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High-throughput multiplex assays with mouse macrophages on pillar plate platforms

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Abstract

It is challenging to rapidly identify immune responses that reflect the state and capability of immune cells due to complex heterogeneity of immune cells and their plasticity to pathogens and modulating molecules. Thus, high-throughput and easy-to-use cell culture and analysis platforms are highly desired for characterizing complex immune responses and elucidating their underlying mechanisms as well. In response to this need, we have developed a micropillar chip and a 384-pillar plate, printed mouse macrophage, RAW 264.7 cell line in alginate on the pillar plate platforms, and established multiplex cell-based assays to rapidly measure cell viability, expression of cell surface markers, and secretion of cytokines upon stimulation with model compound, lipopolysaccharide (LPS), as well as synthetic *N*-glycan polymers. We synthesized novel *N*-glycan polymers that mimic native glycoconjugates and could bind to lectin receptors on RAW 264.7 cells. Interestingly, changes in RAW 264.7 cell viability, expression levels of cell surface markers, and release of cytokines measured from the pillar plate platforms in the presence and absence of LPS were well correlated with those obtained from their counterpart, the 96-well plate with 2D-

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Credit Author Statement

Parnian Bigdelou: Pillar/well plates assays. **Ka Keung Chan:** 96-Well plates assays, **Jinshan Tang:** Synthesis of Glycopolymers. **Kyeong-Nam Yu:** pillar/well plates assays. **Joshua Whited:** Synthesis of Glycopolymers. **Dan Wang:** Cell cultures. **Moo-Yeal Lee:** Conceptualization, Methodology and Supervision on Pillar/well plates assays, Writing-Original draft preparation, Writing- Reviewing and Editing. **Xue-Long Sun:** Conceptualization, Methodology Supervision on Synthesis of Glycopolymers and 96-well plates assays, Writing- Original draft preparation, Writing- Reviewing and Editing.

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Conflicts of interest

The authors have no conflicts of interest to disclose in relation to this manuscript.

Data availability

The authors declare that all the data supporting the results in this study are available within the article and its Supplementary Information.

cultured macrophages. With this approach, we identified that α 2,3-linked *N*-sialyllactose polymer has significant macrophage modulation activity among the *N*-glycan polymers tested. Therefore, we successfully demonstrated that our pillar plate platforms with 3D-cultured macrophages can streamline immune cell imaging and analysis in high throughput in response to compound stimulation. We envision that the pillar plate platforms could potentially be used for rapid characterization of immune cell responses and for screening immune cell-modulating molecules.

Graphical Abstract

Keywords

Micropillar chip; 384-pillar plate; macrophage modulation; multiplex immunoassay; lipopolysaccharide; *N*-glycan polymer

Introduction

The immune system protects the host through the ability to mobilize responses to invading pathogens, toxins, or allergens as immune cells can distinguish self from non-self *via* both innate and adaptive mechanisms [1]. Analytical measurements are required to characterize the state and capability of immune cells, reveal the fundamental biology of immunity, and establish distinct signatures for specific immune responses. In addition, they provide insight into the evolution of disease, and aid the design of clinical diagnostics and interventions [2]. The complex heterogeneity of immune cells and their interactions with pathogens are major challenges to identify immune responses that reflect the state and capability of the immune system [3]. In general, the expression of specific cell surface markers and the profile of cytokines release from the cells are often examined to evaluate a specific immune cell response [4,5]. Conventionally, cell surface markers have been examined by flow cytometry [4], and released cytokines are quantified by an enzyme-linked immunosorbent assay (ELISA) or immunobeads flow cytometry after cell incubation with ligands in microtiter well plates [5]. Overall, a lengthy time necessary for sample preparation could be an issue for both ELISA and bead-based assays. For example, pipetting and centrifuging are often

applied for rinsing and separating cells from culture media, followed by measuring cytokines in the supernatants and cell surface markers in the remnant cells, separately. Multiple steps of cells and media transferring and separation *via* pipetting and centrifuging often lead to losing both cells and media in the process. In addition, a large number of samples are necessary to perform these assays, leading to the high cost of the assays. Another key issue of ELISA is its inconvenience and low throughput, which is unsuitable for screening a large number of samples and their combinations. Furthermore, interferences between capture antibodies could be an issue for bead-based assays. Therefore, tremendous efforts have been made to develop easy-to-use immunoassays. For example, an antibody array-based snap chip [6] and an immune-pillar chip [7] have been developed for rapid and easy to use immunoassays. Nevertheless, these platforms still require multiple steps and complicated settings and thus have not been widely adopted in R&D.

