensors is the mechanosensitive ion channels, which can sense and respond to transient mechanical perturbations(82). The rapid openings and closings of the channels can allow sensitive responses at a local scale, in which ions enter cells only through some sub-locations and cause changes in the proximity(83). Some ions, such as calcium, can also trigger diverse pathways that enable sustained cellular responses(84). The presence of a mechanosensitive ion channel in vertebrates had been evident in electrophysiological measurements. However, the channel was not identified until the discovery of the Piezo1 mechanosensitive ion channel in 2010.

Piezo1, the mechanosensitive ion channel, was discovered in 2010 by screening for potential candidates through knocking down possible targets in Neuro2A cells(85). It is a large

protein (2521 amino acids for human Piezo1), and its structure resembles a trimeric propeller-like shape with three blades, a pore in the middle, and a cap on top(86) (Figure 1.7). The channel does not require additional cellular structures to confer its mechanosensitive properties, as it can be activated by mechanical stimuli even when embedded in acellular liposomes(87). The channel has been found to activate under different mechanical stimuli such as stretch, shear stress, suction, indentation, and substrate stiffness(88) (Figure 1.7). While the exact mechanism for the channel activation is still under investigation, several possible mechanisms have been proposed(88). Under mechanical stretch, the force could transmit through the membrane, resulting in conformational changes in the protein structure that open the pores. Shear stress

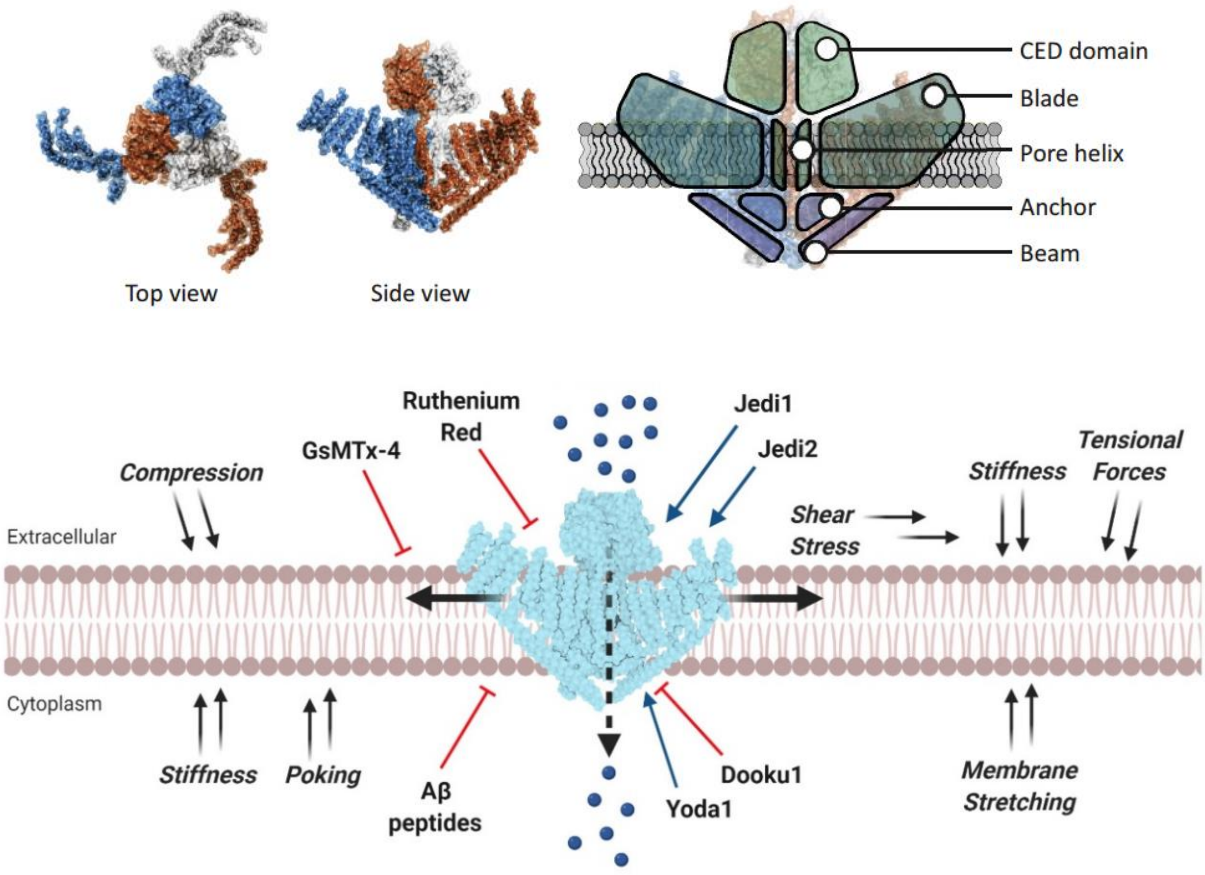

Figure 1.7 The Piezo1 structure and different types of mechanical perturbations that affect its functions
[Adapted from (88) and (146)]

could also displace the cap to open channels. Also, stretches can change the curvature or thickness of the membrane, causing a mismatch in the polarity between the membrane and the transmembrane portion of the protein. This hydrophobic mismatch could then alter protein conformation and activate it. Following activation with a sustained stimulus, Piezo1-dependent current would quickly decay because of the channel inactivation, which is important as channel dysregulation could result in diseases(85,89). While there are still much to be investigated for Piezo1, progress has begun to help us understand the structure and functions of this channel, as well as factors that regulate its activities.

1.4.2 Factors regulating Piezo1 functions

Piezo1 activities are regulated by different cellular components. Since external force transmits directly through the lipid membrane to activate the cell, Piezo1 activation resembles the “force from lipids” concept(82). As a result, membrane properties can affect Piezo1 dynamics. One study explored the capability of different dietary fatty acids to modulate Piezo1 functions and found that margaric acid (MA), commonly found in fish and dairy products, inhibits Piezo1 currents, while several polyunsaturated fatty acids (PUFAs), often in fish oils, alter channel inactivation(90). These fatty acids appear to exert their effects by altering the bilayer physical properties, as MA increased membrane bending stiffness and PUFAs decreased it. In addition, cholesterol content in the lipid bilayer also modulates Piezo1 functions. One study manipulated cholesterol by either depleting it using methyl-b-cyclodextrin (MBCD) or disrupting it via dynasore and found that channel sensitivity, latency, and inactivation mechanics are all affected(91). The results suggested that cholesterol organizations in the membrane help to coordinate the distribution and activities of Piezo1 channels in clusters. Overall, modulation of membrane properties or alteration of Piezo1 cluster distribution via cholesterol manipulation

could both change Piezo1 activities. These findings suggested the possibility that altering membrane composition may also affect how cells sense the surrounding mechanical environments.

On the other hand, the cytoskeleton can also modulate Piezo1 activities. Although Piezo1 operates through a "force from lipids" concept, the forces exerted on the membrane can also be altered by the cytoskeletal network(92). A study comparing Piezo1 activities on an intact membrane and membrane blebs that lack actin connection found that Piezo1 opened at lower pressure in the membrane bleb. This result suggested that the cytoskeleton has a mechanoprotective effect that increases the channel activation threshold(93). On the other hand, actin is necessary for Piezo1 activation in some circumstances, as actin-myosin mediated traction forces evoke channel activities in human neural stem/progenitor cells(83). A recent review article suggests the different roles of the actin cytoskeleton on Piezo1 activities reflects the two mechanisms in which Piezo1 enables mechano-sensing(92). The first mechanism represented the passive, or outside-in mechanotransduction, in which external forces trigger the opening of the channel. In this case, the actin cytoskeleton provides structural support for the cell, thus it stiffens the cell membrane and reduces channel activation. An alternative active, or inside-out mechanotransduction, occurs when the cells actively generate force to probe substrate stiffness. Such active force could also activate Piezo1, and the actin cytoskeleton can facilitate this process and promote channel activities. Thus, the cytoskeleton has diverse roles in regulating Piezo1 function, which also depends on the type of mechanotransduction that is being executed.

Besides membrane and cytoskeletal properties, other factors regulating Piezo1 functions are continuously being found. Extracellular matrix composition may modulate Piezo1 activation, as using an atomic force microscopy cantilever tip to pull the dorsal surface of cells generate

varied Piezo1 activity that depends on the ECM coated on the tip(94). A few chemical compounds also possess the capability to activate or inhibit Piezo1 activation. Several chemicals that act on broad ranges of ion channels, such as the inhibitory GsMTx-4 and Gd^{3+}, exert effects on Piezo1(82). Some substances are more specific to Piezo1, such as Yoda1, which activates the ion channel(95). Recently, another molecule named Dooku1 was synthesized, which was able to counteract the agonist effect of Yoda1(96). There are still much more regulatory mechanisms for Piezo1 that have not been identified yet, and more research would help to discover them and help us understand the different impacts this mechanosensitive ion channel can have on diverse physiological processes.

1.4.3 Physiological importance of Piezo1 mechanosensitive ion channel.

As a mechanosensitive ion channel, Piezo1 possesses important roles in different physiological processes that experience mechanical perturbations. This is especially evident in musculoskeletal and cardiovascular systems, which both experience a significant amount of force. Recent studies revealed that Piezo1 is necessary for normal bone formation, as Piezo1 conditional knockout ($Piezo1^{Ocn/Ocn}$) mice exhibited abnormal bone development such as shorter femur and tibia and decreased bone mass, as well as reduced osteoblast differentiation markers(97). Interestingly, simulated microgravity or mechanical unloading reduced Piezo1 expression, while mechanical stimulation had an opposite effect(97). This observation suggested the role of Piezo1 in mechanical load-mediated bone remodeling. Another tissue subjected to continuous loading and unloading is cartilage. In cartilage, Piezo1 and the related Piezo2 appeared to facilitate sensing of high-strain mechanical loadings(98). Blocking Piezo1 and Piezo2 reduced chondrocyte death following excessive mechanical loads. Based on these results, the authors suggested that Piezo1 may be a potential target to help mitigate the impact of joint

injuries. The studies on Piezo1 have explained important functions in the musculoskeletal system. Future studies on its interaction with other cellular components such as the cytoskeleton, as well as the feedback between mechanical loads and both Piezo1 expression and function, can help us further understand how mechanical force impacts musculoskeletal functions.

Piezo1 also exerts significant impacts on the cardiovascular system. For the heart, the channel is found to mediate calcium and reactive oxygen species signaling in response to mechanical stretch, and it maintained the homeostasis of the signaling regulation(99). Interestingly, Piezo1 overexpression or knockout both contribute to cardiomyopathy, suggesting the need to maintain proper channel functions. Piezo1 also contributes significantly to vascular development(100). Mouse embryo with Piezo1 global knockout died at midgestation, in a time that coincided with vascular development. Interestingly, impaired Piezo1 function also affects lymphatic development and contributes to diseases such as congenital lymphatic dysplasia(101). Further investigation suggested that Piezo1 enables endothelial cells to sense shear stress and contributes to shear-induced endothelial remodeling and realignments(100). Such shear stress sensing capability also enables endothelial cells to monitor blood flow(102). Under heightened physical activity, blood flow increases and causes increased depolarization in endothelial via a Piezo1-dependent manner. This can lead to the activations of voltage-gated ion channels in the surrounding smooth muscle cells, which result in vasoconstriction that is critical for optimal physical performance. On the other hand, the ion channel also helps regulate red blood cell (RBC) volume in response to mechanical perturbations experienced during circulation, such as when cells pass through thin vessels(103). Indeed, genetic mutation of Piezo1 contributes to RBC diseases such as dehydrated hereditary stomatocytosis (DHS)(103). Current research is continuously uncovering additional roles of Piezo1, including its involvement in blood pressure

control. Thus, the Piezo1 ion channel may hold the keys to understanding more aspects of cardiovascular functions and dysregulations.

At cellular levels, Piezo1 mediates several processes involving mechanical sensing of the surrounding microenvironments. There is increasing evidence demonstrating that cells are sensitive toward the surrounding physical environment, such as substrate stiffness, topography, and physical confinement. However, how cells sense these extracellular cues is still under investigation. Recent studies suggested that Piezo1 is involved in these processes. One study found that Piezo1 mediates the differentiation choices for human neural stem cells(104). Such effect is likely due to its activation under cell-generated traction forces, which vary in a stiffness-dependent manner. The ability for Piezo1 to sense substrate stiffness was further supported in another study utilizing micropillar arrays, and the results similarly indicated that such ability was mediated by forces transmitted through both cytoskeleton and cell membrane(105). The same study also demonstrated that substrate roughness modulates Piezo1activites(105). In addition to substrate stiffness and roughness, Pizeo1 can also sense cellular crowding. In epithelial cells, overcrowding led to the extrusion of live cells in regions with the highest level of crowding, and this process depends on Piezo1-mediated signaling(106). Interestingly, in epithelial layers with low cell density, Piezo1 activation under mechanical stretches resulted in cell growth. The authors suggested that differences in cellular Piezo1 localizations between crowded and sparse cultures may contribute to the opposite outcomes(107). Another physical factor that Piezo1 sense is cellular confinement. Hung et. al. found that cells can utilize a Piezo1/Phosphodiesterase 1/Protein kinase A-based pathway to sense cellular confinement during migration and subsequently alter their mode of locomotion accordingly(108). These findings highlighted how Piezo1 can exert different roles in sensing the mechanical environments, either through passive

or active mechanotransduction, and regulate cellular functions. A better understanding of similar processes would improve our understanding of the interaction between cells and their surrounding environments.

1.4.4 Piezo1 and macrophages

The effects of the Piezo1 on macrophage function are not well understood, owing to the recent discovery of the ion channel. However, emerging research has begun to unfold its impacts. Solis et al. described the role of Piezo1 in sensing cyclic stress. Under cyclic pressure, Piezo1 leads to calcium activities that result in endothelin-1 (EDN1) secretion, which then stabilizes hypoxia-inducible factor 1a (HIF1a) and induces pro-inflammatory responses(15). Piezo1 also mediates the mechanotransduction for substrate stiffness. Both our lab and another group demonstrated that Piezo1 induces a stiffness-dependent influx of calcium ions, with more calcium activities observed on cultures with stiffer substrates than on softer substrates (20,109). In addition, works from our lab suggested that mechanical cues, such as static or cyclic stretch, may regulate macrophage responses through the interaction between CD11b and Piezo1(24). Interestingly, decreased expression of CD11b or Piezo1 led to increased expression of the other, suggesting an interplay between the two proteins. Such cross-regulation may modulate macrophage functions in response to mechanical stimuli(24). These studies uncover not only the contribution of Piezo1 in mechanosensing but also its effects on macrophage activations. Piezo1 activation appeared to exert both pro-inflammatory and pro-bactericidal effects, leading to elevated expression of pro-inflammatory markers such as TNFα and iNOS, as well as increased ROS production, phagocytosis capability, and bacterial clearance(20,109). Future work would aim to understand whether Piezo1 modulates additional physical sensing for macrophages, as well as how transient Piezo1-mediated calcium activities translate to more sustained responses.

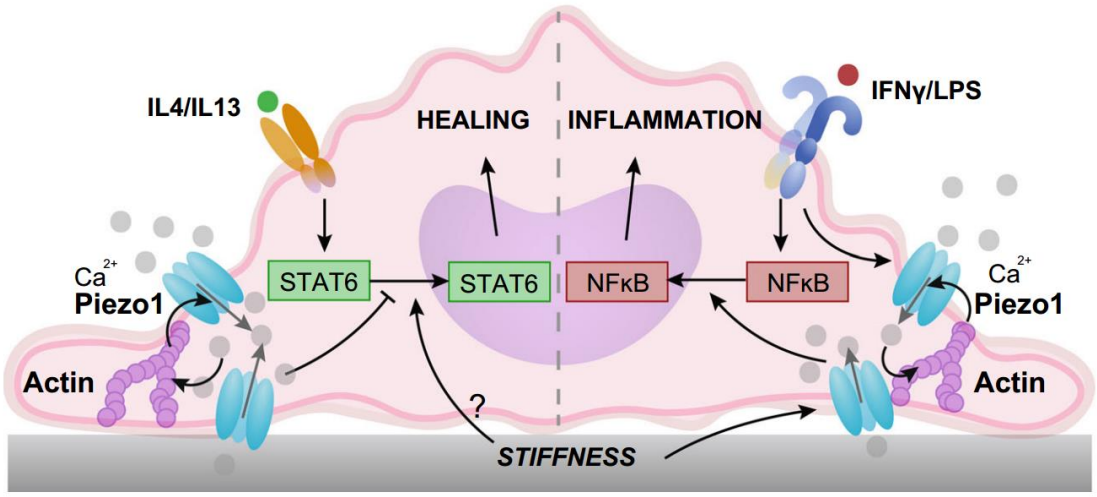

Figure 1.8: The different effects of Piezo1 on macrophage functions [Adapted from (20)]

1.5 Overview of Dissertation Research

Macrophages are sensitive to changes in the surrounding environments. While many studies have investigated how soluble and adhesive factors regulate cellular functions, not many have focused on the effects of cell density. However, cell density can potentially impact macrophage functions, as it can alter the physical and soluble environments outside of cells, as well as changing cellular responses toward those cues. This study aims to investigate how macrophage density can influence cellular functions, with a focus on its roles in regulating pro-inflammatory activations.

