Regulation of macrophage function by the macro- and micro-scale *in vitro* culture environment

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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 and volume of media influence macrophage activation following pro-inflammatory stimulation. We found that in bulk cultures, higher seeding density and lower cell-to-media volume ratio negatively regulated the average TNFα secretion from individual macrophages in response to inflammatory agonists. 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. Overall, this work helps to better understand how variations of experimental parameters broadly 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

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 [1,2]. 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") [3]. 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 causes differential expression of macrophage markers CD11b, CD14, and Ly-6G [4,5]. 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 [4]. 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 [6]. 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 [7]. 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 [8,9]. However, previous studies cover limited subsets of densities ranges [10,11], and direct comparisons between macro-scale cultures in traditional tissue culture wells with micro-scale cultures 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 [12,13]. These advantages have been exploited in the construction of various organ-on-a-chip systems, for example a gut-inflammation-on-a-chip model [14]. 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 a main factor [15]. 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. 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.

RESULTS

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

We first evaluated the effects of cell seeding density, a parameter that often varies among different studies, on macrophage activation by soluble stimuli. BMDMs were seeded at different densities and stimulated with pro-inflammatory ligands, LPS and IFNγ together or LPS alone **[Fig. 1(a)]**. 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 **[Fig. 1(b)]**. 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 **[Fig. 1(c)]**. 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 **[Figs. S1(a) and S1(b)]**.

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 **[Fig. 1(d)]**. 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 **[Figs. S2(a), S2(b), S2(e), and S2(f)]**. 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 2 hours of stimulation and decreased TNFα levels with increasing densities at all of the later time points (6, 12, 24 h) **[Figs. 1(e), S1(c) and S1(d)]**. 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 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 **[Fig. 1(f)]**, 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 [**Fig. 1(g)]**. For macrophages stimulated with LPS alone, a similar increasing trend in IL10 secretion with increasing seeding densities was observed [**Fig. S1(e)]**. However, a biphasic response was not present, and the per-cell IL10 secretion exhibited much less variation across all seeding densities **[Fig. S1(f)]**. 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 [**Figs. 1(h) and S1(g)]**. For the LPS and IFNγ stimulation condition, the per-cell IL10 secretion was similar across the different seeding densities at different time points **[Fig. 1(i)]**. In contrast, higher seeding densities corresponded

to higher per-cell IL10 secretion across the different time points in cells stimulated with LPS alone **[Fig. S1(h)]**. 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 **[Figs. S2(c), S2(d), S2(g), and S2(h)]**. 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 [**Figs. 2(a) and 2(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 [**Figs. 2(c) and 2(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 [**Fig. 2(f)]**. However, per-cell TNFα secretion decreased in culture stimulated in less media volumes, 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 [**Fig. 2(e)]**. 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.

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 [**Fig. 3(a)**]. To ensure the media volume per cell for macrophages in microwells was consistent with our earlier studies, only macrophages in wells containing 1 to 6 cells were analyzed. We observed a wide range of iNOS staining intensity for cells in both microwell and population studies [**Figs. 3(b) – 3(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 [**Fig. 3(f)**]. 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 [**Fig. 3(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 [**Figs. 5(d) and 5(e)]**. This observation agreed with other studies on macrophage activation at single cell level demonstrating precociously activated cells [6,8]. 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 [**Fig. 4A**]. 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 **[Fig. 4B]**. 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 2-6 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.

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 [6,8], 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 [16,17]. 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 [8,18]. 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 [8,19]. Other soluble factors, including IFNβ, nitrogen oxide, and PGE_2, have also been shown to be implicated in macrophage feedback control[19–22]. However, in a recent study, exogenous IL-10 was insufficient by itself to abrogate density-dependent effects on inflammatory activation [6]. 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 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 [6]. 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. 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 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 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[23,24]. 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 [12]. In addition, the ability to integrate different physical stimuli into the system, such as mechanical stretch [25], 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 [26]. 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 [27], and our study reinforced this notion by demonstrating that experimental design strongly influence 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 culture 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.

CONCLUSION

In this study, we report that cell seeding density and cell-to-media-volume ratio, two common culture parameters, 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.

