Microwell-Based Assay Revealed Population Dependent Controls of Macrophage Activation

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Introduction: 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 the regulation of macrophage polarization. Furthermore, recent studies also show that macrophage signaling within a population is critical for coordinating a collective response [1, 2]. However, how such coordination arises from groups of cells, and how the collective behavior of small groups of cells compared to single, isolated cells, remains to be investigated. In this study, we attempt to address this problem by utilizing a microwell-based platform to probe the responses of cells in isolation versus cells in small groups following a pro-inflammatory stimulation. Our study suggests that expression of the inflammatory marker iNOS depends on the communication among groups of cells, and this regulation may also depend on the absolute cell numbers. This experimental platform may help further explore different mechanisms utilized to regulate collective inflammatory responses.

Materials and Methods: Macrophages were seeded into arrays of microwells in sizes of approximately 200x300μm made with PDMS thin films and glass coverslips. After overnight attachment, cells were stimulated with LPS and IFNγ, sealed in the wells, and incubated for 24 hours. Following the treatment, cells in the microwells were fixed and stained for iNOS, and the entire microwell arrays were scanned using an Olympus IX83 microscope. The imaging data were first processed using ImageJ and MATLAB, and then analyzed with R Studio and Microsoft Excel. To ensure that the media volumes per cell were comparable in ranges as that of the bulk studies, only wells containing 1-6 cells were analyzed. For the bulk experiments, cells were seeded in different densities onto 12-well plates containing glass coverslips and followed similar experimental procedures and analysis processes as outlined above.

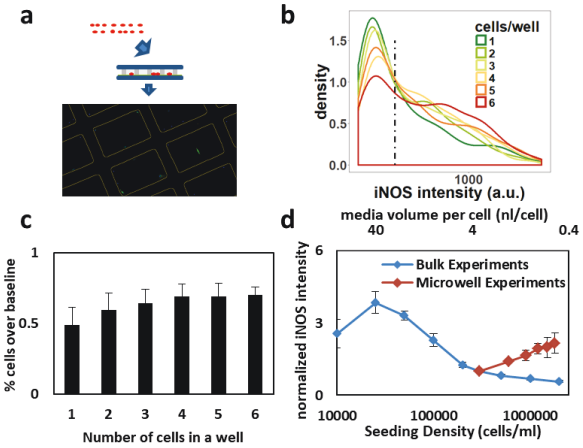

Figure 1: (**a**) A schematic diagram of the design for the microwell experiments (**b**) Density distribution for the sampled iNOS intensity of macrophages in microwells containing 1 to 6 cells (**c**) The percentage of cells with iNOS intensity over the unstimulated baseline with respect to the number of cells in microwells. (Error bar = SEM) (**d**) Normalized median iNOS intensity of macrophages from the microwells containing 1 to 6 cells, as well as from the bulk experiments. (Error bar = SEM) Both curves were matched to have comparable media volumes per cell.

Results and Discussion: We analyzed the expression of iNOS of cells seeded in microwells containing 1-6 cells. When comparing macrophages in microwells containing 1-6 cells, the median cellular iNOS intensity appeared to increase for cells in microwells as the numbers of cells increased. This result was in contrast to the population staining of iNOS, which showed a decreasing trend within the ranges of cell densities that have comparable cell-to-media-volume ratios as those in the microwells. On the other hand, the data also suggested that percentage of cells over unstimulated baseline increased with increasing number of cells in a microwell. Interestingly, such increase appeared to dampen when there were 4 or more cells in a microwell. This effect seemed to mirror the trends observed in population studies, and suggested that intercellular communication could augment or dampen iNOS expression of macrophages depending on the cellular environments or absolute cell numbers.

Conclusions: In this study, we investigated the iNOS expression of macrophages cultured in isolation and in small groups within microwells. Our study suggested that the iNOS expression in macrophages depends on the coordination among the cell populations. In addition, both cellular density and absolute cell numbers may contribute to the macrophage response. Future directions include looking into individual wells to probe the different possibilities that cells may coordinate themselves, as well as incorporating other measurements such as TNFα concentrations to the platform to more comprehensively assess macrophage responses.

Reference: [1] Xue, Q. *Sci. Signal.* 2015(8): ra59 [2] Muldoon, JJ. *Nat Commun.* 2020(11): 878