Chapter two focuses on how soluble factors mediate density-dependent changes in macrophage activation both at macroscale and microscale. On the macroscale, we seeded macrophages at different densities and study their activation under pro-inflammatory stimulation. On the microscale, we seeded cells into pico-liter-sized microwells and examined the effect of cells numbers in a microwell on their polarization. These studies explored how cells coordinate

functions and activations through soluble factors, and how cell density could modify such coordination.

Chapter three investigated the role of the mechanosensitive ion channel Piezo1, a channel known for sensing cell crowding, in modulating the density-dependent effect of macrophage activation. We explored its regulation on macrophage calcium activities and cytokine secretion for cultures in different seeding densities or adhesion times. We also attempted to study the underlying mechanisms, especially the roles of the cytoskeleton. These results supported the idea that physical sensing of the surrounding environment could mediate density-dependent changes in macrophage activation.

CHAPTER 2: Density-Dependent Regulation of Macrophage Function in the Macro- and Micro-scale In Vitro Culture Environment

2.1. Introduction

Macrophages have essential roles in tissue homeostasis, combating infection, and mediating wound healing. They are also involved in the pathogenesis and progression of many chronic inflammatory and autoimmune diseases, including atherosclerosis, rheumatoid arthritis, obesity, and multiple sclerosis (5,110). To perform their myriad of functions, macrophages are exquisitely sensitive to the surrounding environment and can polarize into different phenotypes in response to diverse stimuli, including both soluble and adhesive cues. At the extremes, soluble cues such as the bacterial component lipopolysaccharide (LPS) and interferon-gamma (IFNγ) polarize cells to pro-inflammatory phenotypes (often referred to as "M1"), while interleukin 4 and interleukin 13 (IL4 and IL13) polarize cells to pro-healing phenotype (often referred to as "M2") (26). To study the effects of these soluble cues in macrophage activation, *in vitro* culture systems are often employed and have advanced our knowledge about macrophage activation. These platforms provide well-controlled environments to delineate the effects of different factors. In addition, recent efforts in the development of advanced *in vitro* microsystems can better recapitulate the physiological environment, known as microphysiological systems. These platforms can be used with a wide variety of cell systems including primary human cells, enabling translationally relevant and high throughput studies. However, they also raise new considerations about how the culture environment can potentially influence the function of cells.

Many studies of macrophage activation involve seeding cells into culture wells, followed by stimulation with activating agents and evaluating the expression or secretion of phenotypic markers and cytokines. However, factors including seeding density and media volume per cell

can vary across the many published studies. These factors have been linked to varied behaviors of immune cells. For example, different seeding densities of THP-1 monocytic cells and murine bone marrow cells during differentiation to macrophages cause differential expression of macrophage markers CD11b, CD14, and Ly-6G (75,76). Furthermore, murine macrophages differentiated at different densities responded differently to the stimulation, with cells differentiated at lower densities secreting higher amounts of inflammatory cytokines, including TNFα, IL6, and MIP-1α, and expressed lower anti-inflammatory markers, such as CD206 and Ym1 (75). In another study, murine macrophage cell line RAW 264.7 cultured at higher densities resulted in increased NF-κB signaling, TNFα transcription, and TNFα production (40). These results were attributed to the priming of the macrophages by soluble factors secreted from the cells at the resting state prior to stimulation. Macrophages cultured at higher densities also inhibit mycobacteria growth more effectively than those cultured at lower densities (77). Interestingly, studies separating individual cells into microwells show that cytokine secretion from individual cells in response to inflammatory activators is substantially lower than cells cultured in bulk and allowed to share common paracrine signals (35,42). However, the effects of neighboring cells in small numbers associated with microscale culture, as well as direct comparisons with macro-scale cultures in traditional tissue culture wells have not been established.

Recent efforts to develop more complex microphysiological systems that better recapitulate native tissue environments have revealed additional parameters for consideration in the design of *in vitro* culture studies. The miniaturized dimensions of microfluidic devices have the advantage of using fewer reagents and enabling cultures of small populations or single cells, while also offering the flexibility to design structures and pattern cells or substrates to mimic native cellular environments (111,112). These advantages have been exploited in the

construction of various organ-on-a-chip systems, for example, a gut-inflammation-on-a-chip model (113). However, these tools also introduce new soluble environments for cultured cells, including varied rates of accumulation for endogenous growth factors, reduced media volume conditions, and exposure to other cells. In epithelial cells, differences in the growth rates have been observed for normal mammary gland epithelial cells (NMuMG) cultured in microscopic and macroscopic systems, and accumulation of soluble factor signaling was cited as the main factor (114). Macrophages are known to secrete many cytokines with paracrine effects, and these factors may exert their effects differently in between bulk and micro-scale systems. These issues further necessitate a better understanding of how density or soluble factor-dependent parameters contribute toward the macrophage responses.

Here, we systematically evaluated the roles of different cell culture parameters in the activation of macrophages in both bulk culture and microwells. We examined the effects of cell seeding density and cell-to-media volume ratio on the inflammatory activation of bone-marrow-derived macrophages (BMDMs). We found that lower seeding density and lower cell-to-media volume ratio led to higher TNFα secretion. In addition, studies of small groups of macrophages cultured in microwells also suggest that cellular polarization may also depend on the absolute number of cells in the culture. Our results highlight several factors that needed to be considered when designing cell culture studies for macrophages, both in traditional culture systems and in miniaturized platforms.

2.2. Materials and Methods

2.2.1. Cell isolation and culture

All protocols involving animals were approved by the University of California Irvine's Institutional Animal Care and Use Committee, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALACi). Bone marrow-derived macrophages (BMDM) were obtained by flushing bone marrow cells from the femur and tibia of C57BL/6J mice aged between 6 to 12 weeks, and then treating them with ACK lysis buffer (Life Technology) to remove red blood cells. The cells were then cultured in DMEM media supplemented with 10% heat-inactivated fetal bovine serum (Cytiva), 1% penicillin/streptomycin, 2 mM L-glutamine (both from Life Technology), and 10% macrophage-colony stimulating factor (M-CSF) containing conditioned media for seven days, with a media change on the third day. For experiments, cells were lifted from the plate using cell dissociation buffer (Life Technology) and gentle scrapping for further use.

2.2.2. Cell density studies

BMDM were seeded in 1 ml onto 18 mm glass coverslips in 12 well plates to achieve densities ranging from $5.1x10^5$ to $2.6x10^3$ cells/cm^2. After overnight incubation, cells were stimulated with 10 ng/ml of ultrapure LPS (Invivogen) and IFNγ (R&D System) for 24 hours before supernatants were collected for analysis. To investigate the dynamics of cytokine secretion, separate experiments were set up with cells seeded in 24 well plates at the density of $5.1x10^3$, $7.7x10^4$, and $2.6x10^5$ cells/cm^2. Following stimulation, the supernatant was collected after 2, 6, 12, and 24 hours for analysis. To assess the role of secreted factors in the observed density-dependent effect of macrophages, $2x10^5$ BMDM were seeded onto 12 well plates in 1 ml to achieve a seeding density of 5.1 $x10^4$ cells/cm^2, allowed to adhere overnight, and stimulated with media containing 10 ng/ml of LPS and IFNγ at the volumes of 1 ml, 0.667 ml, and 0.4 ml. This corresponds to the

cell-to-media volume ratio equivalent to cells seeded at $5.1x10^4$, $7.7x10^4$, and $1.3x10^5$ cells/cm^2 in a 12 well plate. After 24 hours, the supernatant was collected for analysis.

2.2.3. Cytokine measurement

Supernatants were analyzed for TNFα and IL10 secretion using the ELISA kits purchased from Biolegend following protocols recommended by the manufacturer.

2.2.4. Fabrication of the microwell membranes

Silicon wafers with arrays of microwells were fabricated using standard photolithography techniques (42). In short, SU8 photoresist (MicroChem) was spin-coated onto a silicon wafer, baked, and then exposed under UV illumination through a custom-designed mask (CAD/Art Services) with patterns of rectangles of 200x300 μm in size. The wafer was subsequently baked before it was developed in the SU8 developer (MicroChem) to create patterns of rectangles with a size of 200x300 μm. To create PDMS microwell membranes for single-cell experiments, PDMS and curing agents were mixed in a 10:1 ratio, degassed in a desiccator, and then spin-coated onto a silicon master with patterns of microwells to create membranes with through-holes at a thickness of around 50μm. Circular PDMS rings were subsequently deposited onto the master to facilitate the separation of the microwell membranes from the master. The PDMS-coated master was then baked in an oven at 65°C overnight for the PMDS to cure. Afterward, the PDMS microwell membranes were carefully peeled off from the master, cleaned with 70% ethanol, and dried in an oven overnight before being used.

2.2.5. Microwell-based cell studies

18 mm coverslips were UVO-treated and then coated with fibronectin (Corning) at room temperature for 1 hour. Afterward, both the coverslips and cleaned PDMS microwell membranes were UVO-treated again before being bonded together and the PDMS ring support being removed. The microwell membrane constructs were then coated with 2% Pluronics F-127 (Sigma Aldrich) for an hour and then washed with PBS for subsequent experiments. The microwell membrane substrates were placed in a 12 well plate and seeded with 1.5 ml of cell suspension at a concentration of 10,000 cells/ml. After overnight incubation, cells in the microwell were stimulated with 10ng/ml of LPS and IFNγ, and the microwells were sealed by applying pressure to hold the substrate against a glass slide using a custom holder. After 24 hours, the microwell substrates were separated from the holder and the glass slide, stained with a fixable dead stain (Life Technology), and then fixed with 4% paraformaldehyde (Electron Microscopy Science) for five minutes before being washed with PBS and blocked with 2% bovine serum albumin (BSA, MP Biomedical) overnight.

2.2.6. Immunofluorescence staining and image analysis

Fixed samples were stained with a rabbit polyclonal anti-iNOS antibody (Abcam) at a dilution of 1:1000 overnight. After washing with 1% BSA three times, samples were stained with Alex Fluor 488 (goat) anti-rabbit secondary antibody (Abcam) at the dilution of 1:1000, and Hoechst 33342 (Life Technologies) at 1:1000, for one hour. Subsequently, samples were washed with 1% BSA three times again and rinsed with phosphate-buffered saline (PBS) before being mounted

onto glass slides with Fluoromount-G (Southern Biotech). Samples were imaged using an Olympus IX-83 (Olympus) epifluorescence microscope, at a magnification of 20X, and the resultant data were processed using FIJI/ImageJ (115). For microwell construct samples, substrates were scanned using Olympus IX-83 to obtain images at green (iNOS), blue (Hoechst), red (dead stain), and brightfield channels. The brightfield channel was used to identify the locations and boundaries of each microwell, while the blue (Hoechst) channel was used to identify the locations of the cells. The latter was also used as a mask to sample the intensity of the iNOS and the dead staining. The fixable dead stain was used to exclude dead cells from the subsequent analysis, and the threshold was determined by taking the 1st percentile of the staining intensity for cells treated with 70% ethanol. After initial image-stitching and processing using the Grid/Collection Stitching plugin in FIJI/ImageJ (116), cell locations were mapped onto well locations using custom MATLAB (Mathworks) codes, and the resultant location and intensity data of cells were analyzed using R Studio.

2.2.7. Statistical analysis

Data were analyzed using one-way ANOVA followed by Tukey's HSD test.

2.3. Results

2.3.1. Macrophage secretion of TNFα and IL10 in response to inflammatory stimuli is density-dependent

To establish a baseline for our studies, we first evaluated the effects of cell seeding density on macrophage activation by soluble stimuli in bulk culture. BMDMs were seeded at

different densities and stimulated with pro-inflammatory ligands, LPS and IFNγ together or LPS alone (Figure 2.1A). In response to LPS and IFNγ, macrophage secretion of TNFα increased as the seeding density increased from $2.6x10^3$ to $2.6x10^5$ cells/cm^2; however, TNFα concentration decreased when the seeding density was further increased from $2.6x10^5$ cells/cm^2 to $5.1x10^5$ cells/cm^2 (Figure 2.1B). Dividing the total amount of TNFα secreted by the number of cells seeded, or per cell TNFα secretion, showed a continuously decreasing trend with increasing cell densities (Figure 2.1C). Stimulation with LPS alone also showed decreasing per cell TNFα secretion with increasing seeding density and increasing overall TNFα concentration with increasing cell seeding density, but no decrease at the highest seeding density (Figure 2.2A, 2.2B).

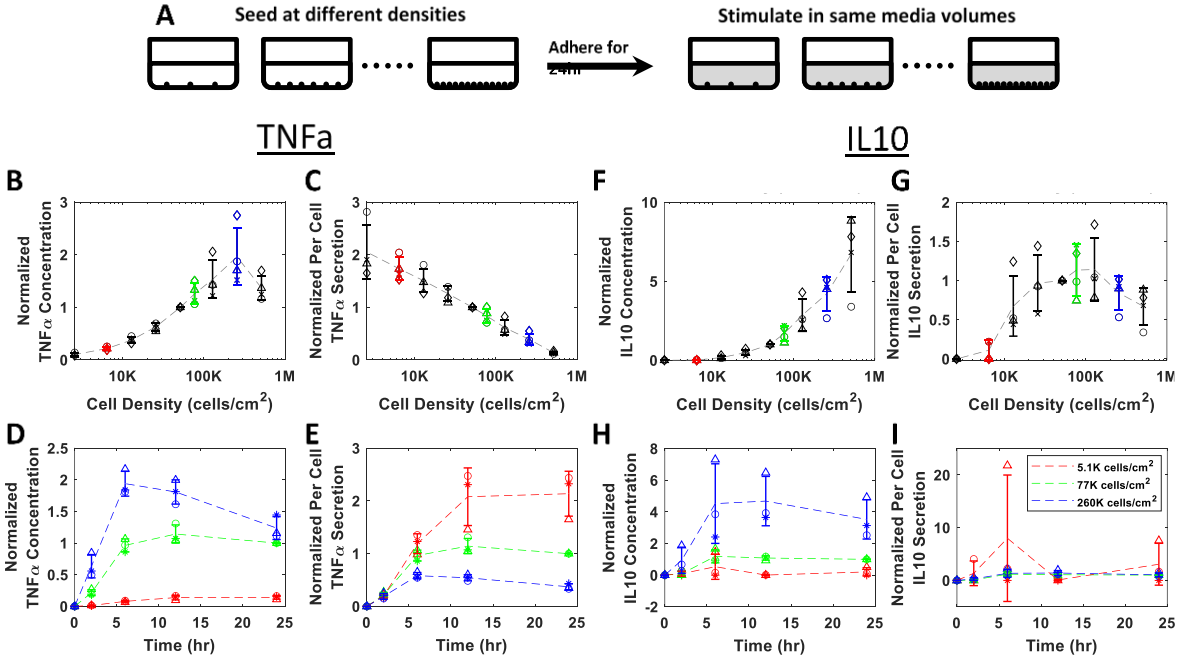

Figure 2.1: Macrophages exhibit density-dependent cytokines secretion under LPS and IFNγ stimulation. (A) Schematic of experiments examining cytokine secretion of macrophages seeded in different densities. (B - E) TNFα concentration (B) and per-cell TNFα secretion (C) of BMDM seeded at different densities on glass, and TNFα concentration (D) and per-cell TNFα secretion (E) at different time points after BMDM seeded at selected densities on polystyrene. (F – I) IL10 concentration (F) and per-cell IL10 secretion (G) of BMDM seeded at different densities on glass, and IL10 concentration (H) and per-cell IL10 secretion (I) at different time points for BMDM seeded in selected densities on polystyrene. (n=4 for figure B, C, F, G, and n=3 for figure D, E, H, I).