Recently, we have developed pillar plate platforms including a micropillar chip and a 384-pillar plate that can facilitate miniature cell cultures as well as high-throughput biochemical and cell-based assays [8,9]. Bioprinted cells in biomimetic hydrogels on the pillar plates were coupled with a microwell chip and a 384-well plate containing growth media, recombinant viruses, test compounds, and reagents for cell culture, test, staining, and imaging *in situ*. Since this approach can simplify the process of 3D cell culture and multiplex cell-based assays, mouse macrophage RAW 264.7 cells have been printed on the pillar plates as an immune cell model and tested with lipopolysaccharide (LPS) and novel synthetic *N*-glycan polymers for immune cell modulation. Our goal in this study was to investigate immune cell responses to modulating compounds on the pillar plates and establish multiplex and miniature immune cell assays in high throughput, alternative to conventional microtiter well plate platforms. Macrophages play significant roles in immune responses and are potential contenders for immunomodulation that may effectively modify immunological processes in cancers and infectious diseases [10–12]. In addition, the synthesized *N*-glycan polymers may mimic the natural *N*-glycan conjugates and could potentially bind to lectin receptors on RAW 264.7 cells for immune modulation applications [13,14]. Therefore, our model system would be well suited for establishing multiplexed, immune cell printing and analysis on the pillar plates and could be potentially used for elucidating the underlying mechanisms of immune responses of macrophages.

Materials and Methods

Materials and reagents

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Deionized water was used as a solvent in all procedures. Polymaleic anhydride-*alt*-1-octadecene (PMA-OD), alginate (alginic acid sodium salt), 0.01% poly-L-lysine (PLL), barium chloride (BaCl₂), and LPS (from *Escherichia coli* 026:B6) were obtained from Sigma Aldrich (St. Louis, MO, USA). All primary antibodies including BD Pharmingen™ FITC rat anti-mouse CD40, BD Pharmingen™ FITC rat anti-mouse CD1d, BD Pharmingen™ FITC hamster anti-mouse CD80, and BD Pharmingen™ FITC mouse anti-mouse I-A[d] were obtained from BD Biosciences (San Diego, CA, USA). Polyacrylamide, *N*-glucosyl polymer, *N*-galactosyl polymer, *N*-mannosyl polymer, *N*-

GlcNAc polymer, *N*-lactosyl polymer, *N*- α 2,6-sialolactosyl polymer, and *N*- α 2,3-sialolactosyl polymer were synthesized in our previously reported method [15]. The micropillar chip, the microwell chip, and the 384-pillar plate were purchased from Medical & Bio Device (MBD) Korea (Suwon, Republic of Korea).

Cell culture and printing on the pillar plates

RAW 264.7 cells from ATCC were cultured in T-75 flasks with DMEM containing 25 mM glucose, 4.0 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin-streptomycin, and 10% fetal bovine serum. For robust attachment of cell spots on the surface of pillar plates, the micropillar chip and the 384-pillar plate were coated with 0.01% PMA-OD in ethanol, an amine-reactive polymer, followed by printing 60 nL of a mixture of 0.0033% PLL and 16.6 mM BaCl₂ on the micropillar chip or printing 1 μ L of the PLL-BaCl₂ mixture on the 384-pillar plate using a microarray spotter (S+ MicroArrayer from MBD Korea). After trypsinization and centrifugation, the suspension of RAW 264.7 cells was mixed with 0.75% alginate and 0.5 mg/mL Matrigel, and then 60 nL of the mixture at a density of 3×10^6 cells/mL (180 cells/pillar) were printed on the micropillar chip with dried PLL-BaCl₂ spots. Similarly, 1 μ L of the cell mixture at a density of 3×10^6 cells/mL were printed on the 384-pillar plate with dried PLL-BaCl₂ spots to obtain 3,000 cells/pillar. After 2–4 minutes of alginate gelation, the pillar plates with RAW 264.7 cells were sandwiched with the microwell chip containing 950 nL of DMEM or the 384-well plate containing 50 μ L of DMEM, and then incubated in a 5% CO₂ incubator at 37°C for 24 hours.