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 $5.1x10^5$ 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.

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 [9]. 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 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 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 5 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 1 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 [28]. 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 [29], 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.

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.

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AUTHOR DECLARATIONS

Conflict of interest

The authors have no conflicts to disclose

Ethics approval

All protocols involving animals were approved by the University of California Irvine's Institutional Animal Care and Use Committee (protocol #AUP-17-85 and AUP-20-47), which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALACi). No human subjects were involved in this study.

DATA AVAILABBLITY

The data that supports the findings of this study are available within the article and its supplementary material. Additional data that support the findings of this study are available from the corresponding author upon reasonable request.

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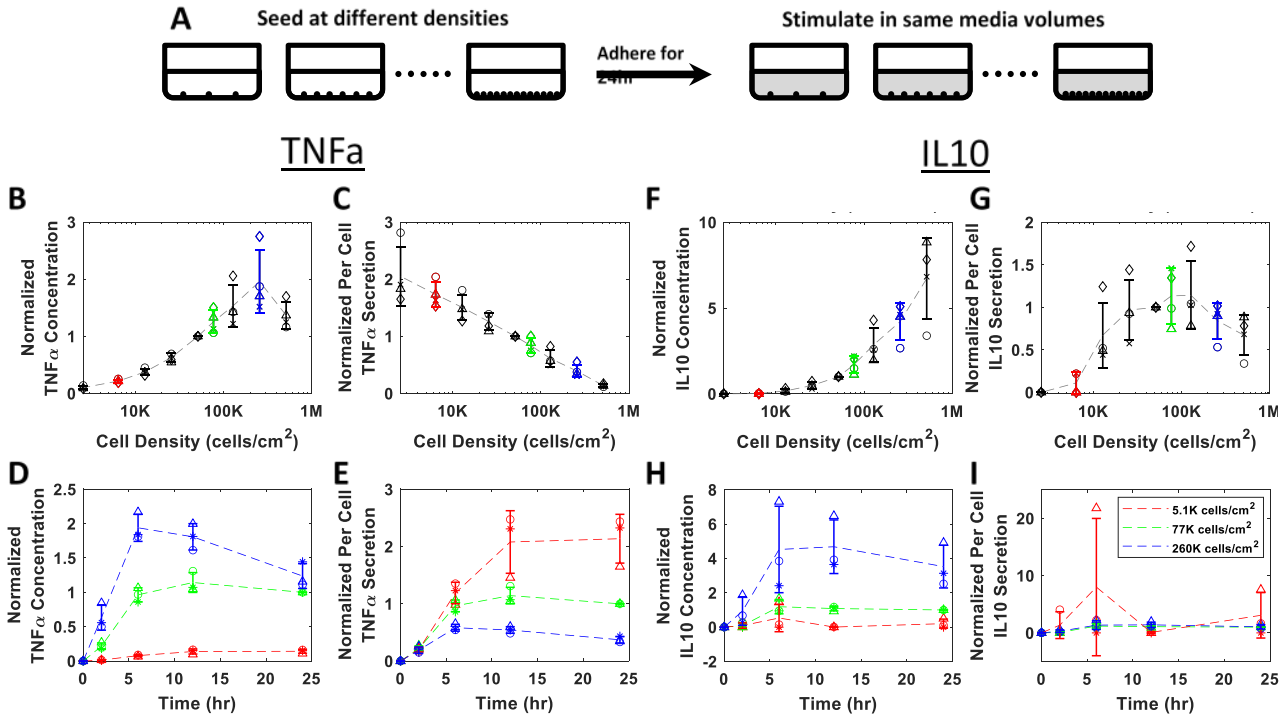


Figure 1: Macrophages exhibit density-dependent cytokines secretion under LPS and IFNγ stimulation. (A) Schematic of experiments examining cytokine secretion of macrophages seeded in different densities. (B - E) TNFα concentration (B) and per-cell TNFα secretion (C) of BMDM seeded at different densities on glass, 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.