To understand the temporal dynamics of the density-dependent TNFα secretion, macrophages were seeded overnight at low ($5.1x10^3$ cells/cm^2), intermediate ($7.7x10^4$ cells/cm^2), and high ($2.6x10^5$ cells/cm^2) densities, stimulated, and the supernatant was subsequently collected at 2, 6, 12, and 24 hours for analysis of secreted cytokines. As expected, the LPS and IFNγ-stimulated macrophages seeded at higher densities exhibited higher TNFα secretion across all time points (Figure 2.1D). Although the general trends between cells seeded on both surfaces were similar, some differences in the fold changes in cytokine secretion at different densities were observed (Figure 2.3A, 2.3B, 2.3E, and 2.3F). Stimulation with LPS and IFNγ or LPS alone both led to similar trends in TNFα secretion when analyzed per cell, with similar TNFα levels across all densities observed after two hours of stimulation and decreased TNFα levels with increasing densities at all of the later time points (6, 12, 24 h) (Figure 2.1E, 2.2C, 2.2D). Overall, macrophages exhibit density-dependent effects on TNFα secretion, with cells seeded in higher

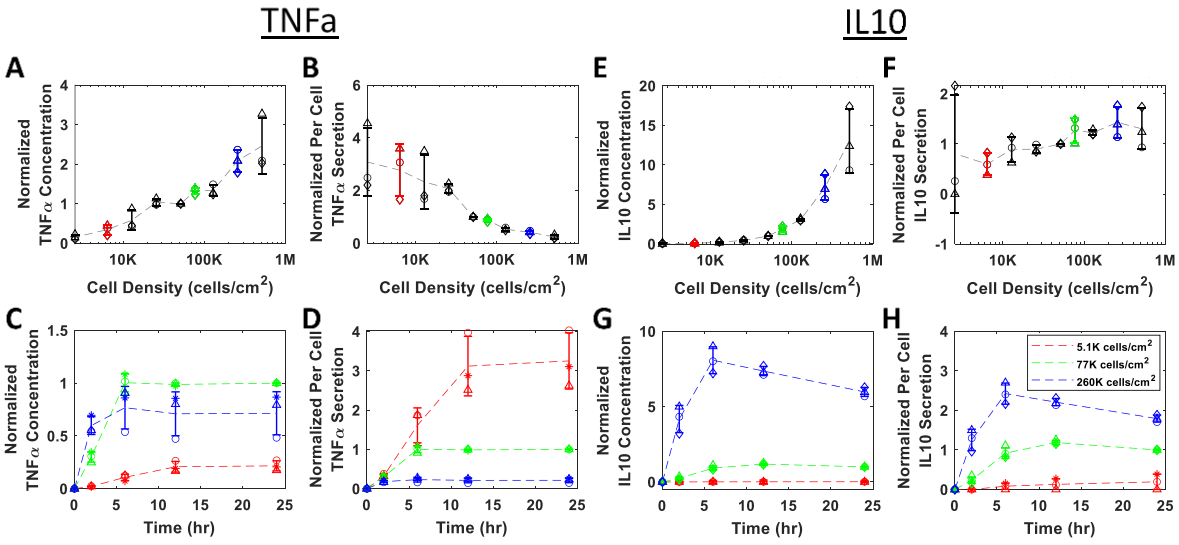

Figure 2.2: Macrophages exhibit similar density-dependent modulation of cytokines secretion when stimulated with LPS only. (A - D) Graphs showing TNFα secretion; (A) concentration and (B) per-cell TNFα secretion of BMDM seeded at different densities (on glass). (C) TNFα concentration and (D) per-cell TNFα secretion at different time points for BMDM seeded in selected densities (on polystyrene). (E - H) Graphs showing IL10 secretion; (E) IL10 concentration and (F) per-cell IL10 secretion of BMDM seeded at different densities (on glass). (G) IL10 concentration and (H) per-cell IL10 secretion at different time points for BMDM seeded in selected densities (on polystyrene). (n=3 for all conditions).

density secreting less TNFα when evaluated per-cell. In addition, density-dependent effects were less apparent at the earlier time points after stimulation but became more pronounced at later stages of the activation.

The effects of seeding density on IL10 secretion were also examined. In macrophages stimulated with LPS and IFNγ, secretion of IL10 increased with increasing densities (Figure 2.1F), but the per-cell IL10 secretion appeared to exhibit a biphasic response, with a peak at around $7.7x10^4$ cells/cm^2 and decreasing with lower or higher seeding densities (Figure 2.1G). For macrophages stimulated with LPS alone, a similar increasing trend in IL10 secretion with increasing seeding densities was observed (Figure 2.2E). However, a biphasic response was not present, and the per-cell IL10 secretion exhibited much less variation across all seeding densities (Figure 2.2F). Evaluation of IL10 at multiple time points showed that higher density cultures exhibited greater concentrations of IL10 in both LPS alone and LPS and IFNγ stimulation conditions across most of the time points examined (Figure 2.1H, 2.2G). For the LPS and IFNγ stimulation condition, the per-cell IL10 secretion was similar across the different seeding densities at different time points (Figure 2.1I). In contrast, higher seeding densities corresponded to higher per-cell IL10 secretion across the different time points in cells stimulated with LPS alone (Figure 2.2H). It is worth noting again that cells were seeded onto tissue culture plastic directly for the time course study, while for the initial experiments on seeding densities, cells were seeded on glass coverslips placed in tissue culture plates. Comparing the trends from the two substrates yielded only moderate differences (Figure 2.3C, 2.3D, 2.3G, and 2.3H). Overall, the secretion of IL10 by macrophages appears to be density-dependent as well. However, these effects may be sensitive to different factors including the batch-to-batch variability in cell donor source, ligands used in stimulation, and culture surfaces.

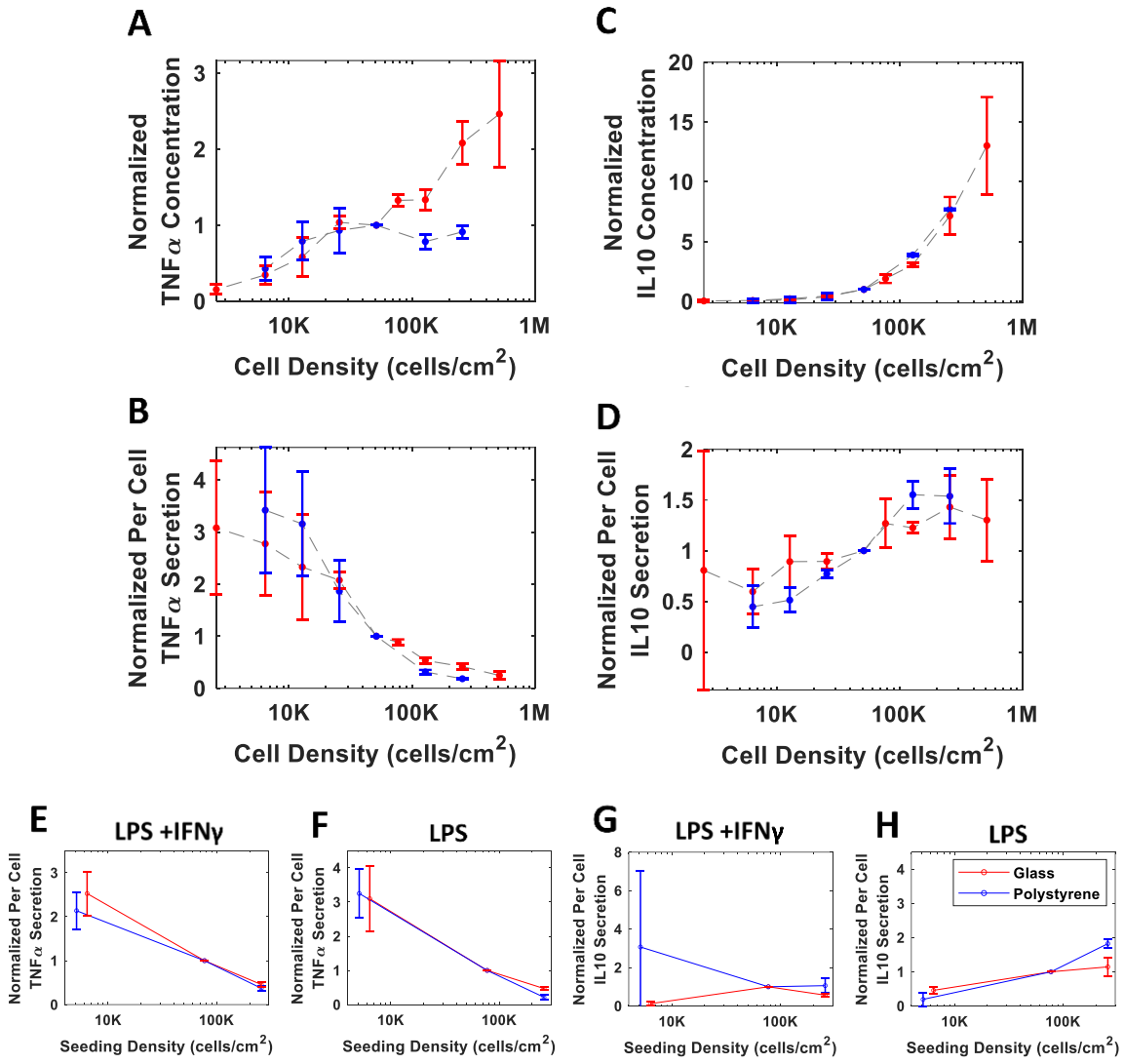

Figure 2.3: Density-dependent effects of LPS-mediated macrophage activation is maintained on different adhesive surface. (A, B) Normalized (A) TNFa concentration and (B) per-cell TNFa secretion of BMDM stimulated with LPS (10ng/ml) on either glass or tissue culture-treated polystyrene surface. (C, D) Normalized (C) IL10 concentration and (D) per-cell IL10 secretion of BMDM stimulated with LPS (10ng/ml) on either glass or tissue culture-treated polystyrene surface. (E, F) Normalized per cell TNFα secretion for cells seeded on glass (red) and TCPS (blue) in different densities stimulated with (E) LPS + IFNg and (F) LPS only. (G, H) Normalized per cell IL10 Secretion for cells seeded on glass (red) and TCPS (blue) in different densities stimulated with (g) LPS + IFNg and (h) LPS only. Data for cytokine secretion from cells on the glass or polystyrene surfaces were normalized to the secretion level at the density of 51K cells/cm^2 for each surface condition. Thus, only the tends between both groups are comparable, and the magnitude between both groups are not comparable. (n=3 for all conditions).

2.3.2. Contribution of culture media volume to the density-dependent effects

The observed density-dependent effects may be a result of paracrine signaling or the physical interactions among cells, which are both enhanced as the seeding density increases. To better control the extent of paracrine signaling, we cultured macrophages within different volumes of media, but all seeded at the same density of $5.1x10^4$ cells/cm^2, which corresponded to the cell-to-media-volume ratio of the cultures seeded at $5.1x10^4$, $7.7x10^4$, and $1.3x10^5$ cells/cm^2 in the previous experiments (Figure 2.4A, 2.4B). As expected, the TNFα and IL10 concentration increased with decreasing volume of cell culture media, or as the cell-to-media-volume ratio was increased (Figure 2.4C, 2.4D). The per-cell IL10 secretion stayed relatively constant across different media volumes, consistent with the IL10 measurements in the corresponding cell-to-media-volume ranges in the previous density experiments (Figure 2.4F). However, per-cell TNFα secretion decreased in culture stimulated in fewer media volumes, with samples from the

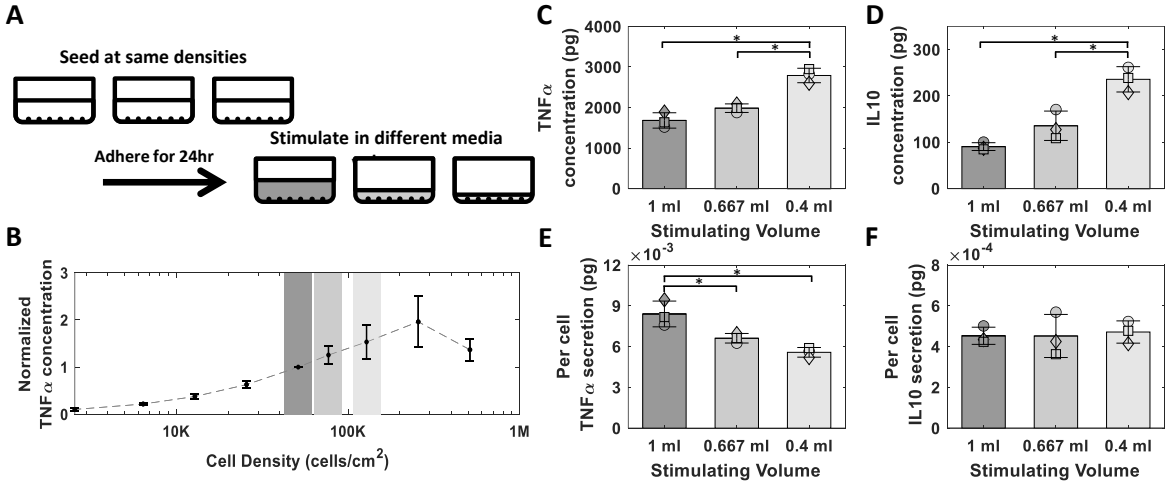

Figure 2.4: **Soluble factors partially contribute to density-dependent effects on macrophage activation.** (A) Schematic of the experiment; cells were seeded at an identical seeding density and cell-to-media-volume ratio, allowed to attach for 24 hours, and then stimulated with LPS and IFNγ at 10ng/ml in different volumes of media (1ml, 0.667ml, 0.4ml) for 24 hours. (B) Graph indicating the selected stimulation volumes (1ml, 0.667ml, 0.4ml) in relation to earlier density experiments in terms of cell-to-media volume ratio equivalence; colors corresponding to bars in C-F. (C, D) (C) TNFα and (D) IL10 concentration of BMDM stimulated with LPS and IFNγ containing media at 1ml, 0.667ml, and 0.4ml. (E, F) per cell secretion of (E) TNFα and (F) IL10 for BMDM stimulated with LPS and IFNγ containing media at 1ml, 0.667ml, and 0.4ml. (n=3 for all conditions, * indicates $p<0.05$; One way ANOVA followed by Tukey HSD Test).

lowest media volume group having almost 30% lower in per-cell TNFα secretion than those in the highest media volume group (Figure 2.4E). Since the number of cells in the culture well was the same for both samples, these results suggested that paracrine interactions among cells partially contribute to macrophage activation in response to pro-inflammatory ligands.

2.3.3. Microwell culture on the coordination among small groups of macrophages and the subsequent inflammatory responses

Our work showed that macrophage activation is dependent on cell density and suggested that soluble paracrine signals play an important role. However, the effects of cell density in small populations of macrophages, which becomes critical as the cell culture platforms are miniaturized, remained unknown. To address this, we isolated individual cells or small groups of cells in arrays of microwell and assessed their response to inflammatory agonists. Here, we evaluated the expression of iNOS, an inflammatory marker, which allowed us to assess the inflammatory levels of individual cells through immunofluorescence staining and compared the expression levels to cells seeded at different seeding densities in bulk culture (Figure 2.5A). To ensure the media volume per cell for macrophages in microwells was consistent with our earlier studies, only macrophages in wells containing one to six cells were analyzed. We observed a wide range of iNOS staining intensity for cells in both microwell and population studies (Figure 2.5B – 2.5E). When comparing the median intensity of iNOS staining for cells in wells containing different numbers of cells, we observed an increase in iNOS expression in response to an increasing number of cells in the microwells (Figure 2.5F). In contrast, a decrease in iNOS expression was observed as cell density increased in tissue culture wells. Normalizing the results from these studies based on equivalent cell-to-media-volume-ratio, we found that the cells

cultured in bulk still decrease in iNOS expression with increasing seeding densities, in contrast to the results in the microwells (Figure 2.5G).

