On-chip immune cell activation and subsequent magnetic bead-based cytokine detection

Patthara Kongsuphol*, Yunxiao Liu, Qasem Ramadan

Institute of Microelectronics, Agency for Science Technology and Research (A*STAR), 11 Science Park Rd, Singapore Science Park II, Singapore, 117685

*Corresponding author: P. Kongsuphol, email: kongsupholp@ime.a-star.edu.sg

Abstract

Cytokine profiling and immunophenotyping offer great potential for understanding many disease mechanisms, personalized diagnosis, and immunotherapy. Here, we demonstrate a time-resolved detection of a panel of cytokine from a single cell cluster using an in situ magnetic immune assay. An array of double-layered microfluidic chambers was fabricated to enable simultaneous cell culture and detection of the induced cytokines at multiple time-points. Each culture chamber comprises two fluidic compartments which are dedicated for cell culture and immune assay, respectively. The two compartments are separated by a porous membrane, which allows the diffusion of cytokines molecules from the cell culture compartment into the immune assay compartment. This structure hence enables capturing the released cytokines without disturbing the cell culture. Functionalized magnetic beads were used as a solid phase carrier for cytokine capturing and quantification. The LPS-induced cytokines were quantified in situ after differential stimulation of U937 monocytes and differentiated macrophages. This study provides a proof-of-concept for future automated system development which will be integrated into a complex cellular system for disease modeling.

1. Introduction

Monocytes and macrophages play an essential role in the immune system and maintaining tissue homeostasis[1]. Upon activation by either endogenous or exogenous factors, monocytes and macrophages release different types of cytokine to regulate and trigger proper immune responses in which these responses can be either defense, inflammation, or tissue remodeling [2,3].

Cytokines mediate many physiological events that contribute to disease development. Due to the importance of cytokines role as pathogenesis biomarker, it is essential to quantify cytokines in an accurate and timely manner even in a minute amount of biological samples. The quantitative detection of cytokine profile and subsequent immunophenotyping holds great promises for a better understanding of many disease mechanisms, personalized diagnostic and immune therapy. As a result of growing knowledge in cytokine, the release of cytokines from immune cells is being used as a parameter for many disease diagnosis and prognosis especially in infectious and autoimmune diseases[4–7]. In cancer research, considerable attention is paid to the possible role of cytokines-based in cancer therapy[8,9].

Currently, enzyme-linked immunosorbent assay (ELISA) is the most accepted method of cytokine detection which enables high sensitivity multiplexed detection however conventional ELISA is a time-consuming process that requires multiple incubations and several washing steps. On-chip cytokine detection offers a much shorter operational time as compare to ELISA[10–14]. Furthermore, On-chip systems allow analysis of minute quantities of patient-derived samples, which is often difficult to analyze using conventional cell culture and detection systems[15]. Therefore, miniaturization of in vitro analytical systems has, thus, attracted significant attention. Zhu et al. [16,17] described a technique that combines cell culture, cell activation, and cytokine detection processes on a single chip offering a miniaturized and efficient

solution for immune cell response monitoring. However, the drawback of this technique is that only one cytokine detection experiment can be carried out from a single cell culture sample. Quantification of cytokines at different time intervals is commonly required for study of cytokine immune response since snap shot measurement does not really reveal the actual immune cell response and multitime-points detections are desired for better monitoring of the cytokine profile over extended periods through. Nonetheless, current ELISA and on-chip cytokine detection technologies do not support multi-points immunoassay detection. Multiple sets of cell culture, stimulations, and treatments must be conducted to match the number of required time points which require a large number of experimental samples and laborious repetitive work.

Here, we describe an integrated microfluidic device for immune cell culture, activation, and subsequent quantification of cytokine released from a single set of cell population at multiple time points in parallel cell culture reactors using a 3d-microfluidic device. Magnetic bead-based capturing was used to trap/recover the cytokines that secreted from the activated immune cells through a porous membrane that physically isolate the cell culture from the bead suspension. This approach reduces unnecessary repetitive work and rules-out any possible inter-experiment error at different time intervals because changes observed from "before", "after" and at subsequent time intervals treatment come from the same set of the cell population.