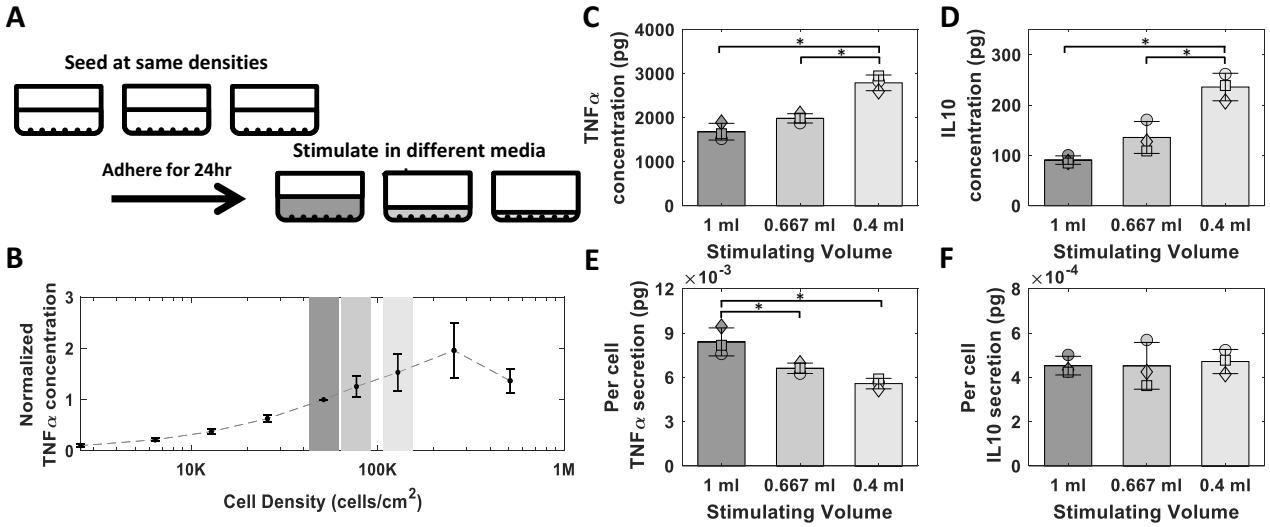


Figure 2: **Soluble factors partially contribute to density-dependent effects on macrophage activation.** (A) Schematic of the experiment; cells were seeded at an identical seeding density and cell-to-media-volume ratio, allowed to attach for 24 hours, and then stimulated with LPS and IFNγ at 10ng/ml 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).