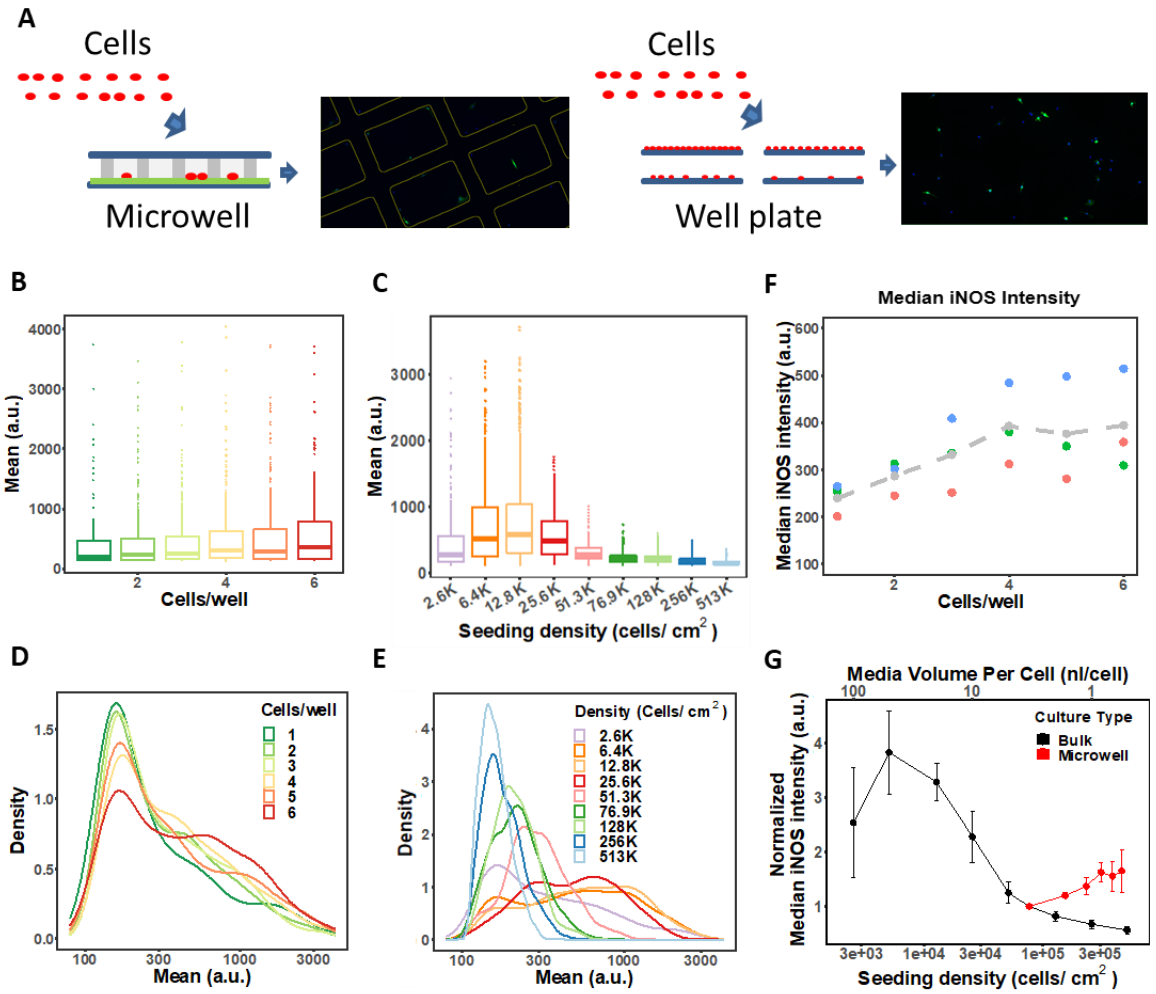

Figure 2.5: Comparison of density-dependent effects in micro- vs. macro-scale cultures. (A) Schematic of the microwell experiment to delineate the effects of single cell vs groups of cells on macrophage activation, and the bulk density experiment for comparison. (B, C) Dot plots of sampled iNOS intensities for cells in (B) microwell experiments with microwells containing 1-6 cells and (C) bulk experiments for seeding densities ranging from $2.6x10^3$ cells/cm^2 to $5.13x10^5$ cells/cm^2. (D, E) Density plots of sampled iNOS intensities for (D) the microwell experiments and (E) the bulk experiments. (F) Median iNOS intensity for population of cells in wells containing 1-6 cells over three experiments. Different colors denote to data from different replicates, and the gray plot represents the mean value. (G) Comparison of the normalized median iNOS intensity of cells from the microwell and bulk experiments. Data were arranged so the cell-to-media volume for results from both experiments were comparable. (n=3 for each condition in panel G).

In both the bulk and microwell studies, it appeared that following the pro-inflammatory stimulation, only a fraction of cells exhibits significant iNOS expression above the baseline

level, which we defined as two standard deviations above the mean of the iNOS signal for the unstimulated population (Figure 2.5D, E). This observation agreed with other studies on macrophage activation at the single-cell level demonstrating precociously activated cells (35,40). To better characterize how interactions among a small group of cells may shift the distribution of the iNOS expression for the cell population, all cells included in the analysis were categorized based on their iNOS staining intensity: non-expressing, 0-20, 20-40, 40-60, 60-80, and 80-100 percentile of iNOS expression (Figure 2.6A). This analysis revealed that when the number of cells in a well increased, the overall cell population generally shifted from below-baseline to higher iNOS expression. To assess whether high iNOS expressing cells in a well could be associated with a higher overall percentage of iNOS expression for cells in the same well, the percentage of iNOS expressing cells in a well was plotted against the staining intensity of the highest iNOS expressing cell in the same well (Figure 2.6B). We found that wells with at least one cell having high iNOS expression appeared to be associated with having higher overall percentage of cells expressing iNOS above baseline. This trend appeared to be maintained for wells containing two to six cells. Together with the bulk culture data, these data suggest that communication among the macrophage population depends not only on the density of the cell population but also on absolute cell numbers. In addition, higher iNOS expressing cells may promote the activation of iNOS in neighboring cells. These factors likely all contribute to the coordinated response of macrophage populations.

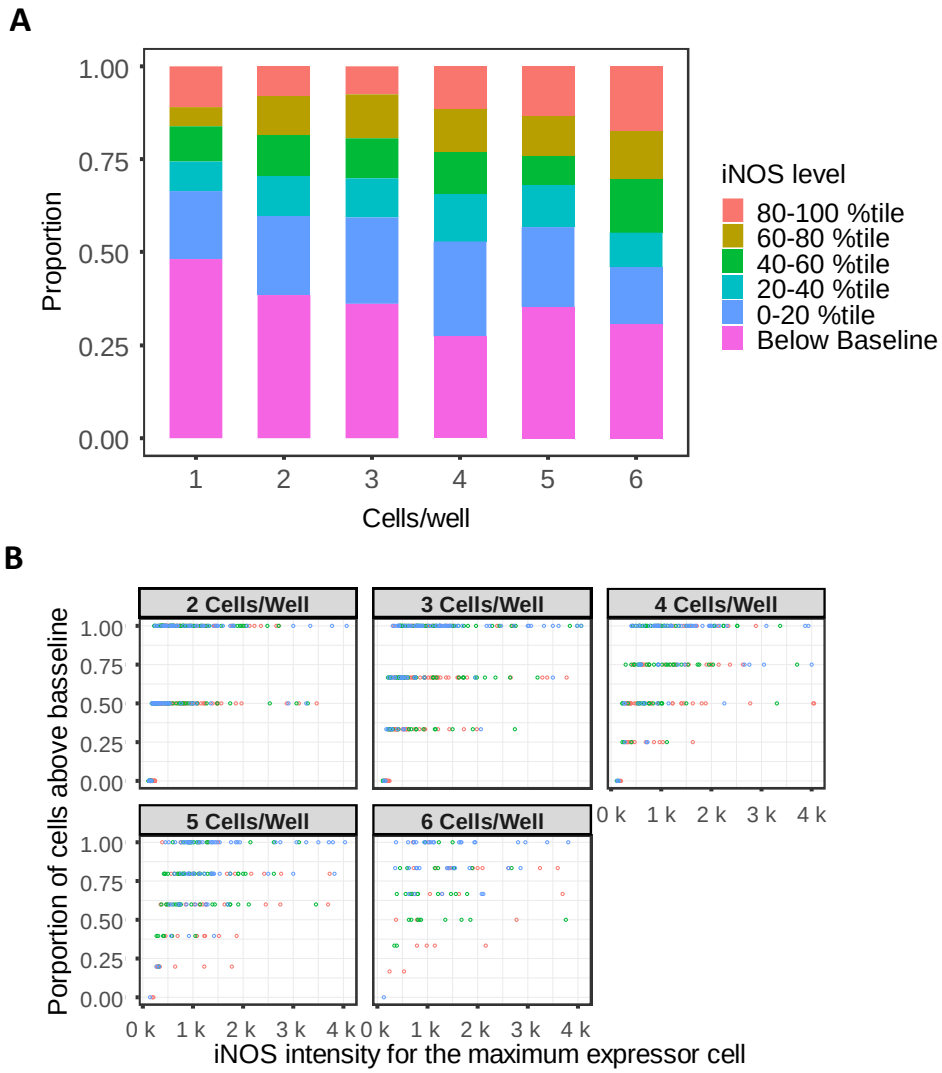

Figure 2.6: Microwell experiments revealed a different mode of density-dependent regulation of macrophage function. (A) A representative sample of distribution of all living cells in wells containing 1-6 cells grouped by their percentile rank. These cells were first separated based on whether their iNOS expression level is above or below the baseline. For those that were above the baseline, they were further separated into 5 groups according to their percentile ranks in iNOS expression (80-100 percentile, 60-80 percentile, 40-60 percentile, 20-40 percentile, and 0-20 percentile). (B) Graphs showing the relationship between the activation level of the highest expressing cell in a well and the percentage of positive iNOS expressing cells in the same well. The data were group based on the number of cells in a well, ranging from 2 – 6 cells, with data from three biological replicates. Each color indicates data from the same biological replicate.

2.4. Discussion

In this study, we identified seeding density and cell-to-media volume ratio as critical *in vitro* culture parameters that influence macrophage activation. Increasing seeding density caused

increasing inhibitory effects on macrophage polarization, as per-cell TNFα secretion exhibited a decreasing trend with increasing seeding densities. Our results contrast with some earlier reports of increased TNFα transcription and production with increasing seeding density (35,40), possibly due to the utilization of macrophage cell lines compared to the primary cells in this study. We also found that seeding density exerted more effects at the later phase of the inflammatory activation than the early phase, since variations were minimal at the earlier time points and became more apparent at the later hours. Soluble factors produced by the cells following stimulation likely contribute to the negative feedback that dampens TNFα secretion, as per cell TNFα secretion was lower when cells were stimulated in lower volume compared to those stimulated in higher volume. This result is consistent with the fact that macrophages are known to secrete inhibitory cytokines such as IL10 to regulate their activation (49,117). Interestingly, cultures within microwells showed a different response, where overall activation increased for small groups of cells compared to cells in isolation. In addition, having at least one cell with a higher iNOS expression level was associated with a higher percentage of iNOS activation in the same microwell, suggesting that highly activated cells may promote the polarization of neighboring cells, possibly through soluble factors. This agrees with previous studies on macrophages and dendritic cells, in which a small subpopulation of precocious or high-secreting cells help coordinate the overall responses of the cell population (35,41). Together, these results suggest that macrophages possess feedback mechanisms that help them tune their functions in response to their population size and density.

Increasing cell density leads to a corresponding secretion of more soluble factors, which can directly feedback to regulate inflammation. For example, IL10 is a well-known negative regulator that is secreted by macrophages stimulated with LPS, and leads to dampening of

inflammation after the initial activation period. Blocking IL10 signaling via IL10 neutralizing antibody, IL10 receptor (IL10R) blocking antibody, or the use of BMDM from an IL-10R deficient mouse all resulted in increased levels of TNFα secretion (35,118). Other soluble factors, including IFNβ, nitrogen oxide, and PGE_2, have also been shown to be implicated in macrophage feedback control(51,78,118,119). However, in a recent study, exogenous IL-10 was insufficient by itself to abrogate density-dependent effects on inflammatory activation (40). This study utilized reporter systems to track NFκB activity and TNFα secretion in RAW264.7 cells, and revealed that cells exhibited density-dependent bimodal activation states following LPS stimulation, which was independent of exogenous IL10. On the other hand, the same study suggested that soluble factors secreted during the resting phase may prime macrophages to respond differently following activation (40). At resting state, high-density culture exhibited higher levels of NFκB than that of low-density culture. In addition, passaging cells at a higher density prior to experimentation, as well as conditioned media from high-density culture, increased reporter expression both with and without stimulation. Together with our results, these findings suggest regulation of cellular responses by density likely involve activities both before and after pro-inflammatory stimulation. How various factors may work together or against each other to orchestrate a collective response will require further study.

Our results reveal complexities in the collective interaction within a population of cells and underscore the need for well-controlled *in vitro* culture systems to characterize these phenomena. When comparing the results from the bulk and microwell culture system, we found that paracrine-based regulation is context-dependent. Among small numbers of macrophages in a microwell, paracrine interactions exerted pro-inflammatory effects. However, at higher cell numbers associated with bulk culture, anti-inflammatory effects resulted. These observations

were only discovered under more precise control of the cellular environment in a microwell system, and the traditional bulk culture system itself is insufficient to evaluate these parameters. In addition, macrophages are sensitive to other environmental conditions, including changes in biophysical cues, which can be better controlled and studied using *in vitro* platforms(15,52). Microphysiological systems possess many favorable characteristics including flexibility in the design of the system, as well as the ability to pattern or control the multicellular architecture cells to better mimic the *in vivo* environment (111). In addition, the ability to integrate different physical stimuli into the system, such as mechanical stretch (120), also allows these systems to study the possibility of macrophages communicating features of physical environments through soluble signals. Further advancements in microphysiological systems may help uncover new mechanisms in the regulation of macrophage activation.

Our studies highlight several important challenges regarding the design for *in vitro* culture studies, especially for micro-scale cultures. First, it is necessary to choose proper culture parameters when designing experiments, since there is a possibility that the experimental results are influenced by specific conditions. Both our microwell studies and other published work showed that the size of the cell population affects the cellular characteristics (121). It is also important to consider these variables when comparing results from different studies. It has been previously noted that reporting of specific culture parameters is necessary to ensure reproducibility (122), and our study reinforced this notion by demonstrating that experimental design strongly influences macrophage behavior and experimental outcomes. These two points suggest both opportunities and challenges for micro-scale cultures: these systems enable better control and modeling of physiological cellular processes, but micro-scale and macro-scale cultures indeed introduce widely different environmental conditions. To facilitate better

comparisons across different studies, further study will be needed to understand how the different culture parameters between both culture formats could affect experimental outcomes.

2.5. Conclusion

In this study, we report that cell seeding density and cell-to-media-volume ratio both affect macrophage activation following pro-inflammatory activation. These results signify the importance of the experimental design in *in vitro* studies of macrophage biology and offer insights into how paracrine interactions among macrophage populations influence their function. The results provided by our study may provide a starting point to help in the design of future studies involving micro-scale culture platforms and ensure that the results could capture the *in vivo* conditions, as well as facilitating the comparison of the results with the established macro-scale culture systems.

CHAPTER 3: Cell Crowding-Mediated Modulates of Macrophage Activation Through a Piezo1-Dependent Mechanism

3.1. Introduction

Macrophages are a versatile cell type that contributes to many important body functions, including wound healing, immune defense, and tissue homeostasis (5). Their plasticity to polarize into different phenotypes enables macrophages to perform these diverse and often tissue-specific roles (123). Such changes in macrophage phenotype are sensitive toward the cues from the surrounding soluble and adhesive environment (8,9,27). The effects of soluble environments have been explored in-depth, and many cytokines known to regulate pro-inflammatory activation, such as TNFα and IL10, have been identified (27). Recent works have also revealed the roles of the physical environment in modulating macrophage activation (8,9). Studies from our lab and others have indicated that elongated cells shape (14), cell confinement (80), substrates stiffness (18–20), and mechanical stretch (24) can downregulate pro-inflammatory activation, while cyclic pressure (15) contribute to inflammatory responses. Although more is known on how macrophages sense the environment, the interaction between cells and the surrounding is dynamic, and changes in cellular characteristics could also alter the macrophage interaction with the extracellular cues.

