Title

Macro- and micro-scale culture environment differentially regulate the effects of crowding on macrophage function.

Journal

Biotechnology and Bioengineering, 121(1)

Authors

Hsu, Ssu-Chieh
Luu, Thuy
Smith, Tim
et al.

Publication Date

2024

DOI

10.1002/bit.28554

Peer reviewed

HHS Public Access

Author manuscript

Biotechnol Bioeng. Author manuscript; available in PMC 2025 February 11.

Published in final edited form as:

Biotechnol Bioeng. 2024 January ; 121(1): 306–316. doi:10.1002/bit.28554.

Macro- and micro-scale culture environment differentially regulate the effects of crowding on macrophage function

Ssu-Chieh J Hsu[1,2], **Wendy F. Liu**[1,2,3,4,5,*]

[1]Department of Biomedical Engineering, University of California, Irvine

[2]UCI Edwards Lifesciences Foundation Cardiovascular Innovation and Research Center, University of California, Irvine

[3]Department of Chemical and Biomolecular Engineering, University of California, Irvine

[4]Institute for Immunology, University of California, Irvine

[5]Department of Molecular Biology and Biochemistry, University of California, Irvine

Abstract

Macrophages hold vital roles in immune defense, wound healing, and tissue homeostasis, and have the exquisite ability to sense and respond to dynamically changing cues in their microenvironment. Much of our understanding of their behavior has been derived from studies performed using *in vitro* culture systems, in which the cell environment can be precisely controlled. Recent advances in miniaturized culture platforms also offer the ability to recapitulate some features of the *in vivo* environment and analyze cellular responses at the single-cell level. Since macrophages are sensitive to their surrounding environments, the specific conditions in both macro- and micro-scale cultures likely contribute to observed responses. In this study, we investigate how the presence of neighboring cells influence macrophage activation following pro-inflammatory stimulation in both bulk and micro-scale culture. We found that in bulk cultures, higher seeding density negatively regulated the average TNFα secretion from individual macrophages in response to inflammatory agonists, and this effect was partially caused by the reduced cell-to-media volume ratio. In contrast, studies conducted using microwells to isolate single cells and groups of cells revealed that increasing numbers of cells positively influences their inflammatory activation, suggesting that the absolute cell numbers in the system may be important. In addition, a single inflammatory cell enhanced the inflammatory state of a small group of cells. Overall, this work helps to better understand how variations of macroscopic and microscopic culture environments influence studies in macrophage biology and provides insight into how the presence of neighboring cells and the soluble environment influences macrophage activation.

Keywords

macrophages; *in vitro* culture; microwells; inflammation

[*]6086 Interdisciplinary Science and Engineering Building, Irvine, CA 92697-2730, wendy.liu@uci.edu; fax: (949) 824-9968.

SUPPLEMENTARY MATERIALS

See the supplementary materials for Figs. S1–S3 showing the effects of cell seeding density with LPS only stimulation, comparison across different adherent surfaces, and additional data from the microwell experiments.

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 (Schultze et al., 2015; Wynn et al., 2013). 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”) (Biswas & Mantovani, 2010). 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, the culture environment often varies across the many published studies. For example, different seeding densities of THP-1 monocytic cells and murine bone marrow cells during differentiation to macrophages causes differential expression of macrophage markers CD11b, CD14, and Ly-6G (Aldo et al., 2013; Lee & Hu, 2013). 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 (Lee & Hu, 2013). 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 (Muldoon et al., 2020). 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 (Boechat et al., 2001). Interestingly, studies separating individual cells into microwells show that cytokine secretion from individual cells in response to inflammatory activators are substantially lower than cells cultured in bulk and allowed to share common paracrine signals (McWhorter et al., 2016; Xue et al., 2015). 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 population or single cells, while also offering the flexibility to design structures and pattern cells or substrates to mimic native cellular environments (Halldorsson et al., 2015; Wikswo, 2014). These platforms have been exploited in the construction of various organ-on-a-chip systems, for example a gut-inflammation-on-a-chip model (Shin & Kim, 2018). 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, with major contributions of soluble factor signaling (Yu et al., 2007). Macrophages are known to secrete many cytokines with paracrine effects, which may exert their effects differently in 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 important parameters that needed to be considered when designing cell culture studies for macrophages, both in traditional culture systems and in miniaturized platforms.

MATERIALS AND METHODS

Cell isolation and culture

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 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.

Cell density studies

BMDM were seeded in 1 ml onto 18 mm glass coverslips in 12 well plates to achieve densities ranging from 5.1×10^5 to 2.6×10^3 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 5.1×10^3, 7.7×10^4, and 2.6×10^5 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, 2×10^5 BMDM were seeded onto 12 well plates in 1 ml to achieve a seeding density of 5.1×10^4 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 5.1×10^4, 7.7×10^4, and 1.3×10^5 cells/cm^2 in a 12 well plate. After 24 hours, the supernatant was collected for analysis. Finally, to assess the effects of IL10 in regulating the density effects, cells were seeded at 6.4×10^3, 2.6×10^4, 7.7×10^4, and 2.6×10^5 cells/cm^2 in a 96 well plate and adhered for overnight. Subsequently, cells were stimulated with 10ng/ml of LPS and IFNγ together with either 20μg/ml of anti-IL10 antibody, 20μg/ml of corresponding isotype antibody (both from BioLegend), and no antibody at all. Supernatants were collected after 24 hours of stimulation to assess for TNFα secretion.