2. Materials and methods

2.1. Chemical and reagents

Reagents and chemicals were purchased from the following sources; N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), leptin, L-glutamine, lipopolysaccharide (LPS) from Sigma-Aldrich (USA); hTNF-α, hIFN-γ, hIL-1β, and hIL-8 recombinant peptides, antibodies, and biotin-conjugated antibodies, streptavidin-HRP solution, TMB solution from Biolegend (USA); phorbol 12-myristate 13-acetate (PMA) from Millipore (Germany) carboxylated magnetic nanoparticles from Sphereotech Inc. (USA); polyethylene terephthalate (PET) membrane from (Corning® Transwell, USA); phosphate buffer saline (PBS) from Biowest (USA); DMEM culture media from Gibco (USA); and polydimethylsiloxane (PDMS) from sylguard® 184 Dow Corning (USA). Other chemicals are of analytical grade from Sigma-Aldrich (USA) or else specified.

2.2. Chip design and fabrication

The microfluidic setup consists of three components (Fig 1A): (i) cell culture chambers panel (ii) immune assay reservoir panel and (iii) magnet assembly for capturing the magnetic bead-cytokine complex (Fig 1A). The microfluidic cell culture panel was fabricated using laser cutting technique in a 1 mm thick poly(methyl methacrylate) (PMMA) plastic which was pre-laminated with double-sided tape (3M, USA) (Fig.1). Cell culture chamber panel (Fig 1Ai and Fig 1B) comprises eight independent fluidic chambers. Each chamber was made of two vertically stacked compartments that are separated by a PET porous membrane with a pore size of 0.4 um (Millipore, USA). In this structure, the porous membrane divided the fluidic chamber into two compartments: the lower compartment which hosts the cell culture and the upper one which hosts the functionalized magnetic beads for capturing the induced cytokines from the cell culture supernatant. In this configuration, cells and magnetic beads are physically separated but fluidically connected, and this allows time-resolved quantification of cytokine from the same cell cluster with minimum disturbance of cells. The fluidic chambers were sealed with a PDMS layer, which allows gas exchange as well as connects the chambers to culture media reservoirs and stimuli injection. 24 reservoir with diameter and depth of 6 and 2 mm respectively are positioned next to the cell culture chambers in which the magnetic immune assay takes place (Fig 1Aii). The magnet assembly comprises 8 NdFeB magnets embedded in a PDMS sheet are arranged with an inter-magnet spacing of 9 mm, such

that matching cell culture chamber arrangement. The magnet parts that protrude from the PDMS are inserted in another PMMA sheet. This structure allows transferring magnetic beads from cell culture chamber to immune assay reservoir by pick-and-release action and also prevents the direct contact between the magnet and the biological sample during experiments. (Fig 1Aiii).

2.3. Cell Culture

Human leukemic monocyte lymphoma cell line (U937) cell (Addexbio Biotechnologies, San Diego, USA) was used as a model of human immune responsive monocytes. Cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics. Cells not older than 30 passages were utilized for experiments. Prior to cell seeding, cell culture chambers panel was exposed to UV light for at least 30 min, washed thoroughly with 70% ethanol, rinsed with copious amount of double distilled water, and incubated with cell culture media for overnight in a cell culture incubator (37°C with humidified atmosphere and 5% CO_2). U937 cells were seeded into the lower compartment and maintained in culture media while the upper compartment was filled with cell-free media. To quantify cell viability, cells were mixed with trypan blue at a 1:1 ratio and counted for living and dead cells using Luna™ (Invitrogen).

2.4. Immune cell differentiation and activation

To induce macrophage cell differentiation, U937 monocytic cells were suspended in culture media containing 100 nM of phorbol 12-myristate 13-acetate (PMA) and incubated for at least 18 hr. Differentiation was confirmed by visual inspection in which the initially suspended monocyte cells adhered to the chamber floor. After cell differentiation, PMA culture media were removed, and macrophage cells were maintained in PMA-free culture media.