Measurement of cytotoxicity on the micropillar chip after treatment with LPS and N-glycan polymers

Synthetic *N*-glycan polymers including *N*-glucosyl polymer, *N*-galactosyl polymer, *N*-mannosyl polymer, *N*-GlcNAc polymer, *N*-lactosyl polymer, *N*- α 2,6-sialolactosyl polymer, and *N*- α 2,3-sialolactosyl polymer were dissolved in DMEM at concentrations of 0, 10, 50, 250, 1250, and 6250 μ g/mL. As a positive control, LPS was dissolved and sonicated in DMEM at 0, 0.4, 2, 10, 50, and 250 μ g/mL. These compounds, dissolved in DMEM, were dispensed into the microwell chip at 950 nL per well (6 doses per compound, 14 replicates per dose). For compound exposure, the micropillar chip with the RAW 264.7 cells was separated and sandwiched onto the microwell chip with test compounds, and then incubated in a 5% CO₂ incubator at 37°C for 24 hours. For live/dead assays, the micropillar chip with the cells was rinsed twice with a saline solution containing 140 mM NaCl and 20 mM CaCl₂ for 10 minutes each, and then stained with 1 μ M calcein AM and 1 μ M ethidium homodimer-1 in the saline solution for 1 hour. This was followed by rinsing twice with the saline solution for 10 minutes each and complete drying for at least 3 hours in the dark. For rapid fluorescent cell image acquisition at 15 frames per second (FPS), the micropillar chip was scanned with an automated epifluorescence microscope (S+ scanner from MBD Korea). Green and red fluorescent cell images were obtained at 4X magnification with the Olympus UPLFLN 4X (numerical aperture (NA) 0.13, f-number 26.5, and depth of field (DOF) $\sim$ 32.3 μ m) (Olympus, Tokyo, Japan), and a green filter (XF404 from Omega Optical) and a red filter (TxRed-4040C from Semrock). The images were batch-processed using ImageJ (NIH) to extract the fluorescent intensity from each cell spot on the micropillar chip. The fluorescent intensities were plotted using S+ Chip Analysis (MBD Korea). The sigmoidal

dose-response curves (variable slope) and IC₅₀ values (i.e., concentration of the compound where 50% of cell viability/growth inhibited) were obtained using the following equation:

$$Y = \text{Bottom} + \left[\frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{LogIC}_{50} - X) \times H}} \right]$$

where IC₅₀ is the midpoint of the curve, H is the hill slope, X is the logarithm of test concentration, and Y is the response (% live cells), starting from the top plateau (Top) of the sigmoidal curve to the bottom plateau (Bottom).

Measurement of cytotoxicity in a 96-well plate after treatment with LPS

For comparison with cytotoxicity data obtained from the pillar chip, cytotoxicity assays were performed with the positive compound, LPS in a 96-well plate. Briefly, RAW 264.7 cell suspension was prepared in DMEM at a cell density of 50,000 cells/mL, 100 µL of this cell suspension was seeded in a 96-well plate at 5000 cells/well, and then the 96-well plate with RAW 264.7 cells was incubated in a 5% CO₂ incubator at 37°C for 24 hours. For compound exposure, the old DMEM medium was removed from the 96-wells, 100 µL of fresh DMEM containing LPS at concentrations of 0, 0.4, 2, 10, 50, 250 µg/mL was added into the 96-wells, and the 96-well plate was incubated in a 5% CO₂ incubator at 37°C for 24 hours. Each LPS test condition consisted of 4 replicates. For live/dead assays, the LPS solution was removed from the 96-wells, and each 96-well with cells was rinsed twice with 100 µL of the saline solution for 10 minutes each and then stained with 100 µL of 1 µM calcein AM and 1 µM ethidium homodimer-1 in the saline solution for 1 hour. Fluorescent cell images were acquired with the S+ scanner using the green and red filters.