Measurement of cytokines

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

Fabrication of the microwell membranes

Silicon wafers with arrays of microwells were fabricated using standard photolithography techniques (McWhorter et al., 2016). 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 200×300 μm in size. The wafer was subsequently baked before it was developed in SU8 developer (MicroChem) to create patterns of rectangles with a size of 200×300 μm. To create PDMS microwell membranes for single-cell experiments, PDMS and curing agent were mixed in 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.

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.

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 (Schindelin et al., 2012). 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 1^{st} 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 (Preibisch et al., 2009), 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.

Statistical analysis

Data were analyzed using one way ANOVA followed by Tukey's HSD Test. For the data in figure 3, Mann-Whitney U test was used for the statistical analysis.

RESULTS

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. We used bone marrow-derived macrophages (BMDM) in our studies, which according to our previous characterizations, exhibited rather tight spread of marker expressions such as F4/80, CD14, as well as integrin receptors αM and β2 (McWhorter et al., 2013). These cells also exhibited low level expressions of M2 markers including arginase-1, CD206, and YM-1. Upon activation with IL4 and IL13, these markers would be upregulated, and in some cases, exhibited a wider spread of expression levels (Hsieh et al., 2019). This reflected the heterogeneous natures of macrophage following activation, as opposed to the more homogeneous expression at basal level. BMDMs were seeded at different densities and stimulated with pro-inflammatory

ligands, LPS and IFNγ together or LPS alone for 24 h (Figure 1a). In response to LPS and IFNγ, macrophage secretion of TNFα increased as the seeding density was increased from 2.6×10^3 to 2.6×10^5 cells/cm^2; however, TNFα concentration decreased when the seeding density was further increased from 2.6×10^5 cells/cm^2 to 5.1×10^5 cells/cm^2 (Figure 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 1c). Raw concentrations measured by ELISA showed similar trends (Figure S1). 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 S2a, S2b). Further tests comparing the LPS-only results conducted on two common culture materials – glass and tissue culture polystyrene revealed that 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 S3a, S3b, S3e, and S3f).

To understand the temporal dynamics of the density dependent TNFα secretion, macrophages were seeded overnight at low (5.1×10^3 cells/cm^2), intermediate (7.7×10^4 cells/cm^2), and high (2.6×10^5 cells/cm^2) densities, stimulated, and the supernatant was subsequently collected at 2, 6, 12, and 24 hours for analysis of secreted cytokines. LPS and IFNγ-stimulated macrophages seeded at higher densities exhibited overall higher TNFα secretion across all time points (Figure 1d). 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 1e, S1c, S1d). Overall, macrophages exhibit density-dependent effects on TNFα secretion, with cells seeded in higher density secreting less TNFα when evaluated per-cell. In addition, density-dependent effects were time-dependent: at the earlier time points (2 h after stimulation), the amount of TNFα secreted by macrophages seeded at different densities was similar, but at later stages of activation (6 h or more), cells seeded at higher densities secreted less TNFα.

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 1f), but the per-cell IL10 secretion appeared to exhibit a biphasic response, with a peak at around 7.7×10^4 cells/cm^2 and decreasing with lower or higher seeding densities (Figure 1g). For macrophages stimulated with LPS alone, a similar increasing trend in IL10 secretion with increasing seeding densities was observed (Figure S3e). However, a biphasic response was not present, and the per-cell IL10 secretion exhibited much less variation across all seeding densities (Figure S3f). Interestingly, in the presence of IFNγ, IL10 increased approximately 7-fold, while in the absence of IFNγ, normalized IL10 concentrations ranged much higher (up to 13-fold). As IFNγ suppresses IL-10 production (Hu et al., 2006), this difference was expected. However, the variation in TNFα concentration across different densities only changed slightly when IFNγ was added (up to 2-fold with IFNγ versus 2.5-fold without). This suggested that although IL10 is a known anti-inflammatory cytokine, it may not be the main contributor for the observed density-dependent effects of macrophage activation.

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 1h, S1g). For the LPS and IFNγ stimulation condition, the per-cell IL10 secretion was similar across the different seeding densities at different time points (Figure 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 S2h). 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 S3c, S3d, S3g, and S3h). 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.

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 5.1×10^4 cells/cm^2, which corresponded to the cell-to-media-volume ratio of the cultures seeded at 5.1×10^4, 7.7×10^4, and 1.3×10^5 cells/cm^2 in the previous experiments (Figure 2a, b). 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 2c, d). 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 2f). However, per-cell TNFα secretion decreased in culture stimulated in less media, with samples from the lowest media volume group having almost 30% lower in per-cell TNFα secretion than those in the highest media volume group (Figure 2e). Since the number of cells in the culture well was the same for both samples, these results suggested that paracrine interactions at least partially contribute to macrophage activation in response to pro-inflammatory ligands.