Recent studies suggest that cells acting collectively in populations of different sizes can alter their sensing and responses to the surrounding environment. For soluble factors, the sharing of some secreted factors is necessary to fully activate macrophages and dendritic cells (35,41), and accumulation of these factors could contribute to a density-dependent regulation of cellular responses (40,78). One study stimulated macrophages seeded in high and low densities and track their responses using intracellular flow cytometry and genetically encoded fluorescent reporters

(40). They found that cells seeded in high densities exhibited stronger activation following stimulation, and the accumulation of secreted factors in the resting phase contributes to this increase (40). We also observed a density-dependent effect of macrophage activation, although with an opposite trend possibly due to the differences in the cell types (RAW vs. BMDM) used in experiments. On the other hand, the physical crowding of cells may also alter their responses to the adhesive environments. Increasing the seeding density of human mesenchymal stem cells has been shown to override the effects of substrate stiffness on the regulation of morphology and proliferation (67). Macrophages crowding also led to lower actin polymerization (80), which could affect how these cells interact with the surrounding environment, as actin structures play an important role in the sensing of physical surroundings (9). Despite the current advancements in these areas, how cell crowding alters the interactions between macrophage and the surrounding physical environments are still being investigated.

The recent discovery of the Piezo1 mechanosensitive ion channels has contributed significantly to our understanding of the interaction between cells and the mechanical cues (124). Piezo1 is a trimeric, transmembrane protein that resembles the shape of a propeller with a pore in the middle of the complex (86). The pore would open in response to mechanical stretch and allow ions to pass through and initiate the downstream signal processes (86). Since its discovery, Piezo1 has been implicated in numerous physiological processes, including regulation of bone formation and normal heart function (97,99), as well as sensing of shear stress and fluid flow in the vascular system (100). Piezo1 has also been implicated in the sensing of crowdedness in epithelial tissue and confinement for migrating cells (106,108). Several groups, including ours, have identified this channel to be important for macrophage mechanotransduction and modulation of the resultant inflammatory responses. These reports demonstrated that cyclic

pressure and stiffer substrates promote Piezo1 activity in macrophages, resulting in effects including pro-inflammatory and antibacterial responses (15,20,109). Interestingly, Piezo1 expression and activity can also be modulated by the environment. Forces presented during the bone formation process have been demonstrated to promote Piezo1 expression (97). In addition, there are suggestions of positive feedback between Piezo1 activity and cytoskeleton dynamics (20,92), and actin polymerization has been known to be affected by environmental factors such as cell confinements (80). Piezo1 appears to be a candidate that could contribute to the effects of cell crowding on macrophage activation, but currently, no studies have explored this possibility.

In this study, we explore the roles of Piezo1 in cell crowding-mediated macrophage activation. We examined the calcium activities, Piezo1 activities, and Piezo1 expression in crowded and non-crowded conditions. We also manipulated Piezo1 expression and function using genetic knockout mice and Yoda1 (95), a Piezo1 agonist, to assess the functional roles of Piezo1 at different crowding levels. The results suggest a dependence of Piezo1 in mediating the crowding-induced regulation of macrophage activation, in which the activity of Piezo1 decreases with increasing cell density. This study provided a novel explanation to the observed density/crowding dependent effect of macrophage, and future studies on elucidating the detail mechanisms can yield more insights on the broader impacts of cell crowding on the sensing and function of macrophage cell populations.

3.2. Materials and Methods

3.2.1. Animal

All protocols involving animals were approved by the University of California Irvine's Institutional Animal Care and Use Committee, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALACi). C57BL/J mice were purchased from Jackson Laboratory. Piezo1$^{\Delta LysM}$ mice were generated with the outlined approach. LysM$^{Cre/Cre}$ (Jackson Laboratories stock no. 004781) mice were first bred with Piezo1$^{flox/flox}$ (Jackson Laboratories stock no. 029213) mice to generate offspring that were heterozygous for both genes. These mice were then bred again with Piezo1$^{flox/flox}$ mice to produce the conditional knockout Piezo1$^{flox/flox}$LysM$^{Cre/+}$ (or Piezo1$^{\Delta LysM}$) mice and the heterozygous control Piezo1$^{flox/+}$LysM$^{Cre/+}$ (or Piezo1$^{fl/+}$) mice. LSL-Salsa6f-Vav1$^{Cre/+}$ mice for calcium imaging were produced following a similar approach. Piezo1P1-tdT mice were kindly provided by the Pathak laboratory.

3.2.2. Cell isolation and culture

Bone marrow-derived macrophages (BMDM) were derived using an existing protocol. In general, the femur and tibia were harvested from mice between 6 to 12 weeks old, and the cells in bone marrow were flushed and treated with ACK lysis buffer to eliminate red blood cells. These cells were then plated with DMEM media containing 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 2mM L-glutamine, and 10% of conditioned media from CMG 14–12 cells to supply macrophage-colony stimulating factor (M-CSF). Cells were fed on day 3 and used on day 6 to day 8 for experiments. In some cases, cells were frozen for use in the future. For seeding, cells were lifted from the plates using cell dissociation buffer (Life Technology) and gentle scraping. They were seeded in different densities (2.56E5 cells/cm^2, 5.13E4 cells/cm^2, and 6.41E3 cells/cm^2 in most of the cases, and 2.56E5 cells/cm^2, 7.79E4

cells/cm^2, 2.56E4 cells/cm^2, and 6.41E3 cells/cm^2 in some cases), left adhered for 2 or 24 hours, and stimulated with 10ng/ml of Lipopolysaccharide (LPS) and interferon-gamma (IFNγ) for 2 or 24 hours prior to sample collection. In some conditions, cells were treated with Yoda1 at concentrations of 300nM and 5μM to assess the effects of Piezo1 on the density-dependent effect of macrophage activation. For studies involving actin quantification, LSL-Salsa6f-Vav1$^{Cre/+}$ tdTomato expressing BMDM were mixed into high, mid, and low densities BMDM cultures to achieve a constant cell density of (2.56E4 cells/cm^2) across all conditions. For studies involving pharmacological manipulation of actin polymerization, cells were treated with DMSO or 500nM of Latrunculin A (LatA) for 15 minutes, before they were stimulated with 10ng/ml of LPS and IFNg for 6 more hours.

3.2.3. Calcium imaging and analysis

Calcium imaging was performed on BMDMs from LSL-Salsa6f-Vav1$^{Cre/+}$ mice using an Olympus Fluoview FV3000RS confocal laser scanning microscope equipped with a high-speed resonance scanner and IX3-ZDC2 Z-drift compensator. Cells were seeded on 35mm glass-bottom MatTeck dishes coated with fibronectin and imaged with an Olympus 40X oil objective (NA 1.25) using excitation line scan at 488nm and 561nm. The temperature of the dishes was maintained at 37°C on the Tokai Hit incubation stage. Data were analyzed using a ratiometric approach on ImageJ. Background subtraction was first performed on the time-lapse images, the subsequent data were then inspected for calcium events, with the locations of the events outlined to generate time-dependent traces of calcium signals. Here, the calcium signal is represented as the G/R ratio, which is the ratio of signals between the calcium-dependent green (GCaMP6f) channel and the calcium-independent red (tdTomato) channel. These data were then processed

using a custom MATLAB script to remove baseline values using a polynomial fit and find calcium events using a threshold-based approach. The recorded numbers of the events were then normalized with the cell counts and the duration of experiments to determine the level of calcium activities. Similarly, the percent active cells were determined by dividing the number of cells exhibiting at least one calcium event by the total cell count. For the analysis of Yoda1 treated cells, outlines were drawn around the cells, and the average G/R ratios for the whole cell were used for quantification. The subsequent data were then analyzed with custom scripts in R.

3.2.4. RNA Isolation and quantitative real-time PCR

Cells were lysed with TRI-reagent (Sigma), and the RNA was isolated using the manufacturer's protocol. cDNA was then produced using a cDNA reverse transcription kit (Applied Biosystem). For qPCR analysis, PerfeCTa® SYBR® Green SuperMix (QuantaBio) was used, and the process was done with a Bio-Rad's CFX96 real-time PCR System. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, and the expressions were normalized to the housekeeping gene *Gapdh*. Following primers were used for analysis: *Piezo1* (Forward: GTTACCCCCTGGGAACATCT, Reverse: TTCAGGAGAGAGGTGGCTGT), *Gapdh* (Forward: GTCAAGCTCATTTCCTGGTAT, Reverse: TCTCTTGCTCAGTGTCCTTGC)

3.2.5. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Science), washed with phosphate-buffered saline (PBS), and then permeabilized with 0.2% of Triton-X 100 for 10 minutes. After another round of wash with PBS, cells were blocked in 2% bovine serum albumin (BSA) (MP Biomedical) overnight. For staining, cells were incubated overnight at 4°C with anti-

RFP antibody (Rockland) in 1:400 dilution and washed with 1% BSA. Cells were then incubated with secondary antibody and washed again with 1% BSA. Afterward, cells were stained for actin and nucleus with Alexa Fluor 488 or 647 phalloidin and Hoechst (Invitrogen) respectively for 30 minutes and washed with PBS. Finally, cells were mounted onto glass slides with Fluoromount-G (Southern Biotech).

3.2.6. Microscopy imaging and analysis

Cells were either imaged with an Olympus IX-83 inverted microscope or ab FV3000 laser scanning confocal microscope. For the quantification of actin polymerization, only tdTomato expressing cells were quantified as it was difficult to trace the outlines of unlabeled cells in crowded states. To perform the analysis, samples were imaged at the blue, red, and green channels to visualize nucleus, tdTomato-expressing cells, and actin staining respectively. The red channel images were used to trace the outlines of the tdTomato-expressing cells in ImageJ, and the actin staining intensities of those cells were then quantified following background subtraction. Subsequent data were analyzed in R using a custom script

3.2.7. ELISA

The supernatants from different cultures were collected, and the TNFα and IL10 concentrations were analyzed using the ELISA Max Standard Set kits from Biolegend following manufacturer recommendations.

3.2.8. Western Blot

Samples were lysed using RIPA lysis buffer (VWR) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The lysate was boiled in water for 10 minutes with Laemmli buffer supplemented with 5% 2-mercaptoethanol to denature the samples. Afterward, the samples were loaded into wells of a 4–15% mini-PROTEANTM precast gel (all from BioRad), and electrophoresis was performed to separate proteins from the loaded lysates. After the procedure, proteins were transferred onto nitrocellulose membrane through the iBlot dry blotting system (Thermo Fisher Scientific) and blocked with 5% non-fat milk for one hour. For Piezo1, samples were loaded into a 4% polyacrylamide gel instead, and the proteins were transferred to the nitrocellulose membrane via a wet-transfer system.

Membranes were then incubated with primary antibody either overnight at 4°C or one hour at room temperature, washed, and then incubated again with secondary antibody conjugated with horseshoe peroxidase. After additional rounds of washing, samples were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged with a Bio-Rad ChemiDoc XRS+ system with Image Lab software.

3.2.9. Statistical analysis

Data were analyzed using a two-tailed Student's t-test followed by Bonferroni correction whenever necessary, a one-way ANOVA followed by Tukey HSD test, or a two-tailed Mann–Whitney U test. A p-value less than 0.05 was considered statistically significant.

3.3. Results

3.3.1. Regulation of Calcium Signaling Activities in Macrophages is Density-Dependent

Cell crowding has been demonstrated to regulate macrophage activation following pro-inflammatory activations in several studies (80). To ensure we could replicate these density-dependent effects for this current study, we stimulated cells seeded in high (2.56E5 cells/cm^2), medium (5.13E4 cells/cm^2), and low (6.41E3 cells/cm^2), densities, and measured the subsequent per-cell TNFα and IL10 secretion (Figure 3.1A). As expected, per-cell TNFα secretion decreased with increasing seeding densities, consistent with previous studies (80). Per-cell IL10 secretion had significant variations among different replicates, and a significant trend was not observed. This also agrees with our previous work. Overall, TNFα secretion exhibited a consistent decreasing trend with increasing cell-crowdedness, and as a result, we would use it as a marker in the subsequent experiments to assess the crowding effects on macrophages under different Piezo1 manipulations.

The Piezo1 mechanosensitive ion channel primarily exerts its effect through modulating cellular calcium activities. We then evaluated whether calcium activities between crowded (2.56E5 cells/cm^2) and non-crowded (6.41E3 cells/cm^2) cultures were different. We utilized bone marrow-derived macrophages from Vav1-Salsa6f mice, which expresses a genetically encoded Salsa6f probe consisting of a tdTomato fluorophore linked to the GCaMP6f calcium sensor via a V5 epitope tag (125). The calcium-insensitive tdTomato signal accounts for the amounts of available sensors, while the calcium-sensitive GCaMP6f sensor fluoresces in green when calcium is present. The ratiometric measurement of the green to red signal ratio (G/R ratio) enables a good quantification of calcium activities that preclude signal variations from processes such as cell movement (125). This result would allow us to effectively track the calcium activities of cells using a live-cell imaging approach.

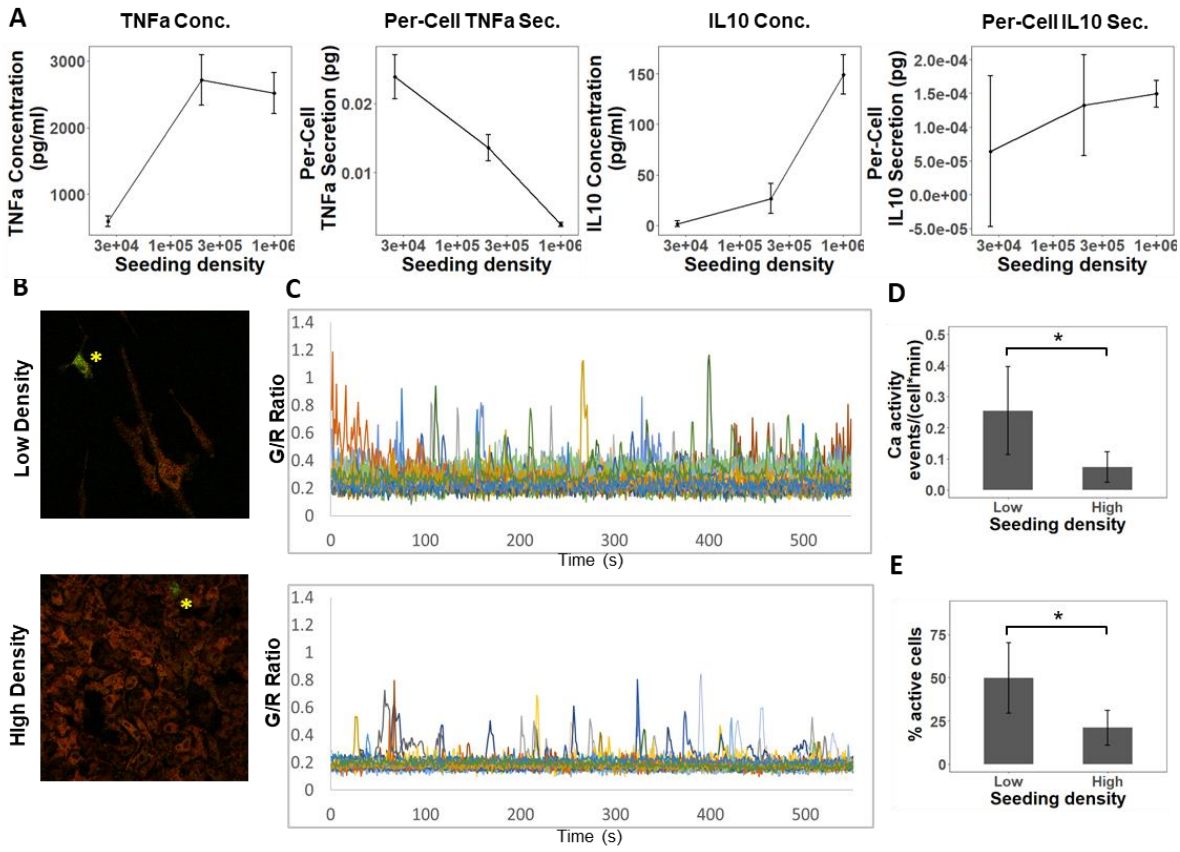

Figure 3.1: Regulation of calcium signaling in macrophages is density dependent. (A) Per-cell secretion of TNFα and IL10 at different seeding densities (B) Images of calcium signals at low density (top) and high density (bottom). Calcium signal is indicated with a yellow asterisk (*). (C) Representative calcium signal traces observed in low density (top) and high density (bottom) field of views. The low-density trace pooled data from 2 field of views to obtain similar number of active cells (24 regions with calcium activities for both conditions) for comparison (D) calcium activities for cells at low and high densities. (E) percent cells with calcium activities at low and high densities. (n=3 biological replicates for data in A, and n=7 fields of views for data in panel D and E, * indicates $p < 0.05$ as assessed using two-way Student t-test)

Following pro-inflammatory stimulations, localized, transient calcium flickers were observed in some cells in both conditions (Figure 3.1B). To better characterize the magnitude and frequencies of these flickers, individual calcium events were identified and traced to assess their temporal dynamics. The results revealed that macrophages in non-crowded conditions appeared to generate more calcium events than cells in crowded conditions (Figure 3.1C). Indeed, non-crowded cells exhibited more frequent calcium events, as well as having a higher percentage of calcium active population than crowded populations (Figure 3.1D and 3.1E).