Measurement of cell surface markers on the micropillar chip after treatment with LPS and N-glycan polymers

The micropillar chip, with RAW 264.7, cells was sandwiched with the microwell chip containing 10 µg/mL of LPS (positive control) and 1250 µg/mL of the N-glycan polymers and incubated in a 5% CO₂ incubator at 37°C for 24 hours. As a negative control, the growth medium alone was used. Each test condition consisted of 4 replicates. The micropillar chip was separated and rinsed twice with Tris-buffered saline (TBS) for 10 minutes each. The cells were fixed in a mixture of ice-cold methanol and acetone at 1:1 ratio for 10 minutes at room temperature. The micropillar chip with fixed cells was rinsed twice with TBS for 3 minutes each, and then incubated with SuperBlock blocking buffer for 1 hour at room temperature. The primary antibodies including FITC-labeled rat anti-mouse CD40, CD1d, CD80, and I-A[d] were diluted in the blocking buffer at 1:50 ratio to obtain the final concentration of 0.01 mg/mL. The cells were stained with 0.01 mg/mL of the primary antibodies at 4°C overnight, followed by staining with 1 µg/mL DAPI for 30 minutes and rinsing twice with TBS for 10 minutes each. After drying the micropillar chip for at least 3 hours in the dark, fluorescent images were acquired from the cells using the S+ scanner and the fluorescent intensity was extracted with ImageJ to measure changes in the expression level of cell surface biomarkers.

Measurement of cell surface markers in a 96-well plate after treatment with LPS

The RAW 264.7 cells in a 96-well plate were exposed to 10 µg/mL of LPS for 24 hours at 37°C, rinsed twice with phosphate-buffer saline (PBS) buffer at pH 7.4 for 5 minutes each, and then fixed in 4% paraformaldehyde for 30 minutes at room temperature. The fixed cells were rinsed twice with the PBS buffer solution for 5 minutes each and incubated with a blocking buffer solution containing 10% BSA and 0.1% sodium azide in the PBS buffer solution for 30 minutes at 4°C. The primary antibodies, including FITC-labeled rat anti-mouse CD40, CD1d, CD80, and I-A[d], were diluted in the blocking buffer at 1:50 ratio to obtain the final concentration of 0.01 mg/mL. The cells were stained with 0.01 mg/mL of the primary antibodies at 4°C overnight, followed by staining with 1 µg/mL DAPI for 10 minutes and rinsing twice with PBS for 5 minutes each. For flow cytometry, a portion of the cells was collected and resuspended in 1% BSA and 0.5% sodium azide in PBS and transferred to Falcon round-bottom tubes. For each test condition, 50,000 events were recorded each time with BD FACSCanto™ II Cell Analyzer, and data were assessed using BD FACSDiva software (BD Bioscience, Mountain View, CA). For confocal imaging, the remaining portion of the cells was collected and resuspended in ProLong™ Gold anti-fade reagent, followed by spotting on microscope slides and image acquisition using Nikon AIRSI confocal microscope. Fluorescent cell images were processed using the NIS Elements AR software.

Measurement of cytokine release from RAW 264.7 cells on the 384-pillar plate after treatment with LPS and N-glycan polymers

The 384-pillar plate with RAW 264.7 cells was sandwiched with the 384-well plate containing 50 µL of 10 µg/mL LPS (positive control) and 50 µL of 1250 µg/mL *N*-glycan polymers and incubated in a 5% CO₂ incubator at 37°C for 24 hours. All compounds dissolved in DMEM were sonicated and filtered through a 0.2 µm membrane. As a negative control, the growth medium alone was used. Each test condition consisted of 4 replicates. The supernatant collected from the 384-well plate was centrifuged at 200 g for 4 minutes and shipped to Eve Technologies (Calgary, Alberta, Canada) for the mouse cytokine 31-plex discovery assay.

Measurement of cytokine release from RAW 264.7 cells in a 96-well plate after treatment with LPS

RAW 264.7 cells cultured in a 96-well plate were exposed to 10 µg/mL of LPS (positive control) and DMEM alone (negative control) in a 5% CO₂ incubator at 37°C for 24 hours. Each test condition consisted of 4 replicates. The cell supernatant collected from the 96-well plate was centrifuged at 200 g for 4 minutes and shipped to Eve Technologies for the mouse cytokine 31-plex discovery assay.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8.0 (La Jolla, CA, USA) to compare the levels of biomarkers expressed and cytokines released from the micropillar chip and the 384-pillar plate. One-way analysis of variance (ANOVA) was used to compare the mean values of the compound obtained from RAW 264.7 cells. Statistically significant differences

between control and test conditions were indicated by * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$ ($n = 4$).