Effects of IL10 on density-dependent effect of macrophage activation

IL10 is an important cytokine involved in dampening macrophage activation, and our results suggested that its secretion was density dependent. To further assess the effects of IL10 in regulating the density-dependent effect of macrophage activation, we used an antibody to block IL10 signaling. Cells were first seeded in different seeding densities (2.6×10^5 cells/cm^2, 7.7×10^4 cells/cm^2, 2.6×10^4 cells/cm^2, 6.4×10^3 cells/cm^2), allowed to adhere overnight, and then stimulated with 10ng/ml of LPS and IFNγ. At the same time, cells were treated with 20μg/ml of anti-IL10 antibody, or 20μg/ml of corresponding isotype antibody and no antibody controls. After 24 hours of incubation, supernatants were collected and measured for TNFα with commercial ELISA kits. We found that neutralizing IL10 with anti-IL10 antibody had limited effects on the density-dependent effects (Figure S4). Treating cells with anti-IL10 antibody did not have noticeable effects on the TNFα secretion across any of the densities tested. Overall, this experiment suggested that although IL10 is an important

negative regulator of macrophage activation, IL10 alone is not sufficient to alter the observed density dependent TNFα inhibition.

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 3a). Our data suggested that iNOS expression was density-dependent (Figure 3c), and there was also a correlation between iNOS expression and per-cell TNFα secretion (Figure S5). 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 3b – e). 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 3f). 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 contract to the results in the microwells (Figure 3g).

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 3d, e). This observation agreed with other studies on macrophage activation at single cell level that revealed precociously activated cells (Muldoon et al., 2020; Xue et al., 2015). 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 4a and Figure S6). 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 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 4b). We found that wells with at least one cell having high iNOS expression appeared to be associated with a 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 other words, cell-cell communication at high cell densities is likely needed to overcome stochastic macrophage activation. This is supported by statistically significant increases in activation with increased cell numbers per well. 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.

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 (Muldoon et al., 2020; Xue et al., 2015), possibly due to the utilization 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 contributes 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 (Howes et al., 2016; Murray & Smale, 2012). Interestingly, culture within a 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 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 (Shalek et al., 2014; Xue et al., 2015). 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 (Gottschalk et al., 2019; Xue et al., 2015). Other soluble factors, including IFNβ, nitrogen oxide, and PGE_2, have also been shown to be implicated in macrophage feedback control(Gottschalk et al., 2019; Jacobs & Ignarro, 2003; Perkins et al., 2018; Postat et al., 2018). However, in a recent study, exogenous IL-10 was insufficient by itself to abrogate density-dependent effects on inflammatory activation (Muldoon et al., 2020). This study utilized reporter

systems to track NFκB activity and TNFa secretion in RAW264.7 cells and revealed that cells exhibited density-dependent bimodal activation states following LPS stimulation, which was independent of exogeneous 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 (Muldoon et al., 2020). At resting state, high density culture exhibited higher levels 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. Importantly, responses to soluble factor accumulation over time likely contributes to their specific signaling properties. In addition, different signaling pathways may also be activated at different time points. As a result, crowding likely has dynamic effects toward macrophage inflammatory throughout various stages of inflammation, as also suggested in previous work (Jain & Vogel, 2018; Meli et al., 2020). Nonetheless, how these 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 population of cells and underscore the need for well-controlled *in vitro* culture systems to characterize these phenomena. Macrophage activation has been found to be highly heterogeneous, as evident in our microwell study. Indeed, a previous study on single-cell cytokine secretion of U937-derived macrophages found that only about 40% of cell secreted TNFα following 100ng/ml of LPS stimulation (Xue et al., 2015). Factors such as stochasticity, existence of sub-populations, and variation of initial states have been suggested to contribute to immune cell heterogeneity (Chen et al., 2019; van Eyndhoven et al., 2021; Xue et al., 2015). Nevertheless, paracrine interactions among a cell population can add further complexities to macrophage functions. 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 cellular environment of a microwell system, and 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 (Solis et al., 2019; Zumerle et al., 2019). 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 (Halldorsson et al., 2015). In addition, the ability to integrate different physical stimuli into the system, such as mechanical stretch (Huh et al., 2010), 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 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 size of the cell population affects the cellular characteristics (Domenech et al., 2009). 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 (Murray et al., 2014), and our study reinforced this notion by demonstrating that experimental design can in fact result in disparate macrophage behavior and experimental conclusions - in our case, cells in bulk tissue culture wells showed density-dependent decrease, whereas cells in microwell showed density-dependent increase, in inflammatory activation. 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 culture indeed introduce widely different environmental conditions. To facilitate better comparisons across different studies, further work will be needed to understand how the different culture parameters between both culture formats could affect experimental outcomes.