Overall, these results suggested a difference in calcium activities between cells in crowded and non-crowded conditions.

3.3.2. Piezo1 is implicated in the density-dependent calcium signaling in macrophages

To determine whether Piezo1 activity is implicated in the crowding-dependent calcium activities, as well as the subsequent signaling outcomes, we proceeded to treat macrophages with a Piezo1 agonist, Yoda1. Using it at a low dose (300nM) enabled us to assess Piezo1 activity in response to outside perturbations. Macrophages were seeded at high (2.56E5 cells/cm^2), medium (5.13E4 cells/cm^2), and low (6.41E3 cells/cm^2) seeding densities, and the resultant calcium activities were recorded after treatments with 300nM of Yoda1. Yoda1 induced density-dependent increases in both local and global calcium signals (Figure 3.2A). Cells with the lowest seeding density appeared to generate the most calcium activities following the stimulation, while cells with the highest density generated the least (Figure 3.2B). In addition, following Yoda1 treatment, low-density culture recorded an average G/R ratio of 0.69, while that for high-density culture was only 0.23 (Figure 3.2C). Around 93% of cells in the sparse conditions had over 50% increase in calcium level over the baseline, but only around 25% of cells in the crowded condition experienced such an increase (Figure 3.2D). These results suggested that macrophages are more likely to generate Piezo1-mediated calcium activity in spare than in crowded cultures.

The functional consequences of Piezo1 in the crowding-dependent effects of macrophage activation were also examined. We first assessed the effects of Piezo1 loss-of-function by utilizing the BMDM from Piezo1$^{\Delta LysM}$ mice, in which Piezo1 expression is ablated in LysM-expressing myeloid cells. Piezo1$^{\Delta LysM}$ BMDM, as well as the control Piezo1$^{fl/+}$ BMDM, were seeded in different densities, left to attach overnight, and then stimulated with LPS and IFNγ overnight. Compared to control BMDMs, cells from the Piezo1$^{\Delta LysM}$ mouse exhibited fewer

variations in TNFα secretion across different seeding densities (Figure 3.2E). We then assessed the effects of over-activating Piezo1 by treating cells in different seeding densities with DMSO or Low (300nM) and High (5μM) doses of Yoda1. High doses of Yoda1 appeared to increase the differences in per-cell TNFα secretion across sparse and dense cultures, while a low dosage of Yoda1 did not exhibit such a trend (Figure 3.2F). Interestingly, a high dosage of Yoda1 did not

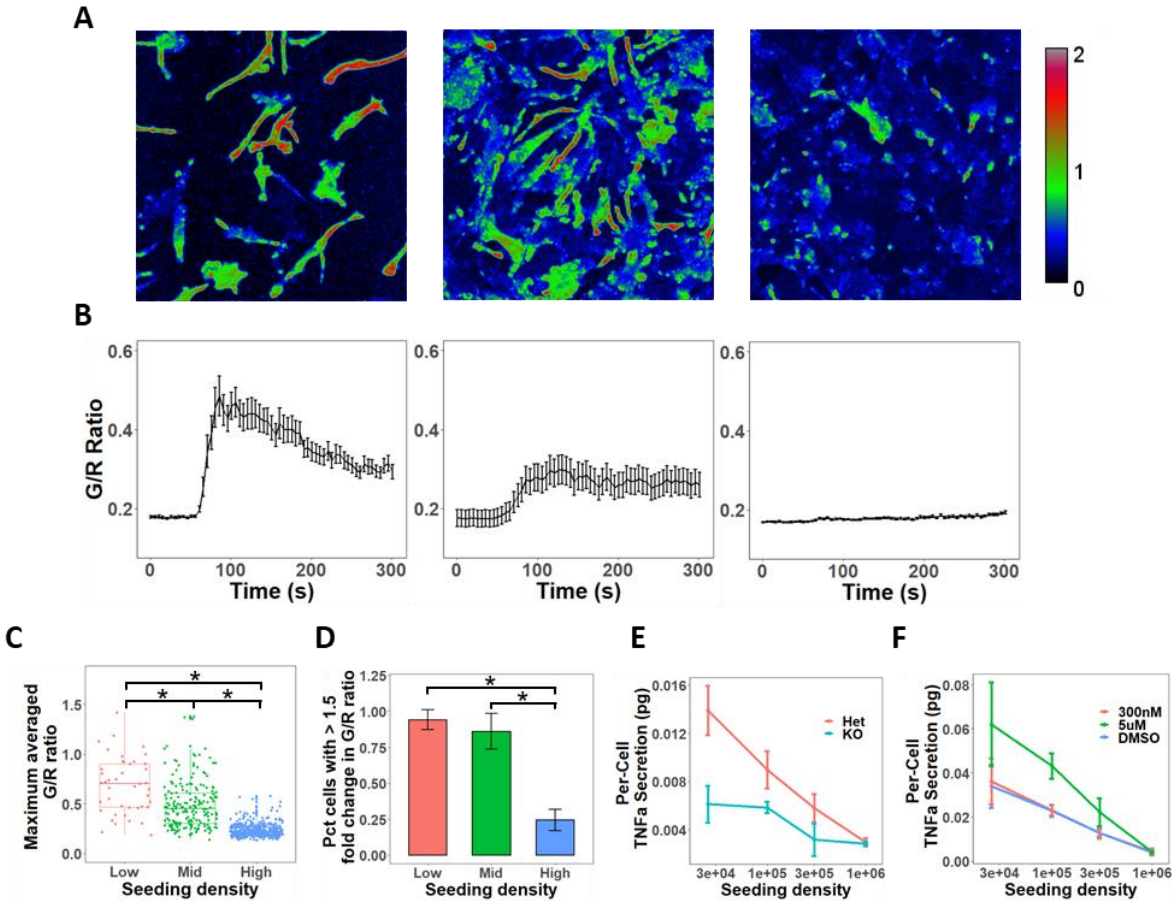

Figure 3.2: Piezo1 is implicated in the density-dependent calcium signaling in macrophages (A) Representative maximum projection of G/R ratio for calcium signal of Yoda1-treated (300nM) cells at different seeding densities. (B) Averaged whole-cell calcium tracing of Yoda1-treated (300nM) cells at different seeding densities. (C) Distribution of maximum average whole-cell G/R ratio for cells seeded at different densities. (D) Percentage of cells with maximum average cell G/R ratio over 1.5-fold of the baseline. (E) comparison of per-cell TNFα secretion between BMDM from Piezo1 knockout and the corresponding heterozygous control at different densities. (F) comparison of per-cell TNFα secretion for cells at different densities treated with either 300nM or 5uM of Yoda1, as well as the DMSO control. (Data in B is presented in mean ± standard error, n=3 fields of view for panel D, n=3 biological replicates for data in panel E and F; * indicates $p < 0.05$ as assessed using two-tailed Mann-Whitney U test for panel C and one-way ANOVA followed by Tukey HSD test for panel D)

affect TNFα secretion two hours after activation, suggesting that Piezo1 activation mainly contributes to TNFα secretion only at the later phases of the pro-inflammatory activation (Figure 3.3). As eliminating the Piezo1 function reduced the crowding-dependent variation and augmenting the Piezo1 function enhanced it, our results demonstrated a role of Piezo1 in the crowding-induced modulation of macrophage activation.

3.3.3. Short adhesion time suppress the functional roles of Piezo1 in macrophage activation

While earlier experiments linked a role Piezo1 in mediating the crowding-induced regulation, it was not known whether changes in the adhesion conditions, such as disrupting the cell-ECM interaction through detachment followed by attachment, may alter such effect. Previous studies (126), as well as our works (Figure 3.4), demonstrated that macrophages with shorter attachment time following seeding secreted less TNFα after stimulation. To investigate

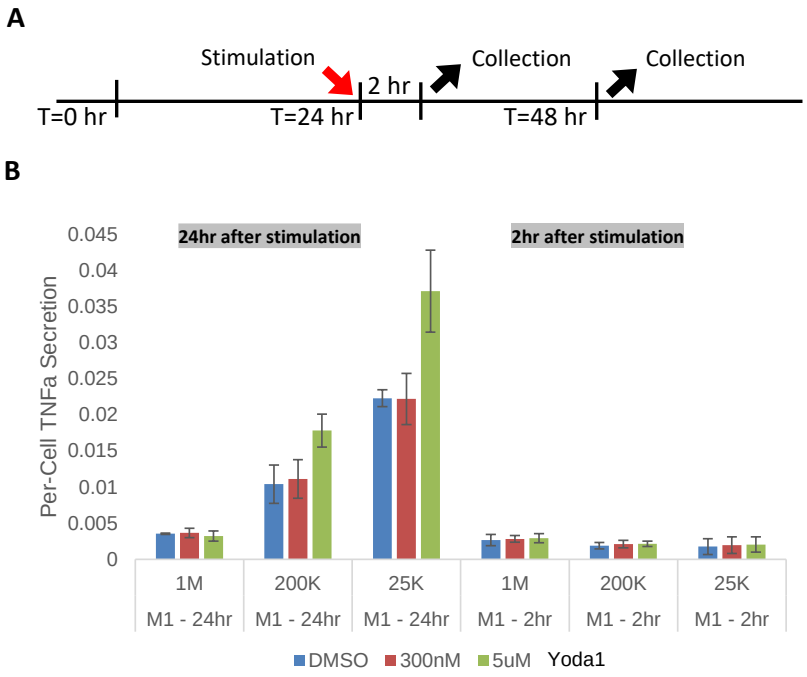

Figure 3.3: Calcium primarily act on the later phase of macrophage activation. (A) schematics of the experiment (B) per-cell TNFa Secretion 2 and 24 hours after stimulation (with 24 hours of adhesion time), with and without Yoda1 (n=3 for data in B)

this effect in different crowding conditions, we seeded cells in high, medium, and low densities, and stimulated cells after short (2 hours) or long (24 hours) attachment time (Figure 3.5a). Shorter attachment times significantly reduced TNFα secretion in samples with medium and low seeding densities, as well as the variations in per-cell secretion across different seeding densities (Figure 3.5b). Surprisingly, repeating the experiment with Yoda1, even at a high dosage, failed to augment the per-cell TNFα secretion across all seeding densities with 2 hours of attachment time (Figure 3.5C). This result suggested that short attachment time can inhibit Piezo1-mediated increases in macrophage activation through an unknown mechanism.

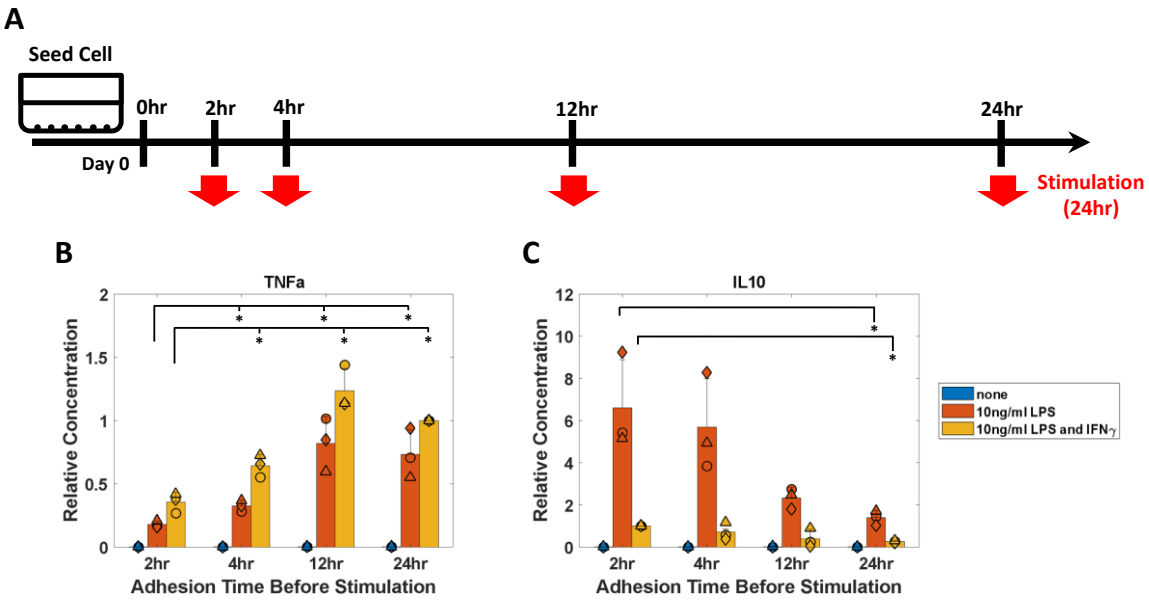

Figure 3.4: Shorter macrophage adhesion times prior to pro-inflammatory stimulation result in altered macrophage activation characterized by lower TNFa secretion and high IL10 secretion: (A) a schematic of the experiment. Cells were seeded at a density of 51K/cm2, allowed to adhere for 2, 4, 12, and 24 hours before being stimulated with 10ng/ml of LPS with or without IFNγ for 24 hours; (B) TNFα and (C) IL10 concentration of LPS and IFNγ activated BMDM stimulated following 2, 4, 12, 24 hours of adhesion time. (n=3 for all conditions, * indicates $p<0.05$; Bonferroni correction for multiple comparison)

3.3.4. Piezo1 expression is not the main cause for the crowding-mediated regulation of macrophage activation

One possible explanation for Piezo1's role in the crowding-dependent regulation of macrophage activation is that total Piezo1 expression may vary with different cells densities and attachment times. To test this hypothesis, Piezo1 expression was evaluated using qPCR, immunofluorescence staining, and western blot. qPCR result demonstrated that after overnight attachment, Piezo1 gene expression was lower in high-density culture than in low-density culture (Figure 3.5G), which appeared to agree with our hypothesis. At 2 hours of attachment time, there were no significant differences in Piezo1 expression among the different seeding densities. However, Piezo1 gene expression appeared to be higher in cultures with shorter adhesion time than those with overnight adhesion time, which contradicted the results from the functional experiments.