Results and Discussion

Cytotoxicity of RAW 264.7 cells against LPS and N-glycan polymers

Prior to testing immune cell responses to novel *N*-glycan polymers, we established cytotoxicity assays with RAW 264.7 cells and LPS on the micropillar chip and in the 96-well plate. RAW 264.7 cell, a mouse macrophage cell line, is often used in immune studies along with LPS, which is an immune stimulator of the cell [16,17]. In this study, RAW 264.7 cells were cultured on the two different platforms, and their responses to LPS stimulation were compared to establish multiplex and miniature immune cell assays on the pillar plates. Alginate used for cell encapsulation on the pillar plates has been known to have no immunomodulation activity on RAW 264.7 cells [18,19]. In addition, Matrigel is often used for 3D macrophage co-culture assays [20,21]. Therefore, a mixture of alginate and Matrigel was selected to encapsulate RAW 264.7 cells on the micropillar chip and the 384-pillar plate in this study (Fig. 1). Briefly, RAW 264.7 cell monolayers in the 96-well plate, as well as bioprinted RAW 264.7 cells in the alginate-Matrigel mixture on the micropillar chip, were exposed to different concentrations of LPS for 24 hours and stained with calcein AM and ethidium homodimer-1 to obtain dose response curves (Fig. 2). As a result, LPS showed dose-dependent cytotoxicity against RAW 264.7 cells on the micropillar chip (Fig. 2A). Green and red dots represent live and dead cells, respectively. This result indicates that RAW 264.7 cells were successfully encapsulated in the mixture of alginate and Matrigel on the micropillar chip and could be cultured over time without basal toxicity issues from the hydrogels and the crosslinker, BaCl_2 . We have already demonstrated that several human cell types can be encapsulated in alginate, Matrigel, fibrin, PuraMatrix, and alginate-Matrigel and cultured on the chip platform for toxicity assessment [8,9,22–24]. Similarly, LPS showed dose-dependent cytotoxicity against RAW 264.7 cells in the 96-well plate (Fig. 2B). Interestingly, the dose response curve obtained from the 96-well plate was well correlated with that from the micropillar chip (Fig. 2C). These results indicate that the micropillar chip can be used for evaluating the viability of RAW 264.7 cells after treatment of large molecules like LPS as an alternative to the 96-well plate. Importantly, our approach can reduce the assay volume as much as 100-fold while obtaining essentially identical data.

With this promising result, we tested cytotoxicity of RAW 264.7 cells on the micropillar chip against a series of monosaccharides, disaccharide, and trisaccharide-containing *N*-linked glycopolymers as biomimetic glycoligands, which include *N*-glucosyl polymer, *N*-galactosyl polymer, *N*-mannosyl polymer, *N*-GlcNAc polymer, *N*-lactosyl polymer, *N*- α 2,3-sialolactosyl polymer, and *N*- α 2,6-sialolactosyl polymer. The *N*-glycan polymers were synthesized from free glycans *via* glycosylamine intermediates followed by acrylation and polymerization *via* cyanoxyl-mediated free radical polymerization (CMFRP), in which no protection and deprotection steps were used (see Supplementary Information, Scheme S1) [15]. Briefly, bioprinted RAW 264.7 cells in the alginate-Matrigel mixture on the micropillar chip were exposed to different concentrations of the *N*-glycan polymers in the microwell chip for 24 hours and stained with calcein AM and ethidium homodimer-1 to obtain dose

response curves. As a result, all *N*-glycan polymers were not significantly cytotoxic against RAW 264.7 cells at a concentration of 6250 µg/mL except for *N*-glucosyl polymer and *N*-α2,3-sialolactosyl polymer, which were non cytotoxic at 1250 µg/mL (Fig. 3 and Supplementary Information Fig. S1 for all dose response curves of the *N*-glycan polymers). Therefore, the dosage of 1250 µg/mL was chosen to evaluate immunomodulation activities of the *N*-glycan polymers in the follow-up experiments.

Changes in the expression levels of cell surface markers after treatment with LPS and *N*-glycan polymers