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 about 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 the future studies involving micro-scale culture platform 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

We thank Xuan My Bui for assistance with processing the data. This work was supported by National Institute of Health (NIH) National Institute of Allergy and Infectious Disease (NIAID) Grant R01AI151301 and National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) Grant R01AR071335, as well as by the National Science Foundation (NSF) Biomechanics and Mechanobiology (BMMB Award number 2035264) and the NSF and industrial members of the Center for Advanced Design and Manufacturing of Integrated Microfluidics (NSF I/UCRC Award number IIP-1362165).

Grant information:

This work was supported by National Institute of Health (NIH) National Institute of Allergy and Infectious Disease (NIAID) Grant R01AI151301 and National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) Grant R01AR071335, as well as by the National Science Foundation and the industrial members of the Center for Advanced Design and Manufacturing of Integrated Microfluidics (NSF I/UCRC Award number IIP-1362165).

REFERENCES

Aldo PB, Craveiro V, Guller S, & Mor G (2013). Effect of culture conditions on the phenotype of THP-1 monocyte cell line. American Journal of Reproductive Immunology, 70(1), 80–86. 10.1111/aji.12129 [PubMed: 23621670]

Biswas SK, & Mantovani A (2010). Macrophage plasticity and interaction with lymphocyte subsets: Cancer as a paradigm. Nature Immunology, 11(10), 889–896. 10.1038/ni.1937 [PubMed: 20856220]

Boechat N, Bouchonnet F, Bonay M, Grodet A, Pelicic V, Gicquel B, & Hance AJ (2001). Culture at High Density Improves the Ability of Human Macrophages to Control Mycobacterial Growth. The Journal of Immunology, 166(10), 6203–6211. 10.4049/jimmunol.166.10.6203 [PubMed: 11342642]

Chen Z, Lu Y, Zhang K, Xiao Y, Lu J, & Fan R (2019). Multiplexed, Sequential Secretion Analysis of the Same Single Cells Reveals Distinct Effector Response Dynamics Dependent on the Initial Basal State. Advanced Science, 6(9). 10.1002/advs.201801361

Domenech M, Yu H, Warrick J, Badders NM, Meyvantsson I, Alexander CM, & Beebe DJ (2009). Cellular observations enabled by microculture: Paracrine signaling and population demographics. Integrative Biology, 1(3), 267–274. 10.1039/b823059e [PubMed: 20011455]

Gottschalk RA, Dorrington MG, Dutta B, Krauss KS, Martins AJ, Uderhardt S, Chan W, Tsang JS, Torabi-Parizi P, Fraser ID, & Germain RN (2019). IFN-mediated negative feedback supports bacteria class-specific macrophage inflammatory responses. ELife, 8, 1–23. 10.7554/eLife.46836

Halldorsson S, Lucumi E, Gómez-Sjöberg R, & Fleming RMT (2015). Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices. Biosensors and Bioelectronics, 63, 218–231. 10.1016/j.bios.2014.07.029 [PubMed: 25105943]

Howes A, Taubert C, Blankley S, Spink N, Wu X, Graham CM, Zhao J, Saraiva M, Ricciardi-Castagnoli P, Bancroft GJ, & O'Garra A (2016). Differential Production of Type I IFN Determines the Reciprocal Levels of IL-10 and Proinflammatory Cytokines Produced by C57BL/6 and BALB/c Macrophages. The Journal of Immunology, 197(7), 2838–2853. 10.4049/jimmunol.1501923 [PubMed: 27549173]

Hsieh JY, Keating MT, Smith TD, Meli VS, Botvinick EL, & Liu WF (2019). Matrix crosslinking enhances macrophage adhesion, migration, and inflammatory activation. APL Bioengineering, 3(1). 10.1063/1.5067301

Hu X, Paik PK, Chen J, Yarilina A, Kockeritz L, Lu TT, Woodgett JR, & Ivashkiv LB (2006). IFN-γ Suppresses IL-10 Production and Synergizes with TLR2 by Regulating GSK3 and CREB/AP-1 Proteins. Immunity, 24(5), 563–574. 10.1016/j.immuni.2006.02.014 [PubMed: 16713974]

Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Yuan Hsin H, & Ingber DE (2010). Reconstituting organ-level lung functions on a chip. Science. 10.1126/science.1188302

Jacobs AT, & Ignarro LJ (2003). Cell density-enhanced expression of inducible nitric oxide synthase in murine macrophages mediated by interferon-β. Nitric Oxide - Biology and Chemistry, 8(4), 222–230. 10.1016/S1089-8603(03)00027-2 [PubMed: 12895431]

Jain N, & Vogel V (2018). Spatial confinement downsizes the inflammatory response of macrophages. Nature Materials, 17(12), 1134–1144. 10.1038/s41563-018-0190-6 [PubMed: 30349032]

Lee CM, & Hu J (2013). Cell density during differentiation can alter the phenotype of bone marrow-derived macrophages. Cell and Bioscience, 3(1), 1. 10.1186/2045-3701-3-30 [PubMed: 23286669]