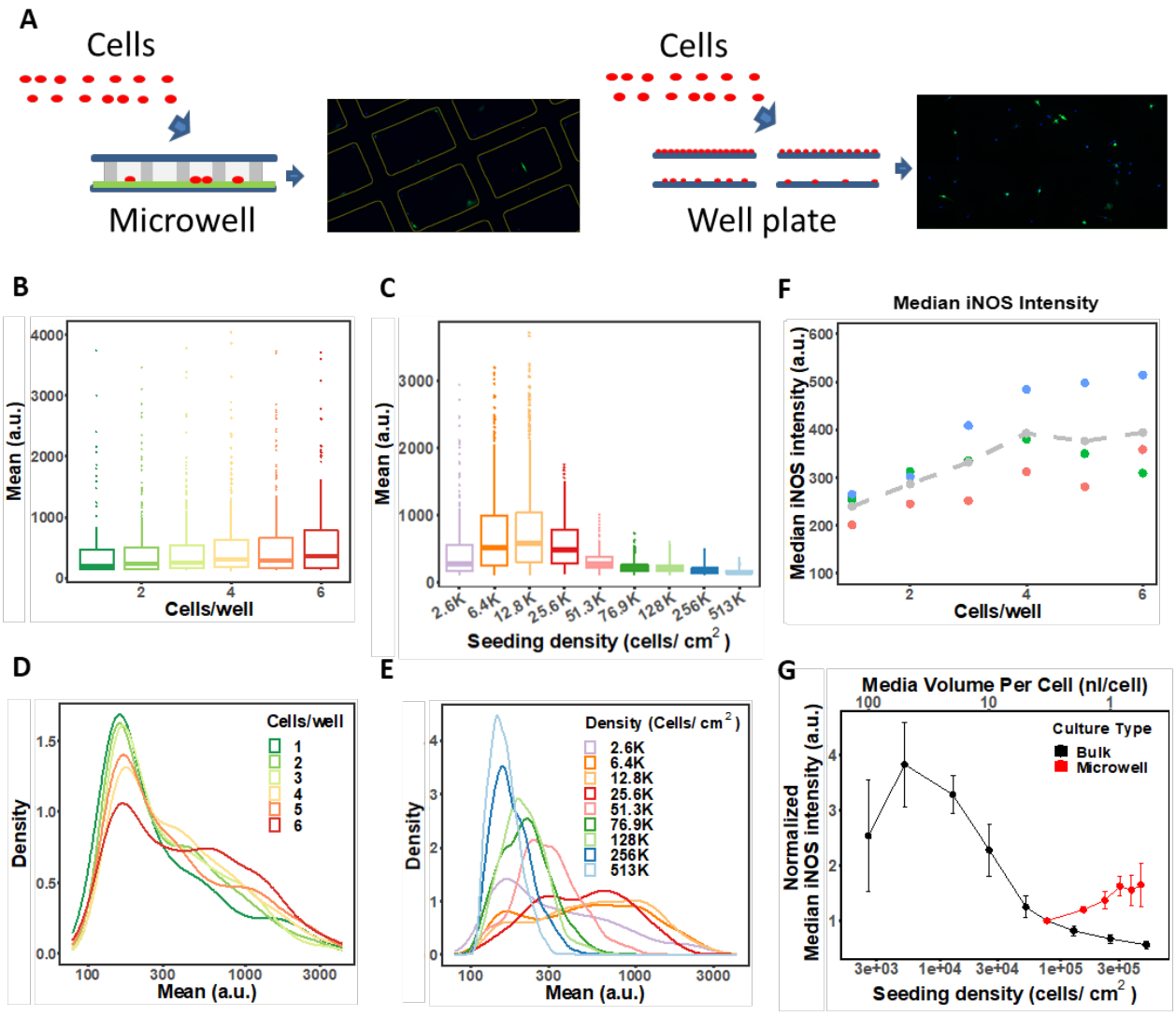


Figure 3: Comparison of density-dependent effects in micro- vs. macro-scale cultures. (A) Schematic of the microwell experiment to delineate the effects of single cell vs groups of cells on macrophage activation, and the bulk density experiment for comparison. (B, C) Dot plots of sampled iNOS intensities 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.