Interestingly, Piezo1 protein expression presented yet a different trend than that obtained at the gene level. To reliably detect the Piezo1 protein, experiments were performed using BMDMs derived from the Piezo1P1-tdT mice with its endogenous Piezo1 protein fused to tdTomato (83). Samples collected from the experiments were then probed using an anti-RFP antibody. For cultures with overnight adhesion time, results from immunofluorescence staining and western blot did not suggest decreased Piezo1 expression in high-density cultures (Figure 3.5D-3.5F). Quantification of the immunofluorescence images even suggested that the crowded population expressed more Piezo1 than the sparse population (Figure 3.5E). For cells with 2 hours of attachment time, Piezo1 expression appeared to be lower than those with overnight attachment, but there were no noticeable differences in Piezo1 expression across different seeding densities. Interestingly, Piezo1 appeared to aggregate in the cytoplasm of crowded cells, as opposed to the more evenly spread distributions in non-crowded states. We attempted to assess Piezo1 function by performing calcium imaging on macrophages with short and long

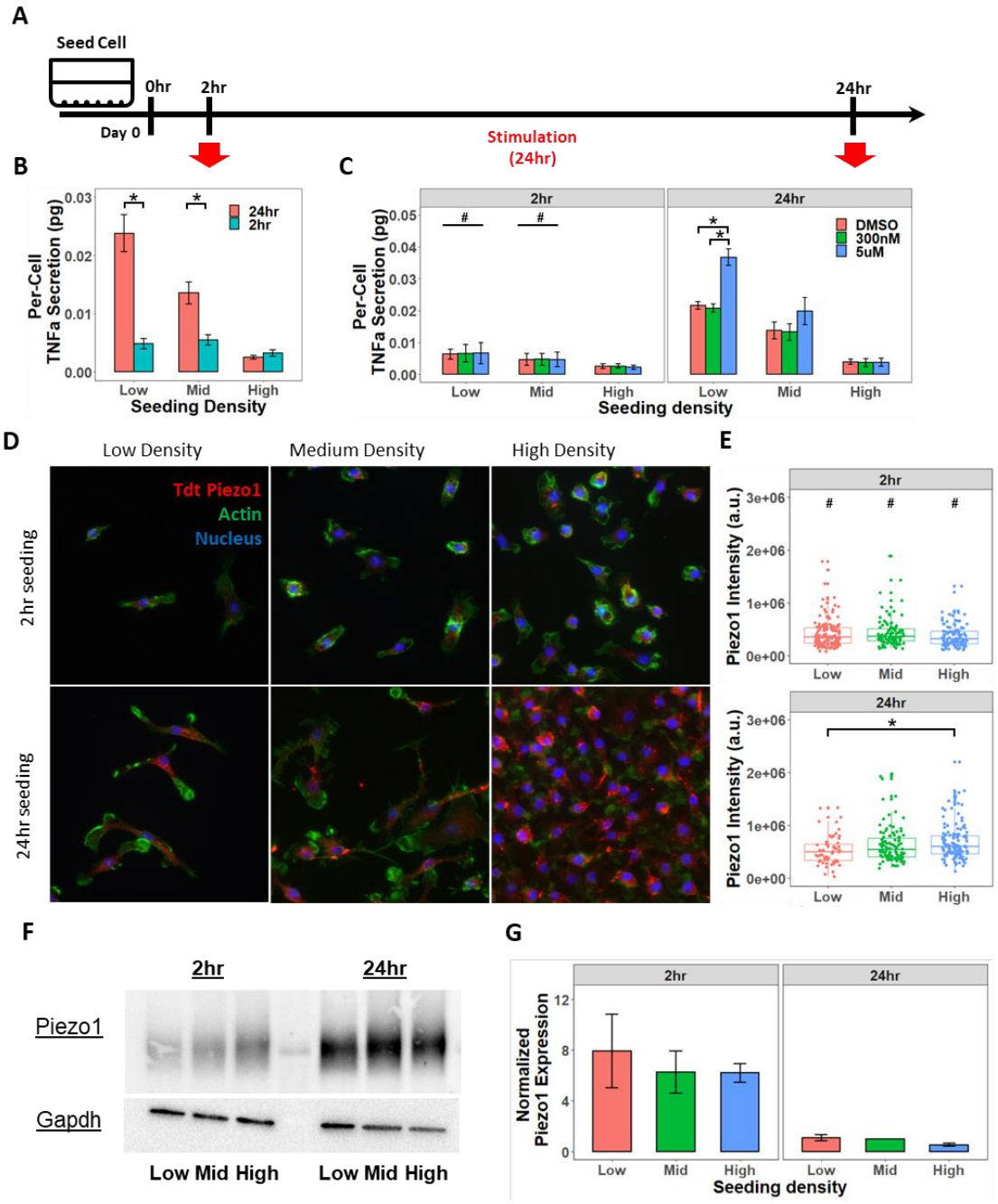

Figure 3.5: short adhesion time may reduce the influence of Piezo1 on the density-dependent calcium signaling. (A) A schematic of the experiments to test the effects of cell adhesion time on cell function. (B) Per cell TNFα secretion of macrophages in different densities and stimulated 2 hours or 24 hours after seeding. (C) per-cell TNFα secretion for Yoda1 or DMSO treated macrophages stimulated two hours following seeding (D, E) Representative images (D) and quantification (E) of tdTomato-Piezo1 expression for cells in different seeding densities and with short (2hr) and long (2hr) attachment times. (F, G) Representative Western Blot analysis (F) and gene expression (G) of tdTomato-Piezo1 expression for cells in different seeding densities and attachment times. (n=3 biological replicates for data in panel B, C and G, data in panel E were from a single experiment; # indicates $p < 0.05$ when comparing samples with the same seeding density between 2hr and 24hr, and * indicates $p < 0.05$ as assessed using two-tail Student t-test for Panel B, one-way ANOVA followed by Tukey HSD test for panel C, and two-tailed Mann-Whitney U test for panel E)

attachment time and treated with a high dosage of Yoda1 (Figure 3.6). For cells with short adhesion time, due to the low throughput nature of the calcium imaging, we could not record calcium activity for all conditions rapidly. The actual recording time ranged between two to six hours after seeding. Regardless, robust calcium events were recorded in all conditions, including those with shorter adhesion time, indicating that Piezo1 is likely functional even with a short attachment time. As Piezo1 exerts its function at protein levels, we concluded from the protein level results that Piezo1 expression is not the main cause for the crowding-induced variation in

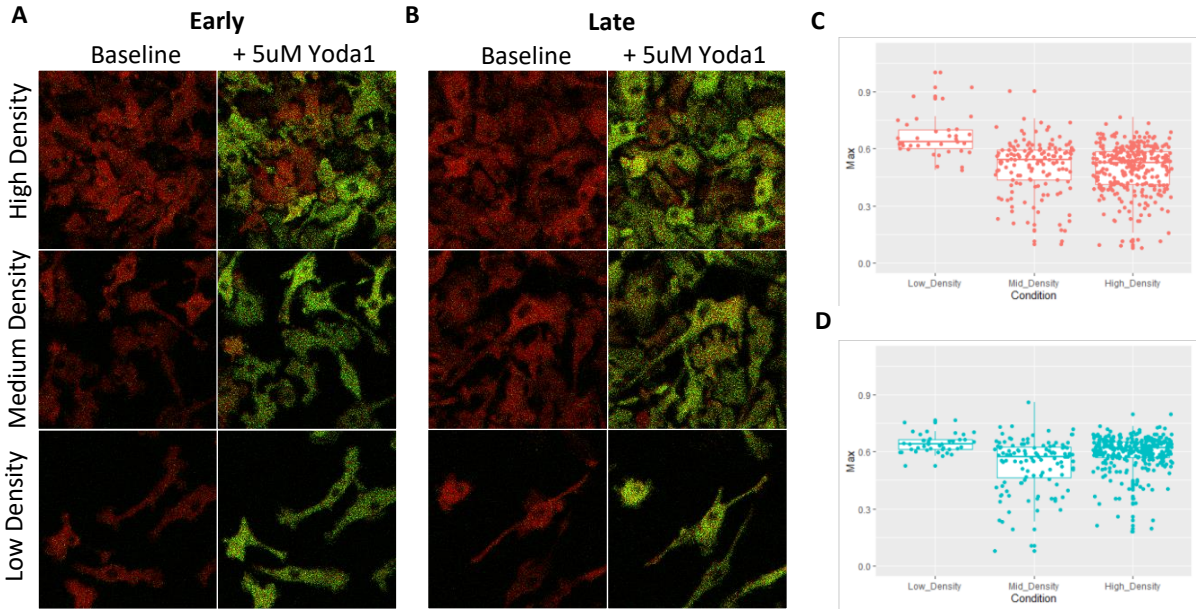

Figure 3.6: Macrophages respond to high dose of Yoda1 (5uM) at both earlier and later attachment time
(A, B) Live cell calcium imaging of macrophages at different seeding densities (A) early (2-6 hours) and (B) late (>24hr) after seeding. (C, D) Maximum G/R Ratio for Yoda1 treated cells (C) early (2-6 hours) and (D) late (>24hr) after seeding. Data from one imaging experiment

calcium signaling. Other unknown mechanisms, which may involve pathways downstream of calcium signaling, may be responsible for the altered effects of Piezo1 in macrophage activation.

3.3.5. Actin polymerization may contribute to the observed density-dependent calcium signaling

Actin polymerization could contribute to the Piezo1-mediated calcium modulation in a crowded environment. Confinement-induced reduction in actin polymerization has been demonstrated to downregulate late-phase inflammatory responses (80), and our earlier studies also suggested a positive correlation between actin expression and Piezo1 channel activities (20). To address this possibility, we first assess the degree of actin polymerization in cultures of different crowding levels. Macrophage cultures with different crowdedness were fixed and stained with phalloidin 2 and 24 hours after seeding, and the whole-cell actin expression level was quantified. Small amounts of Salsa6f cells were mixed into the seeded culture across all seeding densities, which helped visualize cellular outlines and facilitated boundary tracing especially in crowded cultures, as the cell boundaries were often hard to identify. The results suggested that cells with 24 hours of attachment time had greater variations in actin staining between high and medium or low densities than cells with 2 hours of attachment time (Figure 3.7A, 3.7C). These results appeared to match the corresponding trends in TNFα secretion for cells of different attachment times and seeding densities (Figure 3.5B). Interestingly, high-density culture did not have significant differences in actin polymerization between 2 or 24 hours of attachment time.

We then tested the hypothesis that disrupting actin polymerization would alter the crowding effects. Latrunculin A was used to disrupt actin polymerization, and cultures with different seeding densities were fixed and stained for phalloidin one hour after drug treatment. A similar approach of mixing Salsa6f cells was used to facilitate outlining cellular boundaries. We observed that latrunculin A induced a robust decrease in actin staining intensity across all seeding densities (Figure 3.7B, 3.7D). For functional experiments, DMSO treatment groups exhibited significantly higher secretion of per-cell TNFα in low and medium-density cultures 6

hours after stimulation, when compared with that of high-density culture (Figure 3.7E). Interestingly, variations in per-cell TNFα secretion among different seeding densities did not reach statistical significance for the latrunculin A-treated group. As latrunculin A is known to alter actin polymerization by destabilizing it respectively, we believe that disruption of actin polymerization can regulate the crowding-induced effect. However, the exact mechanism still needs further investigation.

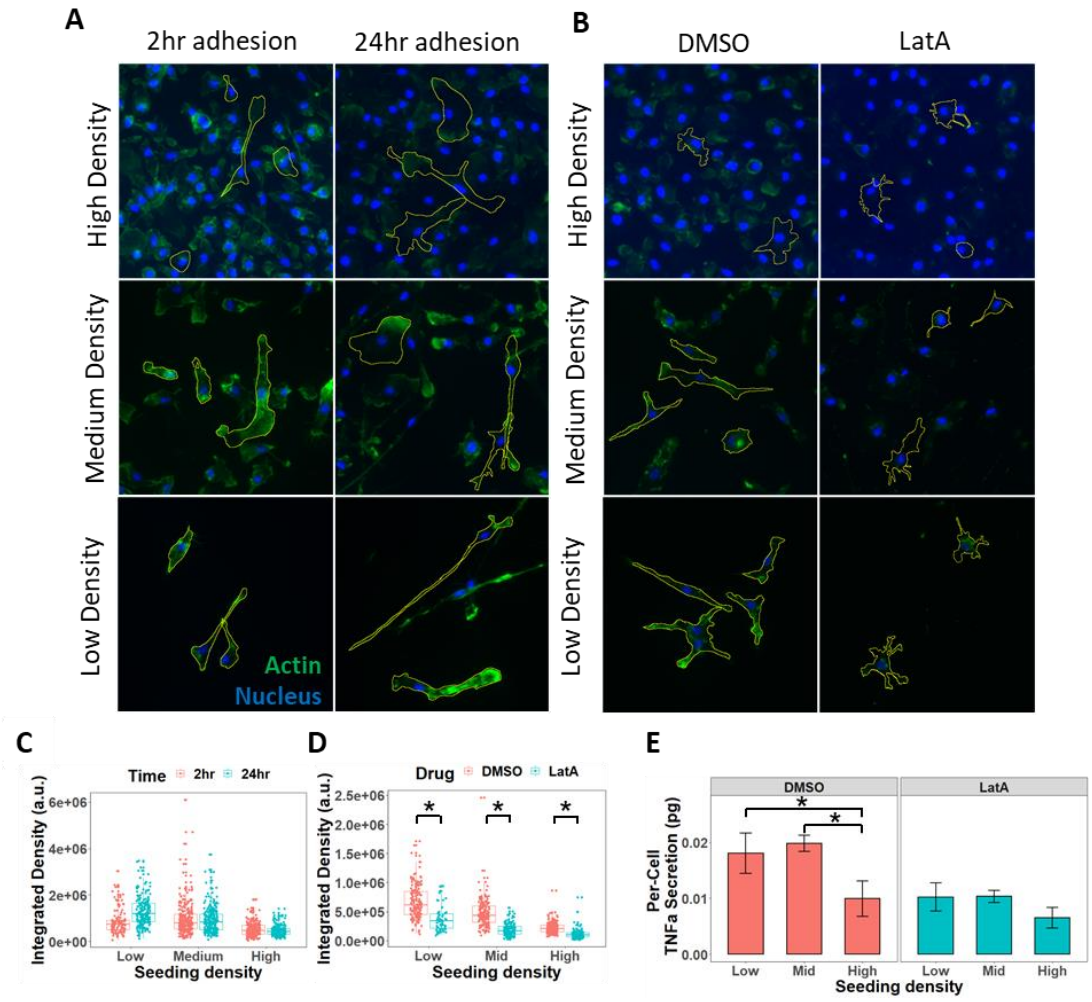

Figure 3.7: Actin polymerization may be involved in the observed density-dependent calcium signaling. (A) Representative actin staining images of cells at different densities. (B) Representative images of DMSO and Latrunculin treated cells at different seeding densities. (C) Quantification of actin staining intensity of macrophages seeded at different densities from a representative image. (D) Quantification of cell area and total actin staining for the drug-treated cells seeded in different densities and seeding time. (E) Per-cell TNFα secretion of macrophages treated with DMSO and Latrunculin, and seeded at different densities. (n = 3 for the data in Panel E; * indicates $p < 0.05$ as assessed using two-tailed Mann-Whitney U test for data in Panel D and one-way ANOVA followed by Tukey HSD test for data in Panel E)

3.4. Discussion

Increasing evidence highlighted the different properties between crowded and sparse macrophages populations. In this study, we demonstrated that Piezo1, a mechanosensitive ion channel, contributes to the crowding-dependent regulation of macrophage activation. Crowding decreased calcium activities in macrophages following pro-inflammatory activation, and Piezo1 is implicated in this process. Literature has reported density-dependent differences of calcium oscillation tendencies in HeLa and HEK293 cells, suggesting that density may have broad influences on calcium activities of diverse cell types (127). We also showed that differential Piezo1 activities may regulate crowding-dependent variations in macrophage activation, as knocking out Piezo1 reduced the crowding effect and chemical activation augmented it. Our finding reaffirms the reported correlation between Piezo1 activity and macrophage activation (20). Interestingly, since short adhesion time abrogated this effect, there may be additional adhesion-related mechanisms regulating the signaling downstream of Piezo1. Finally, we demonstrated a role of actin polymerization in this effect, as destabilizing actin polymerization using latrunculin A inhibited crowding-mediated variations in TNFα secretion. Our results suggested that the crosstalk between actin organization and Piezo1 may help regulate the crowding-dependent effect on macrophage activation. Overall, this study explored another mechanism that enables cell crowding to regulate macrophage function, and further highlighted the potentially broad effects of crowding on different cellular processes.