Bacterial-derived lipopolysaccharides (LPS) is an endotoxin that is known to induce a strong immune response in the human body. Cell stimulation experiments were carried out 24 hr after cell seeding or differentiation by replacing the culture media with a fresh one that contain LPS with specific concentration and incubated for at least 18 hr before carrying out cytokines detection. To further examine cytokine immune response of monocytes and macrophages leptin and glutamine were used in parallel with LPS to induce inflammatory cytokine secretion.

2.5. In situ magnetic bead-based immune assay

200 µl of carboxylated magnetic beads (0.31 µm, 0.5%w/v) were pre-cleaned with PBS for 3 times followed by incubating with mixture of 0.4 M EDC and 0.1 M NHS in PBS at room temperature for 30 min. The EDC/NHS mixture was removed from the magnetic beads, and the beads were incubated with 200 µl of 20 µg/ml desired cytokine antibody for 1 hr at room temperature. Unbound antibodies were removed from the beads, and the beads were resuspended in 1%BSA (dissolved in PBS) and stored at 4°C until use. Prior to use, antibody-conjugated magnetic beads were washed and resuspended in PBS.

Fig.2 schematically shows the magnetic-bead based cytokine capturing procedure and the subsequent detection. After cell differentiation and activation, the PDMS lid was detached from the cell culture panel and 10 µl of antibody-conjugated magnetic-beads was added to the upper compartment of the cell culture chamber, which contains the cell culture supernatant, and incubated for 1 hr in cell culture incubator. During incubation, the cell culture panel was covered with the PDMS lid to prevent media and other reagents evaporation. Then, the magnetic beads were collected using the magnet assembly (Fig 2ii) and transferred into the immune assay panel by detaching the magnet assembly from the magnetic PMMA cover sheet (Fig 2iii). The cell culture panel was then placed back in the cell culture incubator (Fig 2iv) for further other subsequent examination of cytokines. The collected magnetic beads were washed three times with PBS with 1% tween20 buffer (PBST) and incubated with a biotin-conjugated secondary

antibody and HRP solution for 30 min. The magnetic beads were then washed three times with PBST and incubated with Tetramethylbenzidine (TMB) substrate for 15 min. Finally, the reaction was stopped using 2 M H_2SO_4 and signals were detected at 450 nM using Enspire® Multimode Plat Reader (PerkinElmer, USA) (Fig 2v).

3. Results and Discussions

We first focused on maintaining viable cell culture on the chip. U937 cells were seeded into the lower compartments of cell the culture panel and were counted for cell growth and viability for over one week period. Results show that cell normally proliferated (Fig 3A) and maintained a viability rate of more than 90% (Fig 3B) during the experimental period. The detection of LPS-induced TNF-α released from monocytes (untreated U937 cell), and macrophages (differentiated U937 cell) were then tested. U937 monocyte cells were induced to differentiate to macrophages using PMA. Morphological differences of monocytes and macrophages are shown in Fig 4A and B. Undifferentiated monocytes maintain suspension state while the macrophage adhered to the bottom surface of the fluidic compartment after ~24 hr of PMA treatment. Cell adhesion was confirmed by flushing the chamber with a steady flow of culture media where cells remain attached against the media flow. Results from Fig 4C show that baseline of TNF-α was slightly higher in macrophages comparing to that from monocytes. Upon LPS stimulation, no change in TNF-α level was observed from monocytes whereas a significant increase in TNF-α level was seen from macrophages. Control experiments were conducted in parallel using 96-well cell culture inserts and ELISA technique. Results obtained from the cell culture plate show similar trend of TNF-α secretion as compared to the chip. Fig.4D shows the TNF-α secretion after treating U937 cells with different LPS doses. TNF-α secretion was found to be proportional to LPS concentration. Our data are also in line with reported data[18].