Prior to assessing potential immune responses of *N*-glycan polymers, we established immunofluorescence assays on the micropillar chip and rapidly measured changes in cell surface markers on RAW 264.7 cells after LPS treatment. LPS has been known to induce expression of specific cell surface markers and secretion of cytokines from RAW 264.7 cells [16]. Flow cytometric studies indicate that cell surface markers including CD40, CD80, and class II major histocompatibility complex (MHC) antigens were upregulated on LPS-treated RAW 264.7 cells [16]. To establish a proof of concept study, the immune response of RAW 264.7 cells to LPS on the micropillar chip was examined by sandwiching the micropillar chip with RAW 264.7 cells onto the microwell chip containing 10 µg/mL of LPS and staining the cells with FITC-labeled primary antibodies including rat anti-mouse CD40, CD1d, CD80, and I-A[d]. As a result, LPS induced high levels of CD-40, CD-80, CD1d, and class II MHC markers expression (Fig. 4A), which were well consistent with the flow cytometric data from literature [16]. To further validate the results from the micropillar chip, we conducted immunofluorescence assays in a 96-well plate and examined the same cell surface markers by flow cytometry and confocal microscope analysis. Except CD1d, all other surface markers data from both flow cytometry (Fig. 4B) and confocal imaging (Supplementary Information Fig. S4) were aligned well with the data from the micropillar chip, thus confirming that the micropillar chip platform can be a practical tool for examining cell surface markers upon compound treatment.

With successful establishment of immunofluorescence assays on the micropillar chip, we measured potential immune responses of *N*-glycan polymers. In this study, we focused on Siglecs which recognize sialyl glycoconjugates for immune response and are potential targets for immunomodulation for the treatment of cancers as well as inflammatory, autoimmune, allergic, and infectious diseases [25]. Synthetic sialyl glycoconjugates may affect the expression of cell surface antigens on macrophages but have not been fully investigated so far. We selected *N*-α2,3-sialolactosyl polymer and *N*-α2,6-sialolactosyl polymer as Siglecs-targeting biomimetic glycoligands. In addition, *N*-glucosyl polymer, *N*-galactosyl polymer, *N*-mannosyl polymer, *N*-GlcNAc polymer, and *N*-lactosyl polymer were synthesized for comparison. The modulation effects of these *N*-glycan polymers on RAW 264.7 cells were evaluated on the pillar plate platforms by measuring the expression levels of cell surface biomarkers and the secretion levels of cytokines, respectively. Briefly, the micropillar chip with RAW 264.7 cells was sandwiched onto the microwell chip containing 1250 µg/mL of the *N*-glycan polymers and stained with FITC-labeled primary antibodies including rat anti-mouse CD40, CD1d, CD80, and I-A[d]. As a result, the *N*-sialolactosyl polymers (compounds **9** and **10**) induced significantly high expression levels of CD40,

CD80, CD1d, and class II MHC markers (Fig. 5), as compared to DMEM (negative) and LPS (positive) controls. In particular, *N*- α 2,3-sialolactosyl polymer (**9**) showed the highest induction of all four cell surface markers, indicating its strong macrophage modulation activity. This result is correlated well with precedent reports that macrophage Siglecs prefer binding glycans with a Neu5Ac α 2-3Gal β 1 sequence [26]. In addition, *N*-galactosyl polymer (**5**) induced higher expression level of CD80 and class II MHC cell surface markers. This could be because it may target macrophage galactose binding lectin (MGL) as well, which is a C-type lectin on macrophages [27]. MGL is an oligomeric type II transmembrane protein and serves as an important immune cell target as well [28]. These results indicate that the *N*-glycan polymers have a strong immunomodulation effect on RAW 264.7 cells. The *N*-sialolactosyl polymer may serve as a lead for continuing study to develop Siglecs ligands with improved binding affinity and selectivity towards Siglecs, which would represent invaluable tools and candidates for exploring therapeutic opportunities of sialic acid-mediated Siglecs recognition.

Changes in secretion of cytokines after treatment with LPS and N-glycan polymers

With the established immunofluorescence assays on the micropillar chip, we profiled cytokines released from RAW 264.7 cells by printing RAW 264.7 cells in alginate on the 384-pillar plate, a large-size micropillar chip, and sandwiching it onto a 384-well plate containing 10 μ g/mL of LPS for 24 hours. The 384-pillar plate was introduced due to a relatively large volume of samples (50 μ L) required for the mouse cytokine 31-plex discovery assay. For validation of the 384-pillar plate, RAW 264.7 cell monolayers were cultured in a 96-well plate and exposed to 10 μ g/mL of LPS for 24 hours. The solutions in the 384-well plate (complementary to the 384-pillar plate) and the 96-well plate were collected to determine the secretion levels of cytokines by the mouse cytokine 31-plex discovery assay. As a result, LPS induced the production of TNF- α , IL-6, and IL-10 significantly, but a minimum amount of VEGF production from RAW 264.7 cells on the 384-pillar plate as well as in the 96-well plate (Fig. 6). All these data indicate that alginate and Matrigel used for encapsulation of RAW 264.7 cells on the micropillar chip and the 384-pillar plate do not stimulate the cells, and the pillar plate platforms are well suited for rapidly evaluating the immune response of RAW 264.7 cells with compounds. Importantly, the cytokines secreted into the 384-well plate were collected and measured directly after removing the 384-pillar plate containing RAW 264.7 cells, avoiding multiple washing steps in conventional ELISA assays, with greater efficiency and speed.