McWhorter FY, Smith TD, Luu TU, Rahim MK, Haun JB, & Liu WF (2016). Macrophage secretion heterogeneity in engineered microenvironments revealed using a microwell platform. Integrative Biology (United Kingdom), 8(7), 751–760. 10.1039/c6ib00053c

McWhorter FY, Wang T, Nguyen P, Chung T, & Liu WF (2013). Modulation of macrophage phenotype by cell shape. Proceedings of the National Academy of Sciences of the United States of America, 110(43), 17253–17258. 10.1073/pnas.1308887110 [PubMed: 24101477]

Meli VS, Atcha H, Veerasubramanian PK, Nagalla RR, Luu TU, Chen EY, Guerrero-Juarez CF, Yamaga K, Pandori W, Hsieh JY, Downing TL, Fruman DA, Lodoen MB, Plikus M. v., Wang W, & Liu WF (2020). YAP-mediated mechanotransduction tunes the macrophage inflammatory response. Science Advances, 6(49), 1–14. 10.1126/sciadv.abb8471

Muldoon JJ, Chuang Y, Bagheri N, & Leonard JN (2020). Macrophages employ quorum licensing to regulate collective activation. Nature Communications, 11(1), 1–14. 10.1038/s41467-020-14547-y

Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO, Mege JL, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, … Wynn TA (2014). Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. Immunity, 41(1), 14–20. 10.1016/j.immuni.2014.06.008 [PubMed: 25035950]

Murray PJ, & Smale ST (2012). Restraint of inflammatory signaling by interdependent strata of negative regulatory pathways. Nature Immunology, 13(10), 916–924. 10.1038/ni.2391 [PubMed: 22990889]

Perkins DJ, Richard K, Hansen AM, Lai W, Nallar S, Koller B, & Vogel SN (2018). Autocrine–paracrine prostaglandin E 2 signaling restricts TLR4 internalization and TRIF signaling. Nature Immunology, 19(12), 1309–1318. 10.1038/s41590-018-0243-7 [PubMed: 30397349]

Postat J, Olekhnovitch R, Lemaître F, & Bousso P (2018). A Metabolism-Based Quorum Sensing Mechanism Contributes to Termination of Inflammatory Responses. Immunity, 49(4), 654–665.e5. 10.1016/j.immuni.2018.07.014 [PubMed: 30266340]

Preibisch S, Saalfeld S, & Tomancak P (2009). Globally optimal stitching of tiled 3D microscopic image acquisitions. Bioinformatics. 10.1093/bioinformatics/btp184

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, & Cardona A (2012). Fiji: An open-source platform for biological-image analysis. In Nature Methods. 10.1038/nmeth.2019

Schultze JL, Schmieder A, & Goerdt S (2015). Macrophage activation in human diseases. Seminars in Immunology, 27(4), 249–256. 10.1016/j.smim.2015.07.003 [PubMed: 26303100]

Shalek AK, Satija R, Shuga J, Trombetta JJ, Gennert D, Lu D, Chen P, Gertner RS, Gaublomme JT, Yosef N, Schwartz S, Fowler B, Weaver S, Wang J, Wang X, Ding R, Raychowdhury R, Friedman N, Hacohen N, … Regev A (2014). Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. Nature, 510(7505), 363–369. 10.1038/nature13437 [PubMed: 24919153]

Shin W, & Kim HJ (2018). Intestinal barrier dysfunction orchestrates the onset of inflammatory host-microbiome cross-talk in a human gut inflammation-on-a-chip. Proceedings of the National Academy of Sciences of the United States of America, 115(45), E10539–E10547. 10.1073/pnas.1810819115 [PubMed: 30348765]

Solis AG, Bielecki P, Steach HR, Sharma L, Harman CCD, Yun S, de Zoete MR, Warnock JN, To SDF, York AG, Mack M, Schwartz MA, dela Cruz CS, Palm NW, Jackson R, & Flavell RA (2019). Mechanosensation of cyclical force by PIEZO1 is essential for innate immunity. Nature, 573(7772), 69–74. 10.1038/s41586-019-1485-8 [PubMed: 31435009]

van Eyndhoven LC, Singh A, & Tel J (2021). Decoding the dynamics of multilayered stochastic antiviral IFN-I responses. In Trends in Immunology (Vol. 42, Issue 9, pp. 824–839). Elsevier Ltd. 10.1016/j.it.2021.07.004 [PubMed: 34364820]

Wikswo JP (2014). The relevance and potential roles of microphysiological systems in biology and medicine. Experimental Biology and Medicine, 239(9), 1061–1072. 10.1177/1535370214542068 [PubMed: 25187571]

Wynn TA, Chawla A, & Pollard JW (2013). Macrophage biology in development, homeostasis and disease. Nature, 496(7446), 445–455. 10.1038/nature12034 [PubMed: 23619691]

Xue Q, Lu Y, Eisele MR, Sulistijo ES, Khan N, Fan R, & Miller-Jensen K (2015). Analysis of single-cell cytokine secretion reveals a role for paracrine signaling in coordinating macrophage responses to TLR4 stimulation. Science Signaling, 8(381), ra59. 10.1126/scisignal.aaa2155 [PubMed: 26082435]