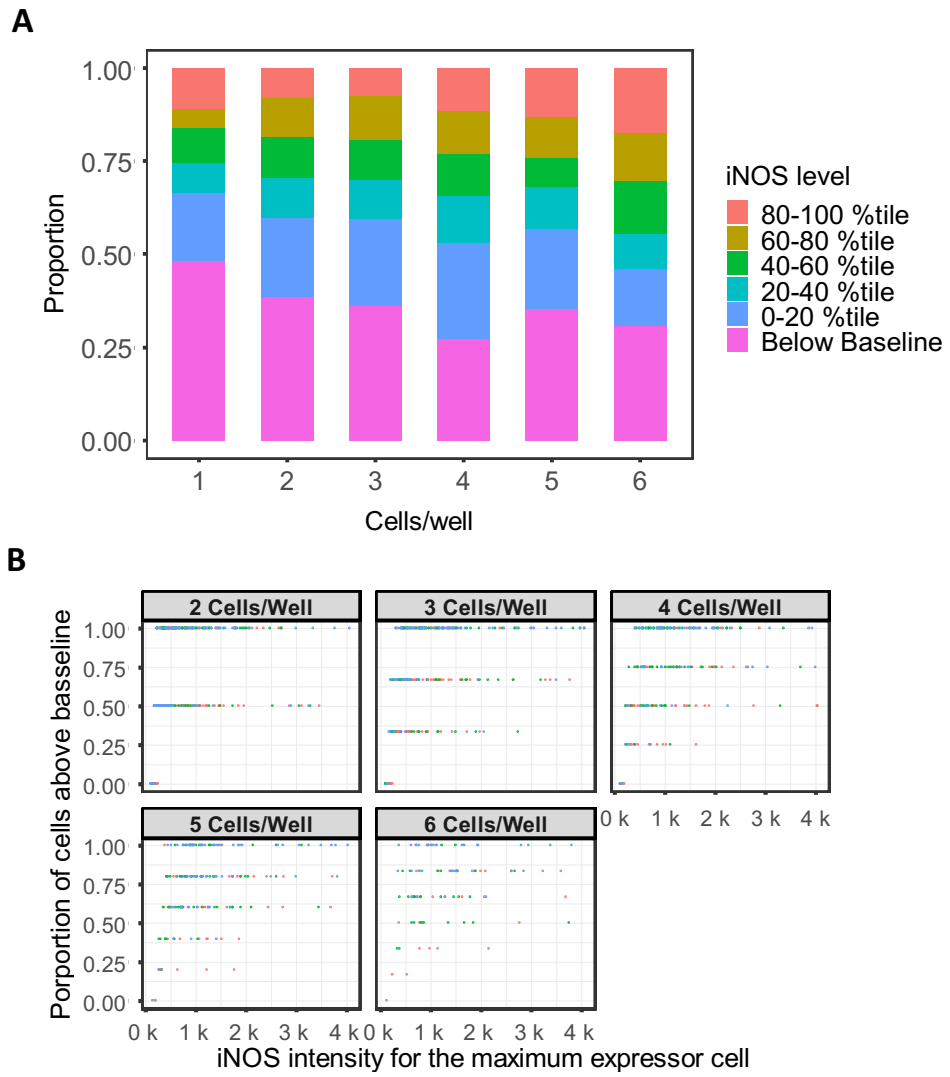
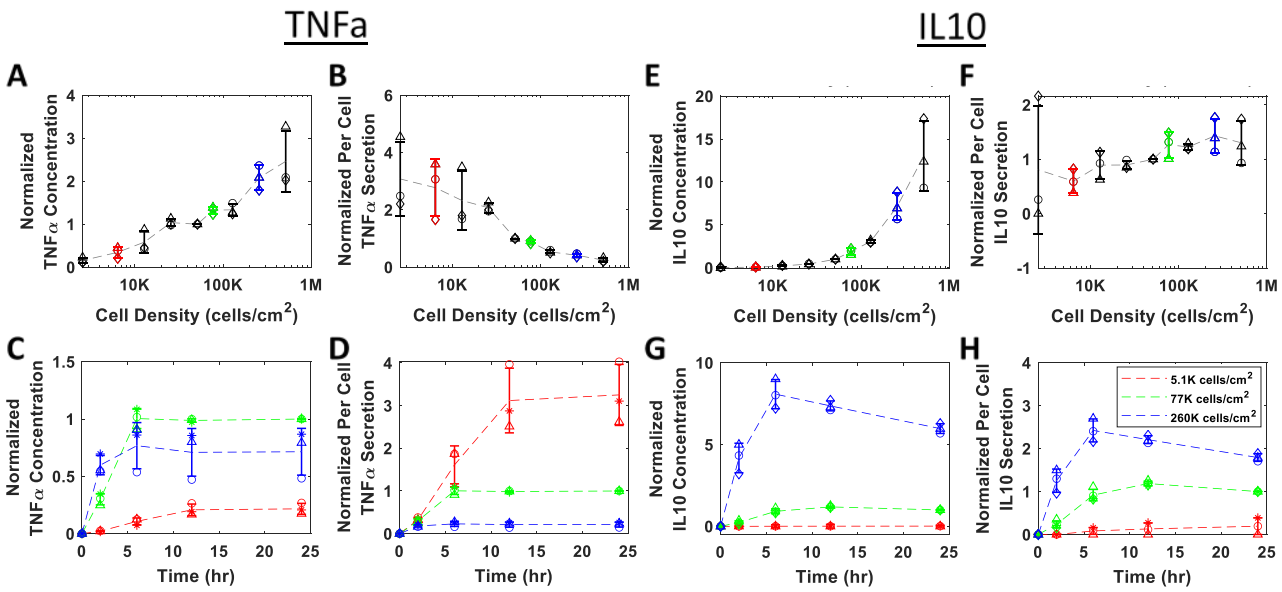