While Piezo1 is known to facilitate the sensing of cellular confinement and cell crowding, our study suggested that cell crowding could also feedback to regulate Piezo1 functions. Recent works demonstrated the abilities of extracellular cues to modulate Piezo1 expression and function. During bone formation, Piezo1 expression in osteoblasts increased in

response to the mechanical loading (97). In epithelial cells, Piezo1 activations via crowding in high-density culture and stretching in low-density culture yield different results, leading to cell ejection and cell division respectively (107). The different spatial distributions of Piezo1 appeared to contribute to such an effect, in which Piezo1 largely clustered in the cytoplasm in dense culture and localized in both plasma membrane and the cytoplasm in spare culture (107). In our study. we also observed aggregates of Piezo1 in dense culture, and it would be interesting to assess whether such localization could contribute to the crowding effects in macrophages. On the other hand, membrane characteristics and cytoskeletal structures can also affect Piezo1 functions. Previous studies have suggested that cell crowding can increase membrane rigidity and decrease actin polymerization in a cell population (66,80). Assessing the correlation among cell density, membrane properties, actin polymerization, and Piezo1 activity may yield novel insights on Piezo1 and its regulation.

In addition to the regulation through Piezo1, our study indicated that crowding may exert Piezo1-independent effects on macrophage activation. We observed that while macrophages do express Piezo1 two hours after seeding, Piezo1 induced negligible effects on macrophages with short attachment time, and the crowding effect was also diminished. Although the cause is unknown, several factors could contribute to crowding-dependent regulations independent of Piezo1. Actin polymerization is a parameter known to modulate macrophage polarization in response to the physical confinement of cells, including crowding (80). Crowding could also alter the soluble environments, which can feedback to modulate the subsequent inflammatory responses (40). In addition, increasing cell density affects endocytosis, and endocytosis can modulate macrophage activation, as seen in an example that investigated TRPM7-mediated regulations of macrophage activation (63,128). These and other Piezo1-independent mechanisms

may work in synergy with each other, as well as with Piezo1, to exert complex effects on macrophage functions. A better understanding of how these factors crosstalk with each other could yield new insights on macrophage regulation.

Overall, our study reinforced the notion that macrophages exhibit different characteristics and functions between crowded and sparse states. In literature, dense macrophage populations exhibited altered immune response including enhanced iNOS expression and inflammatory activation in RAW 264-7 cells, dampened late-stage activation in BMDM, as well as better control on mycobacterial growth (77,78,80). However, cell crowding can also alter other aspects of macrophage behaviors. The decreased Piezo1 activity in crowded populations suggested that crowded macrophages may sense and interact with the mechanical cues differently than sparse cells, as Piezo1 regulates cellular mechanotransduction. In addition, crowded macrophage populations can have altered metabolic processes via a NO-mediated mechanism that inhibited cellular respiration and decreased ATP: ADP ratio (119). These findings demonstrated that cell crowding could exert broad spectrums of effects on diverse aspects of macrophage biology, and such alterations may have *in vivo* significance. Indeed, higher tumor-associated macrophage density has been associated with a worse prognosis for several types of cancer (129). Although the interplay between macrophages and the tumor environments is largely responsible for such effect(130), high cell density itself could also contribute to the outcome. Future works on the mechanisms contributing to the crowding-induced effects on macrophages may provide novel insight into the regulation of macrophage functions, and this can help equip us to better treat diseases involving macrophage dysfunctions.

CHAPTER 4: Conclusions and Future Direction

Our study gave a glimpse into how population density could regulate macrophage responses through both soluble factors and physical sensing. While it is a general rule of thumb in *in vitro* cell culture that cell confluence could affect cell phenotype, not many studies have systematically studied this issue. Such area could be relevant for macrophages, as macrophage density could vary significantly in different physiological states, and high macrophage density is also associated with some pathological conditions such as in some types of cancers(131). Our study joined other works in exploring this emerging field, and future advancement in this area could yield more insights into macrophage regulation and potentially also into mechanisms involving macrophage-related dysfunctions.

While our studies added more insights into the effects of cell density/cell crowding on macrophage functions, there are still several limitations for the study. First, although our bulk experiments demonstrated that soluble factors could modulate density-dependent macrophage responses, we cannot decipher details of such cell-cell communication. It is a challenge to identify potential cytokines contributing to this effect, as well as distinguishing the effects of cellular uptake from reduced cytokine secretion. Several approaches are available to investigate the components in macrophage secretome that contribute to the density effect, including multiplex immunoassay and mass spectroscopy(132). Another approach involves the use of data from RNA sequencing to predict the signaling compounds(133). This method poses some unique advantages, as it does not require prior knowledge to guess potential candidates, and the analysis on transcripts abundance for cytokines such as TNFα, IL6, and IL10 may help distinguish cellular uptakes from the true effect of density-dependent modulation. On the other hand, accumulated metabolic wastes may also contribute to the density-dependent effects, and our

experimental setup cannot separate such factors from other pathways linked to secreted proteins. To remove metabolic waste, some studies concentrated the conditioned media using ultracentrifugation with low molecular weight cutoff(134). This could mitigate the effects of metabolic wastes, although other low molecular weight compounds may potentially be involved in the regulation as well. Thus, careful experimental planning is required to prevent confounding factors from complicating the results.

Another challenge in our study is the inefficiency of the single-cell microwell system. Our system lacked the capability for multiplexing, which is a common problem for immunofluorescence staining. The multiplex capability for this technique was constrained by the limited numbers of species that antibodies can be sourced from, as well as the spectral overlap from different fluorophores. (135). Some methods exist to bypass this limitation, including the use of quantum dots, which have narrower excitation/emission spectrums so more targets could be probed(58). Other methods have also been developed to accommodate higher numbers of staining targets, including those utilizing different chromogenic, fluorescent, metal, and nucleic acid-based substrate(135,136). On the other hand, our system utilized a passive approach to load cells into microwells, and such a method can only achieve a maximum of 36% single-cell loading efficiency, with lower percentages for loading higher numbers of cells into the well(137). A more active approach could be used instead to increase efficiency. A recent study utilized a device combining a DEP array at the bottom and a microwell array on top(138). The DEP array was first used to trap single cells, and then the device was flipped to transfer captured cells into microwells. Similar devices could be modified to load multiple cells into a well, and this could greatly improve the loading efficiency of the system.

Finally, the significant differences in cell numbers between crowded and sparse conditions, as well as the low throughput nature of the calcium imaging, limit the studies on the role of Piezo1 in the density effect. Current equipment can only image one field of view per run, and each run takes at least 5-10 minutes to complete. Thus, to obtain enough cells in the sparse conditions to generate reliable data, many fields of view are necessary. This would necessitate a substantial amount of time to complete the calcium imaging and cause calcium imaging at early time points to become impractical, as much time would have passed between the first and last fields of acquisition. To address this shortcoming, the role of Piezo1 may be indirectly inferred by examining the differential gene expressions for targets involved in macrophage function and downstream of known calcium pathways induced by the ion channel. Genetic knockdown of Piezo1 can further distinguish the Piezo1-dependent signaling cascade from those caused by other mechanisms. These methods could circumvent the difficulty in performing calcium images at early time points, and they could enable future studies to better understand the role of Piezo1 in modulating density-dependent macrophage activations.

Our studies revealed the different effects macrophage density can have on cellular functions, but there are still many questions to be answered. First, we still lack understandings of how cell crowding could affect the physical interaction between cells and the surrounding environments. As cell crowding reduced Piezo1-mediated calcium activities, this observation suggested that a crowded population may sense the environment differently from that of the sparse population. Future works could assess how mechanical sensing of macrophages, such as that for different substrate stiffness or mechanical stretch, could vary between dense and sparse populations. On the other hand, we also observed that macrophages seeded in different densities exhibited different cellular morphology following pro-inflammatory activation. This is especially

evident in very sparse culture, in which cells stayed in a relatively elongated shape as compared to the more rounded morphology seen in cultures with regular seeding density. Intriguingly, the shape transition occurred around the seeding density of 25.6K cells/cm^2. Cells at this density were not crowded and physical interactions appear to be not prevalent. This suggested the

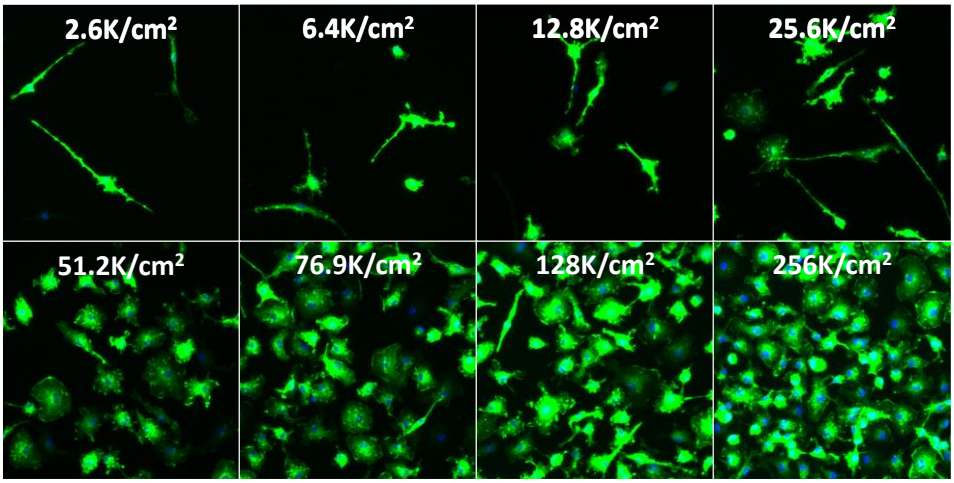

Figure 4.1 Morphology of bone marrow-derived macrophages 24 hours after 10ng/ml LPS + IFNγ stimulation. Cells were stained with iNOS. The staining was enhanced individually to enhance contrast and help identify cellular boundaries.

possibility that paracrine signaling may have broader regulatory effects on macrophages and can regulate some physical properties such as cell shape.

Microwell-based systems could help investigate the potential roles of soluble factors in regulating macrophage physical interactions with the environment. In our current work, we utilized a microwell platform to investigate how soluble factors coordinate macrophages during pro-inflammatory activation. This platform, which consists of a top detection slide, middle microwell membrane, and a bottom substrate for cell adhesion, could be modified to manipulate the cellular mechanical environments. The bottom substrate could be switched to PDMS, which can be tuned to exhibit different levels of stiffness, and the compliant nature of PDMS may also enable mechanical stretching of the device. These modifications could facilitate studies on the heterogeneity of macrophage responses toward varying substrate stiffness and mechanical

stretches, as well as allowing investigators to determine whether cells in isolation exhibit different responses than cells in small groups. On the other hand, the microwell platform could also be modified to study calcium signaling at the single-cell level. As studies have indicated that intercellular calcium signaling exists among macrophage populations(52), the ability to isolate single cells and small groups of cells can help understand whether such intercellular signaling may also contribute to the density-dependent calcium activities observed in our studies. Overall, the development and application of microwell platforms could yield insights on how different physical or soluble cues, as well as the crosstalk among them, could regulate diverse aspects of macrophage phenotype and function.

While our studies revealed different characteristics of the density-dependent macrophage activation, the underlying mechanisms are still not known. Some of our results may offer insights to address this question. In the study on the per-cell secretion of TNFα at different time points, we discovered that cultures with different seeding densities have comparable per-cell TNFα secretion at the early 2-hour time point. This finding was consistent even with the addition of Yoda1, the Piezo1 agonist. Although further validation may be needed, the results suggested that cell density may mainly exert its effects on the later phase of macrophage activation. Some pathways are known to act later in the pro-inflammatory responses, including pathways downstream of MRTF-A and TRIF. MRTF-A regulates expressions of several late-stage pro-inflammatory genes following LPS activation(80), while TRIF mediated a delayed modulation of NFκB dynamics in the TLR4 pathway(37). In addition, MRTF-A has been implicated in confinement and crowding-mediated macrophage activation, and TRIF signaling can be modulated by calcium activities(80,139). Further studies on these pathways, or other relevant

signaling cascades, may help uncover the regulatory mechanisms governing the density-dependent effect of macrophage activation.

Finally, we hope to assess whether macrophage density could alter the cellular metabolic state and investigate how such changes may modulate macrophage functions. Macrophages are known to switch their metabolic states in response to different stimuli. Under pro-inflammatory (M1) or pro-healing (M2) activation, these cells would reprogram to utilize glycolysis or oxidative phosphorylation for energy production respectively(140). Increasing evidence suggests that macrophage metabolism is an important regulator of cellular functions, as it not only affects how cells utilize energy but also has broader effects on the regulation of transcription and post-transcriptional processing, which can lead to altered immune functions(140). Indeed, studies have probed the possibility of utilizing macrophages metabolism as a therapeutic target for diseases such as cancer and lupus nephritis(141,142). Interestingly, research on other cell types suggested that cellular density may alter the metabolic states of the population(143,144). One study found that the high-density culture of primary fibroblasts exhibited lower metabolic activity than the low-density culture(143). This difference decreased in other types of fibroblasts with increasing loss in growth control. Thus, metabolic regulations of macrophages may be another mechanism to exert density-dependent control on cellular activation. Recently, a method based on fluorescence lifetime microscopy has been developed to noninvasively probe cellular metabolic states at the single-cell level(145). This method could help study the metabolic states of macrophages under different levels of crowding. Through a collaboration with Dr. Michelle Digman's lab in UC Irvine, we performed pilot studies utilizing this approach and found that metabolic states of macrophages following stimulation could differ between crowded and sparse cultures (Figure 4.2). Future works on the associations among macrophage function, cellular

metabolism, and seeding density would yield more insights into the diverse regulation of macrophage functions, and potentially provide novel targets for therapeutic interventions.

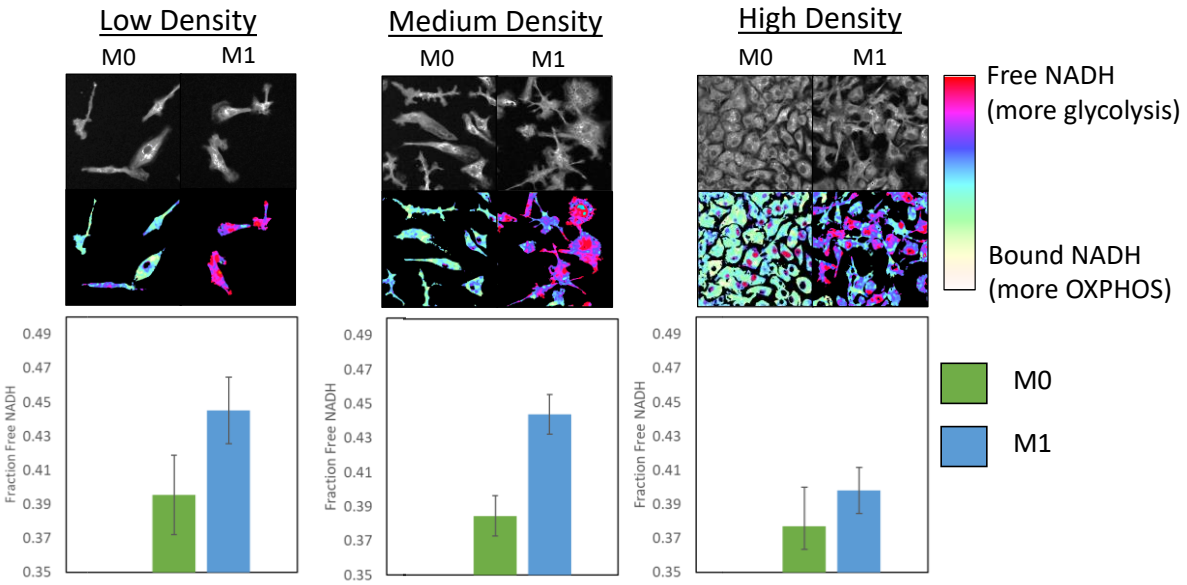

Figure 4.2 Initial assessment of the metabolic states for LPS and IFNγ activated macrophages seeded in different cell densities. Cellular metabolic states were measured 24 hours after stimulation with 10ng/ml of LPS and IFNγ. A higher fraction of free NADH is corresponded to a more glycolytic state.

Overall, our studies on the density-dependent effect of macrophage activation have established an important groundwork in understanding how cell density regulates macrophage functions. We provided evidence demonstrating that cell crowding could alter macrophages activation both through soluble signaling or physical interactions. Specifically, we identified the differential functions of soluble communication in cultures with low and high cell numbers. We also identified the mechanosensitive ion channel, Piezo1, in mediating at least part of the density effect. Nevertheless, much of the underlying mechanism for the density-induced regulation of macrophages remains unknown, and the physiological significance of this effect is still not clear. It is hoped that future works would uncover more contributing factors and help elucidate the physiological relevance of this density-dependent macrophage activation.

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