Yu H, Alexander CM, & Beebe DJ (2007). Understanding microchannel culture: Parameters involved in soluble factor signaling. Lab on a Chip, 7(6), 726–730. 10.1039/b618793e [PubMed: 17538714]

Zumerle S, Calì B, Munari F, Angioni R, di Virgilio F, Molon B, & Viola A (2019). Intercellular Calcium Signaling Induced by ATP Potentiates Macrophage Phagocytosis. Cell Reports, 27(1), 1–10.e4. 10.1016/j.celrep.2019.03.011 [PubMed: 30943393]

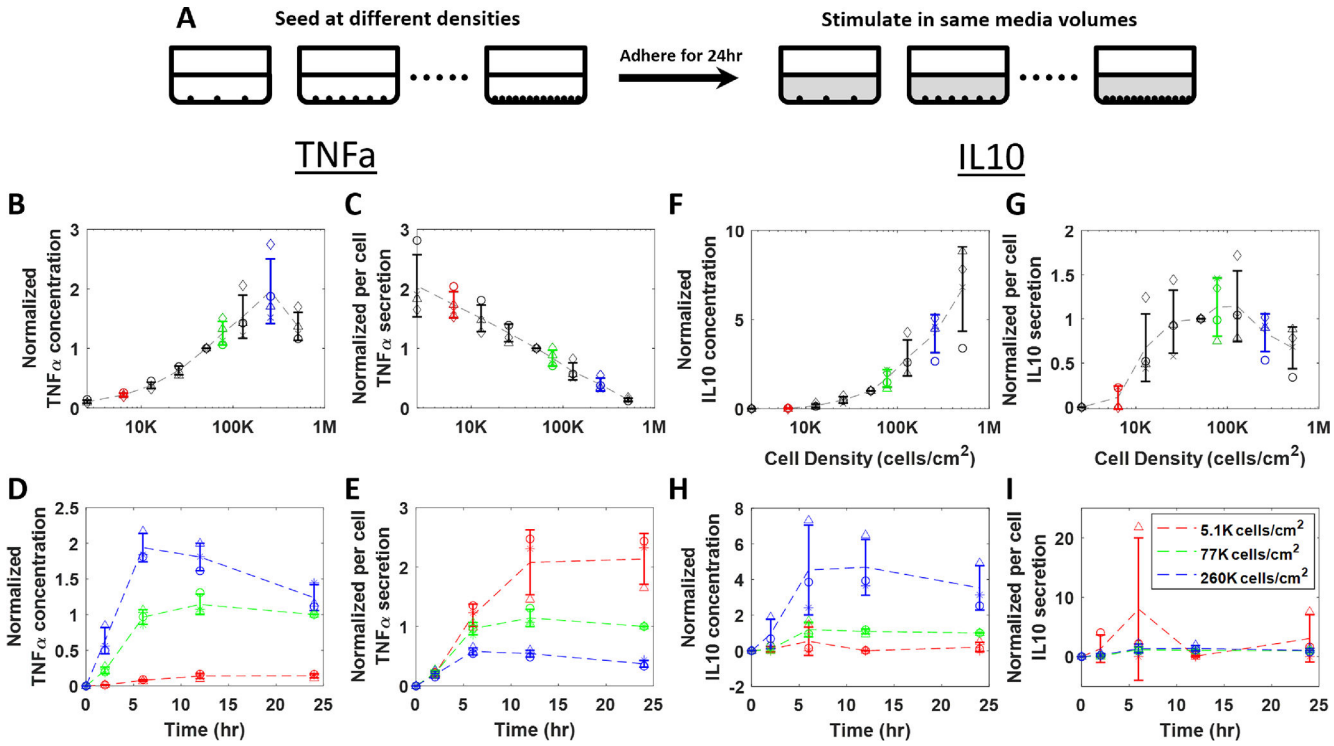

Figure 1: Macrophages exhibit density-dependent cytokines secretion under LPS and IFNγ stimulation.

(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 24 hours after stimulation, 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 24 hours after stimulation, 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. For figure B, C, F, G, data were normalized to the value at the concentration of around 51000 cells/cm^2. For figure D, E, H, I, data were normalized to the value at 24-hour time point with a concentration of around 77000 cells/cm^2.