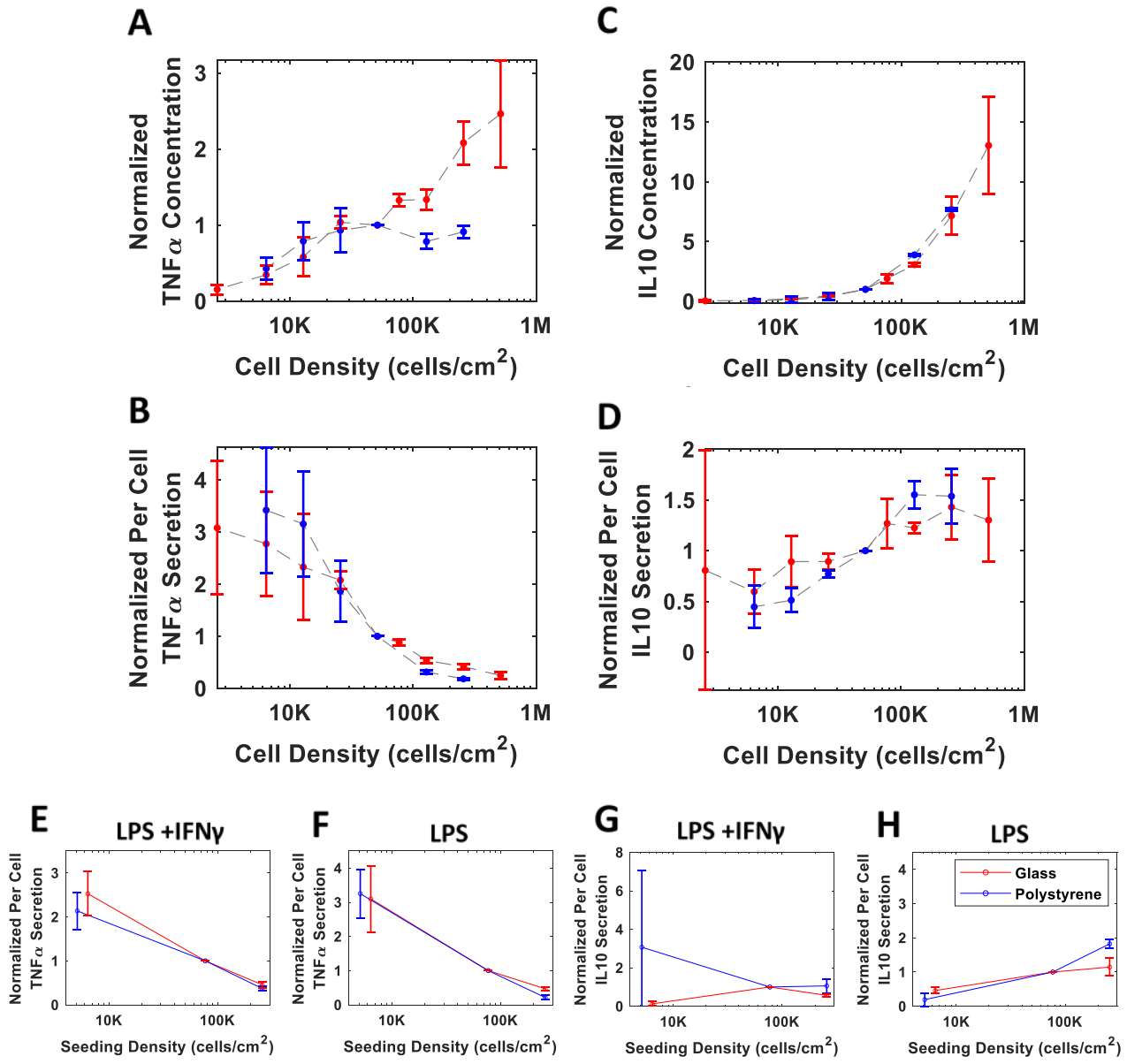