The feasibility of differential stimulation and subsequent detection of a panel of cytokines, namely, TNF-α, IFN-γ, IL-1β, and IL-8 in parallel on a single device was then examined. Results show that macrophages are more sensitive to LPS stimulation comparing to undifferentiated monocytes in which all cytokine panel released from macrophages show higher value as compared to monocytes (Fig 5A). Beside LPS, the effect of different stimuli on cytokine secretion was also examined on monocytes and macrophages. Leptin and glutamine are reported to induce pro-inflammatory cytokine secretion from immune cells[19,20] therefore in this experiment secretion of cytokine was induced in parallel using leptin, glutamine, and LPS. Multiple time-points detection of cytokine was performed from each treatment group from a single chip to monitor the time-dependent changes in cytokine level from a single set of cell population. Only IFN-γ was used in this detection experiment. The results revealed that macrophages are more sensitive to most of the applied stimuli. Higher level of IFN-γ release was observed in macrophages when treated with LPS and glutamine compared to that in monocytes (Fig 5B) while similar level of IFN-γ release was observed in monocytes and macrophages when treated with Leptin. The time-dependent variation of IFN-γ expression was monitored by measuring the cytokine level at three-time points with a time interval of 24 hours. Results show that in LPS treated group of both cell types, IFN-γ increased in every detected time points in which more notable changes were observed in macrophages. In a leptin-treated group the same trend of change in IFN-γ release was observed in both cell types. This includes an increase in IFN-γ after 48 hr and decreases after 72 hr. In glutamine treated group, monocytes showed a decrease in IFN-γ in every detected time point whereas macrophages showed an increase in IFN-γ at 48 hr and decrease at 72 hr.

4. Conclusions

The feasibility of simultaneous on-chip immune cell culture and cytokine detection was demonstrated. The double-layered microfluidic device allows parallel and multiple time-points cytokine detection from a single small set of the cell population. Magnetic beads-based assay was utilized for cytokine capture and

detection. Using U937 monocyte as a model of immune-responsive cells, the results reveal that macrophages were more sensitive to inflammatory stimuli in which higher level of pro-inflammatory cytokine secretions were detected compared to undifferentiated monocytic cells. Multiple time-points cytokine detection from a single set of cell population was demonstrated from on-chip U937 cell culture treated with an array of different stimuli. In this study, the chip was fabricated in, a rather large, millimeter scale as a proof-of-concept demonstration. In future work, micro-scale device will be fabricated with an integrated magnetic bead manipulation and efficient fluid utilization for automated and time-resolved cytokine profiling on a chip.

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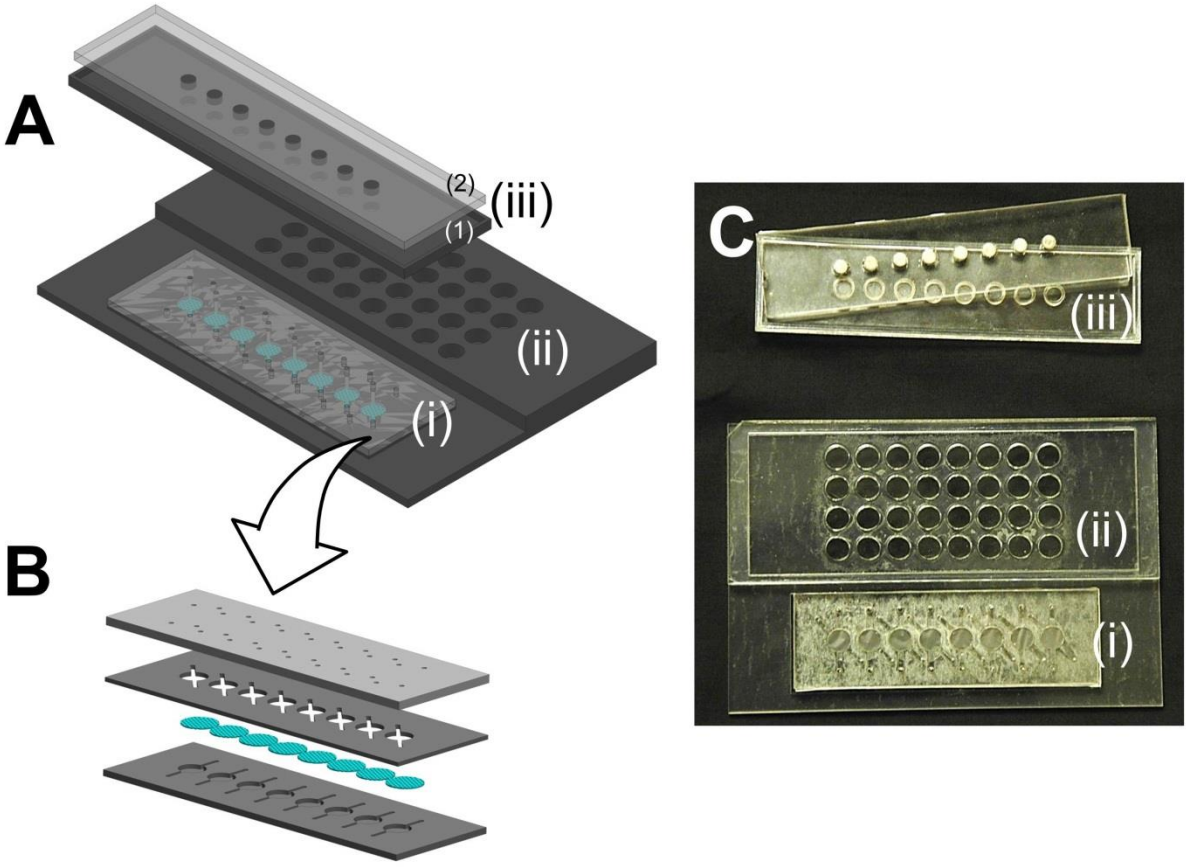