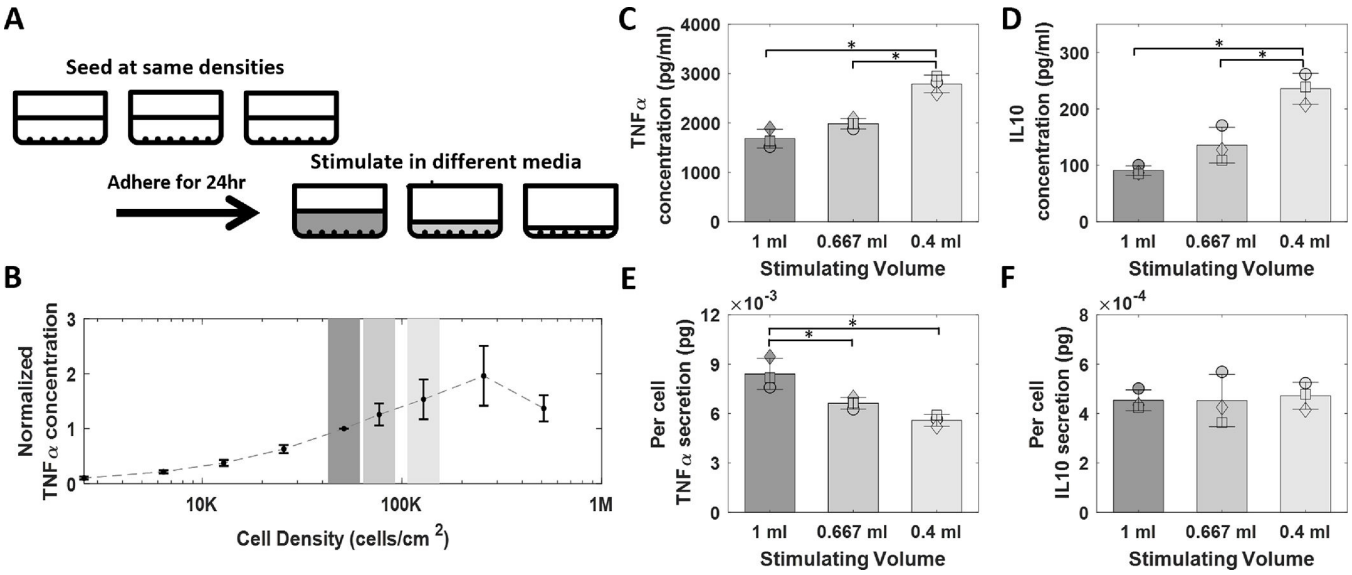

Figure 2: Soluble factors partially contribute to density-dependent effects on macrophage activation.

(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).