Similarly, cytokine secretion from RAW 264.7 cells after treatment with 1250 μ g/mL of the *N*-glycan polymers was assessed on the 384-pillar plate as it is another key feature in macrophages activation [29]. Sialic acid-containing macromolecules were reported to affect the secretion of cytokine IL-6 and IL-10 from murine and human macrophages [30,31], indicating the potential of exploiting inhibitory signaling by macrophage Siglecs as a tool for dampening inflammatory [32]. The samples collected from the 384-well plate were examined by the mouse cytokine 31-plex discovery assay to measure the levels of cytokine secretion. As a result, most *N*-glycan polymers induced secretion of anti-inflammatory cytokines such as IL-6, IL-10, and VEGF (Fig. 7). Among them, *N*- α 2,3-sialolactosyl polymer (**9**) induced IL-6 secretion significantly (Fig. 7A), *N*-galactosyl polymer (**5**)

induced IL-10 (Fig. 7B) and VEGF (Fig. 7C) secretion mildly, and *N*-glucosyl polymer (**4**) and *N*-GlcNAc polymer (**7**) induced VEGF secretion (Fig. 7C). These results further confirmed the immunomodulatory effect of the *N*-glycan polymers on RAW 264.7 cells. Thus, the 384-pillar plate could be used for high-throughput screening of cytokine secretion activities of synthetic glycoligands on macrophages. The 384-pillar plate-based assay may enable simultaneous quantitation of multiple secreted cytokines and could be an invaluable high-throughput tool for the comprehensive study of immunomodulation.

Conclusions

In summary, we have established multiplex immune cell assays including cell viability, the expression of cell surface markers, and the secretion of cytokines on the pillar plate platforms upon stimulation of mouse macrophages with LPS and the *N*-glycan polymers. Our pillar plate platforms offer unique advantages over conventional microtiter well plate platforms for high-throughput screening of immune responses. First, the immune cell encapsulation protocols established in alginate droplets on the micropillar chip and the 384-pillar plate allowed convenient growth medium change without damaging the cells during culture, prevented cell loss during extensive rinsing steps, and could be well suited for encapsulation of non-adhesive immune cells. Second, high-throughput, high-content cell imaging facilitated *in situ* on the pillar plate platforms allowed rapid assessment of cytotoxicity and cell activation by compound treatment. Finally, the pillar plate platforms required relatively small amounts of cells, compounds, and reagents for the assays, which would be best suited for testing compounds in a scarce quantity. Currently, we are developing immunofluorescence assays on the pillar plates with multicolor-labeled antibodies (e.g., Alex488-anti-CD40 antibody, Alex402-anti-CD1D antibody, Alex532-anti-CD80 antibody, etc.) to detect multiplex features of immune cells. Therefore, we envision that our pillar plate platforms could be potentially used for a wide range of immune cell-based assays for basic research, development, clinical diagnosis, and screening applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ELISA	enzyme-linked immunosorbent assay
LPS	lipopolysaccharide
MHC	class II major histocompatibility complex

PLL	poly-L-lysine
PMA-OD	polymaleic anhydride- <i>α</i> / <i>l</i> -1-octadecene
TBS	Tris-buffered saline
PBS	phosphate-buffer saline

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Highlights

An easy-to-use immunoassay with pillar/well platform using mouse macrophages was established.

The new immunoassay can streamline cell imaging and analysis of immunoresponse in high throughput

A multiplex cell assay allows rapidly measurement of cell surface markers and secreted cytokines.

α 2,3-linked *N*-sialyllactose polymer was confirmed to modulate macrophage with the new assay.