Fig 1. Microfluidic cell culture and cytokine detection chip. A) The chip comprises (i) cell culture chamber panel, (ii) immune assay reservoir panel and (iii) magnet assembly for capturing the magnetic bead-cytokine complex. The magnetic assembly comprises two sub-components; (1) PMMA cover sheet and (2) magnet embedded PDMS sheet. B) Exploded view of cell culture chamber panel which comprises eight independent microfluidic cell culture chambers. The components are (from bottom to top) lower fluidic compartment, PET membrane, upper fluidic compartment, and PDMS cover. C) Picture of the actual device. Remarks (i), (ii), and (iii) correspond to structures denoted in Fig 1A.

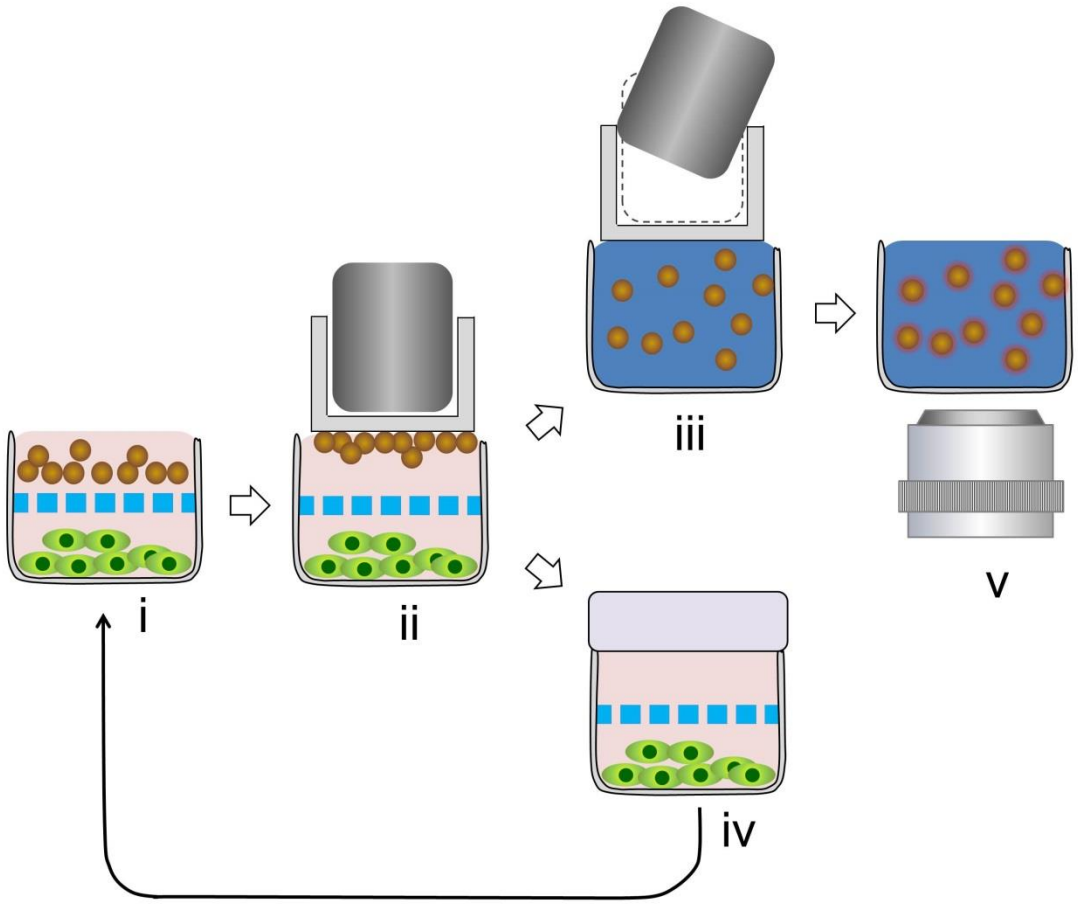