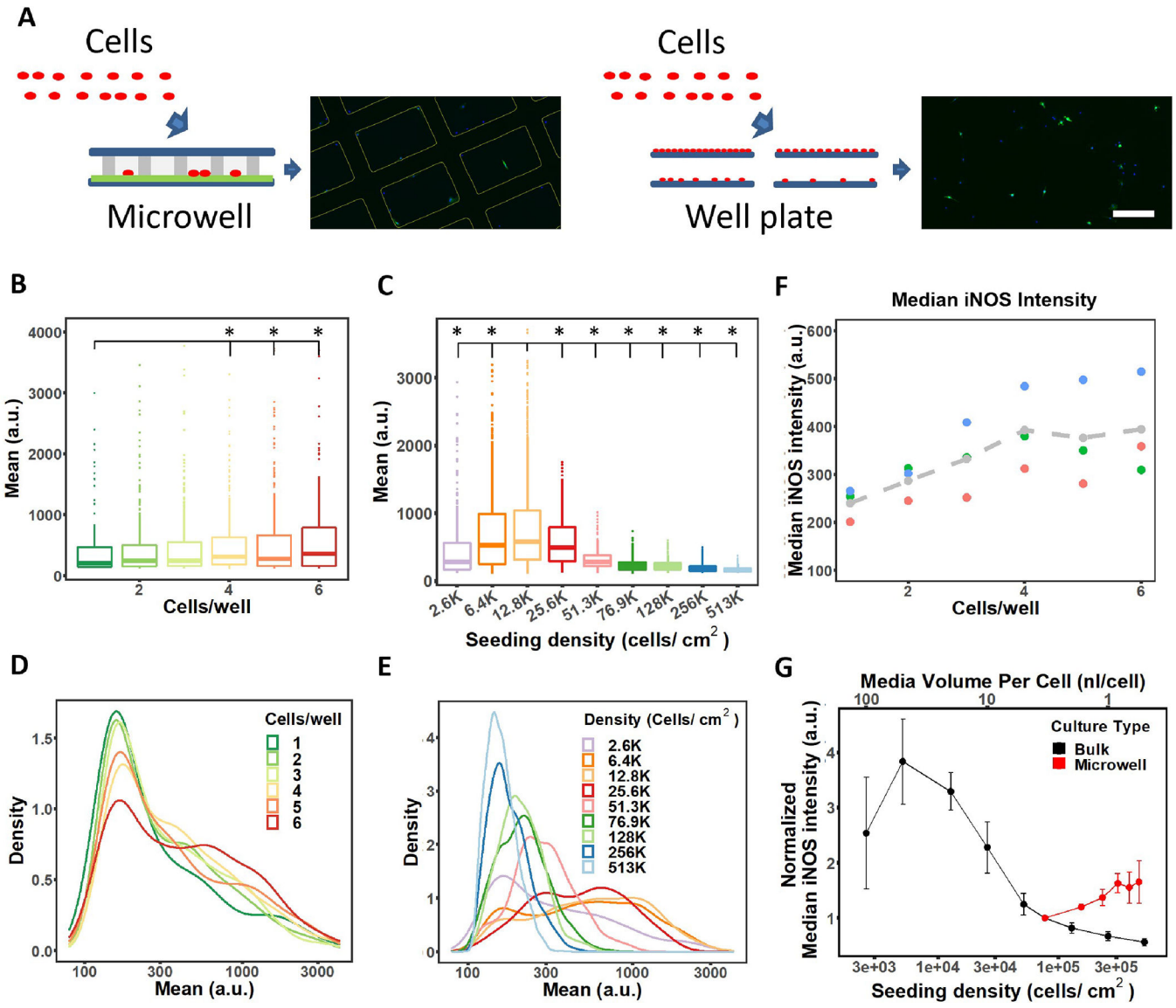

Figure 3: Comparison of density-dependent effects in micro- vs. macro-scale cultures.

(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. Scale bar denotes 200μm. (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 2.6×10^3 cells/cm^2 to 5.13×10^5 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.

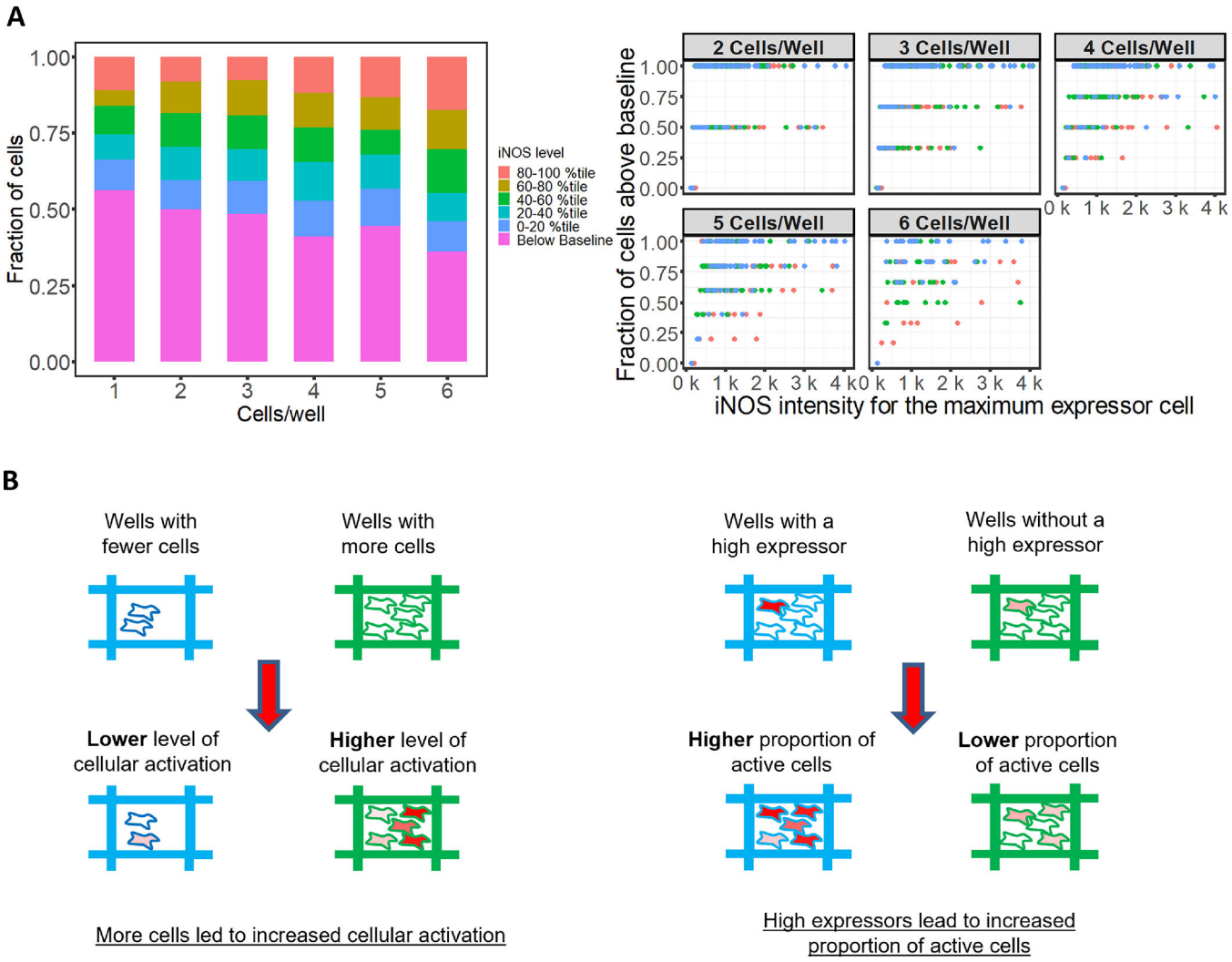

Figure 4: Microwell experiments revealed a different mode of density-dependent regulation of macrophage function

. (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.