(A) A picture of micropillar/microwell chips and 384-pillar/well plates. A single micropillar chip contains 532 micropillars (0.75 mm pillar diameter, 1 mm pillar height, and 1.5 mm pillar-to-pillar distance) whereas a single 384-pillar plate contains 384 pillars (2.0 mm pillar diameter, 10.4 mm pillar height, and 4.5 mm pillar-to-pillar distance). The pillar plates can be separated and sandwiched onto new well plates containing test compounds and reagents for various miniature biochemical and cell-based assays. (B) Schematics for profiling of immune cell responses using the pillar plate platforms. RAW 264.7 cells in alginate are printed on the pillar plates using a microarray spotter and cultured by sandwiching the well plates with growth media and test compounds. High-content cell imaging can be performed on the pillar plates to assess changes in cell viability and measure the expression level of both cell surface and intracellular markers after compound exposure. Cytokines secreted from the activated cells into the well plates can be profiled by microbead antibody array/flow cytometry.

Figure 2. Cytotoxicity of RAW 264.7 cells on the micropillar chip and in the 96-well plate after treatment with lipopolysaccharides (LPS).

(A) Images of RAW 264.7 cells treated with LPS on the micropillar chip. The diameter of the entire cell spot was 0.75 mm. (B) Images of RAW 264.7 cells treated with LPS in the 96-well plate. Green and red dots represent live and dead RAW 264.7 cells after staining with 1 μM calcein AM and 1 μM ethidium homodimer-1. (C) Dose response curves obtained from RAW 264.7 cells on the micropillar chip ($n = 14$) and in the 96-well plate ($n = 4$).

Figure 3. Cytotoxicity of RAW 264.7 cells on the micropillar chip after treatment with *N*-glycan polymers:

(A) *N*-α2,3-sialolactosyl polymer and (B) *N*-α2,6-sialolactosyl polymer. The green and red dots represent live and dead RAW 264.7 cells stained with 1 μM calcein AM and 1 μM ethidium homodimer-1 (scale bar = 200 μm). Dose response curves were obtained from 14 replicates per dosage.

Figure 4. Expression levels of cell surface markers on RAW 264.7 cells after treatment with the growth medium (negative control) and LPS (positive control):
(A) the micropillar chip and **(B)** the 96-well plate. To determine statistically significant differences between no LPS control and LPS exposure conditions, one-way ANOVA analysis was performed, and the results were indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$ ($n = 4$). No indication means $p > 0.05$.

Figure 5. Expression levels of cell surface markers on RAW 264.7 cells after treatment with *N*-glycan polymers.

Fluorescent intensity of (A) CD40, (B) CD80, (C) CD1d, and (D) Class II MHC makers were determined by staining RAW 264.7 cells with FITC-labeled primary antibodies. The numbers indicate RAW 264.7 cells on the micropillar chip treated with: (1) growth medium alone, (2) LPS, (3) polyacrylamide, (4) *N*-glucosyl polymer, (5) *N*-galactosyl polymer, (6) *N*-mannosyl polymer, (7) *N*-GlcNAc polymer, (8) *N*-lactosyl polymer, (9) *N*- α 2,3-sialolactosyl polymer, and (10) *N*- α 2,6-sialolactosyl polymer. To determine statistically significant difference between growth medium control and *N*-glycan polymer exposure conditions, one-way ANOVA analysis was performed, and the results were indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$ (n = 4). No indication means $p > 0.05$.

Figure 6. Secretion of cytokines after treatment of RAW 264.7 cells with LPS.

Changes in cytokine release from RAW 264.7 cells on the 384-pillar plate and in the 96-well plate were measured in the presence of the growth medium alone (LPS -) and LPS (LPS +). To determine statistically significant difference between no LPS control and LPS exposure conditions, one-way ANOVA analysis was performed, and the results were indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$ ($n = 4$). No indication means $p > 0.05$.

Figure 7. Secretion of cytokines after treatment of RAW 264.7 cells with *N*-glycan polymers. The concentrations of (A) IL-6, (B) IL-10, and (C) VEGF were determined by the mouse cytokine panel assay. The numbers indicate RAW 264.7 cells on the 384-pillar plate treated with: (1) growth medium alone, (2) LPS, (3) polyacrylamide, (4) *N*-glucosyl polymer, (5) *N*-galactosyl polymer, (6) *N*-mannosyl polymer, (7) *N*-GlcNAc polymer, (8) *N*-lactosyl polymer, (9) *N*- α 2,3-sialolactosyl polymer, and (10) *N*- α 2,6-sialolactosyl polymer. One-way ANOVA analysis indicated as follows: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$ ($n = 4$). No indication means $p > 0.05$.