Fig 2. Diagram explaining in-situ magnetic beads-based work flow. (i) PDMS cover was removed, and antibody-conjugated magnetic beads were added to the upper fluidic compartment of the cell chamber. (ii) After 1 hr of incubation, magnetic beads were collected using magnetic assembly and (iii) released into immune assay reservoir panel by detaching magnet embedded PDMS sheet from the PMMA cover sheet. (iv) The cell culture panel was sealed back to its PDMS cover for continuation of cell culture. (v) The collected magnetic beads were then labeled with biotin-conjugated antibody and HRP for signal detection. Arrow denotes process repetition for multiple time-points cytokine detection from the same set of cell population.

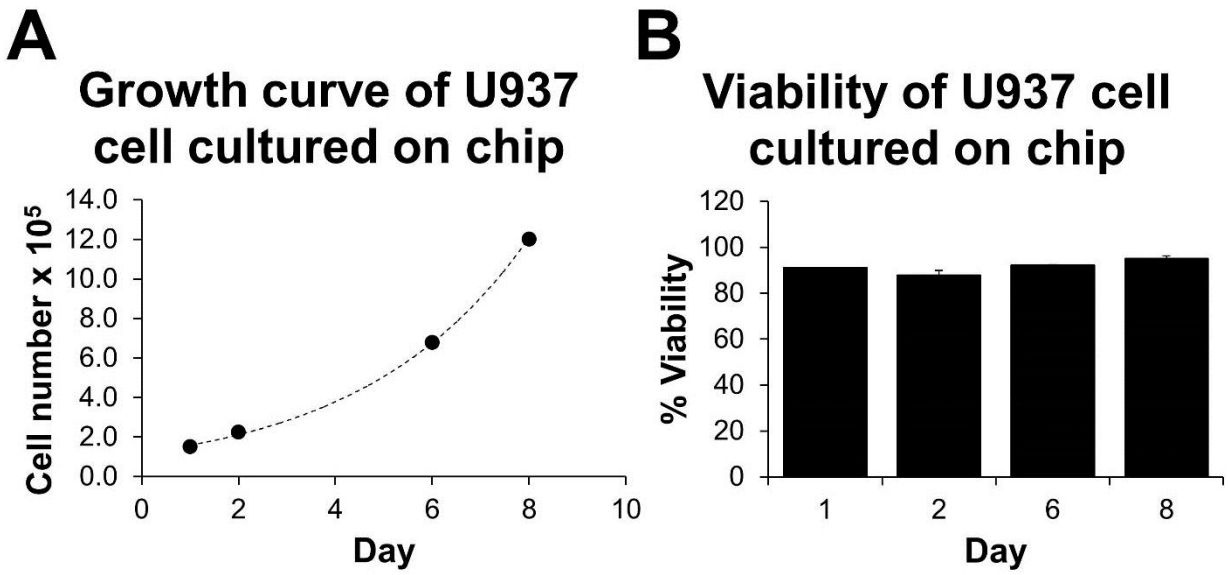

Fig 3. On-chip cell growth and viability. A) Cell growth curve and B) viability of undifferentiated U937 cell cultured on chip obtained from 8 days culture period.