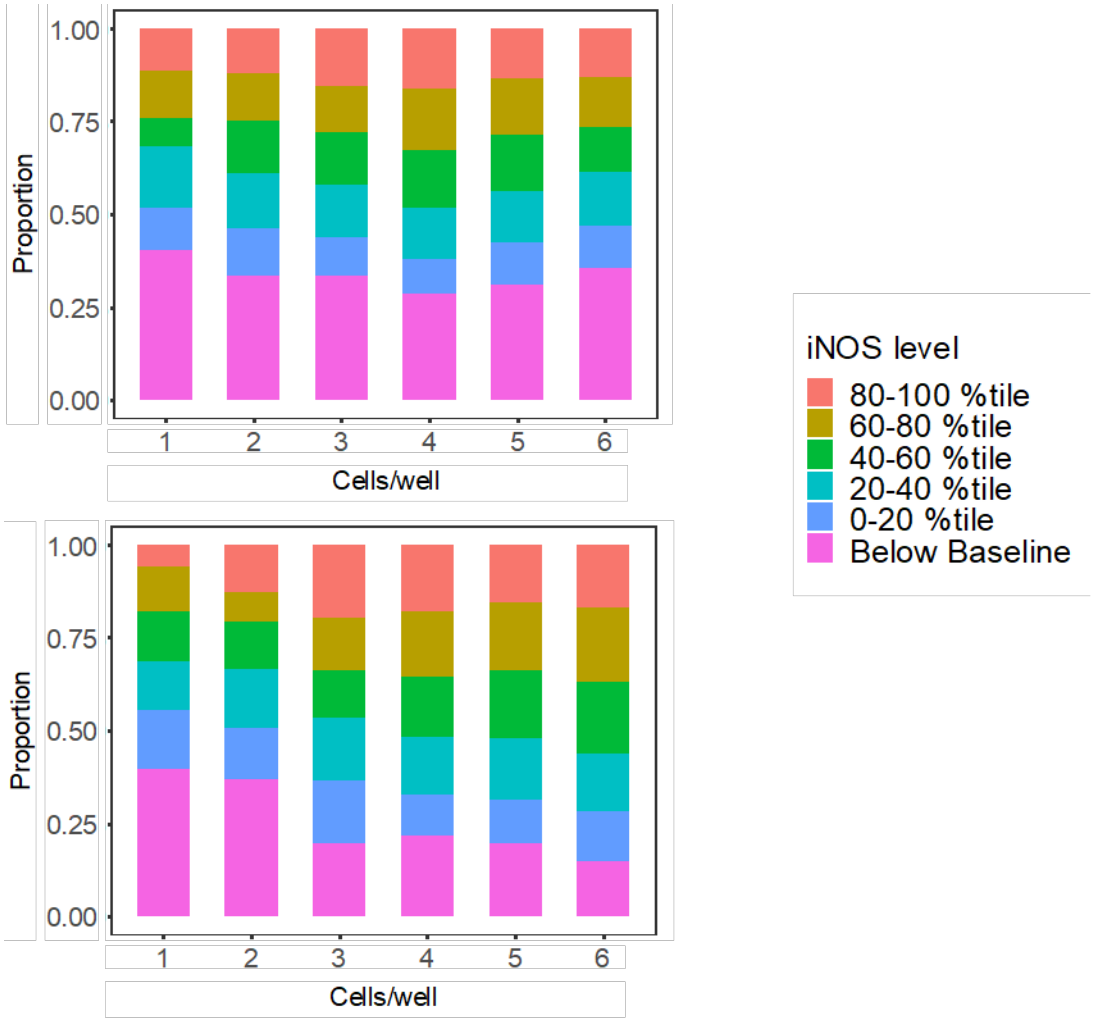
Figure 4: Microwell experiments revealed a different mode of density-dependent regulation of macrophage function. (A) A representative sample of distribution of all living cells in wells containing 1-6 cells grouped by their percentile rank. These cells were first separated based on whether their iNOS 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.



Supplement figure 1: 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.



Supplement figure 2: 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.



Supplement figure 3: Compared to cells in isolation, cells in small groups generally have higher expressions of iNOS. Distribution of all cells in wells containing 1-6 cells; these cells were separated based on their iNOS expression levels. For cells 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, 0-20 percentile). Each graph represents a different experimental replicate.