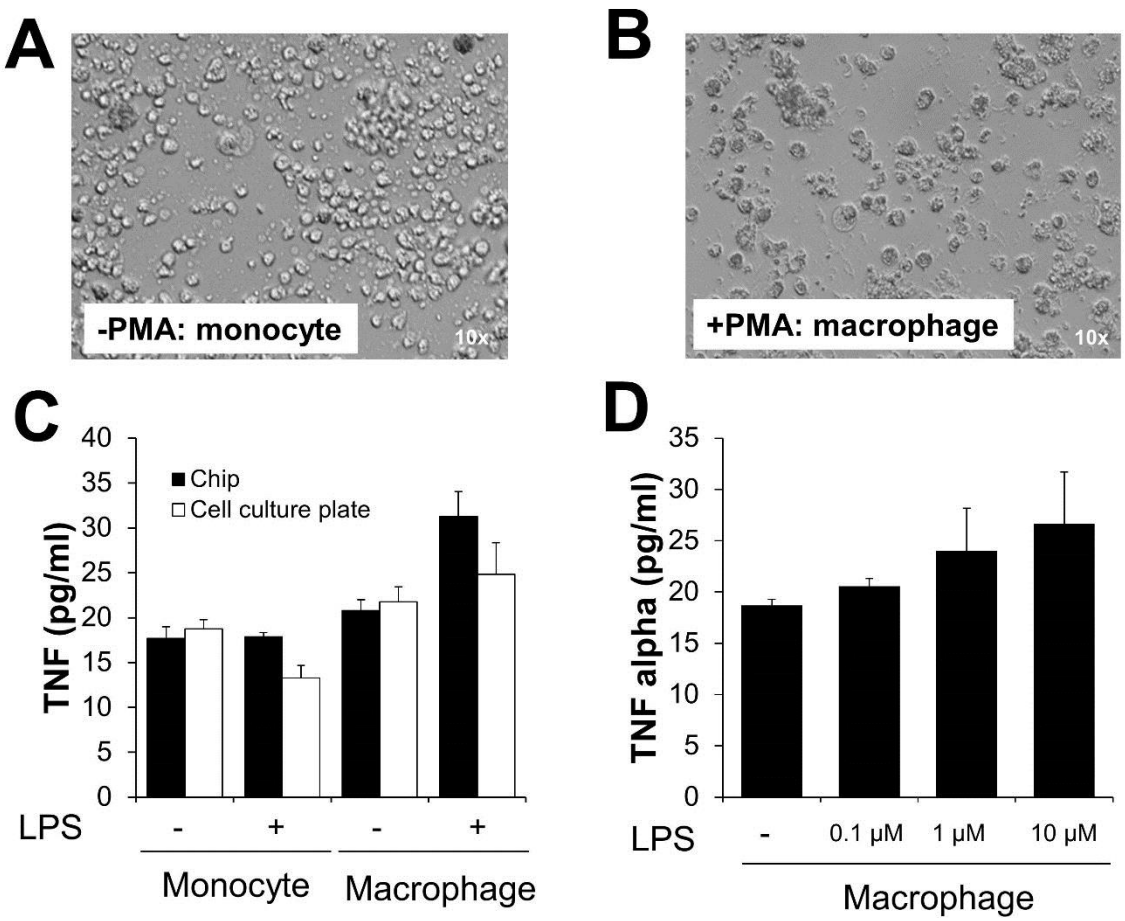

Fig 4. On-chip cytokine detection. Pictures of A) unstimulated U937 monocytes and B) PMA stimulated macrophages cultured on chip. C) TNF-α released from monocytes and macrophages treated with LPS detected from microfluidic immune cell culture and cytokine detection chip (filled bar) and cell culture plate and ELISA technique (open bar). D) On-chip detection of TNF-α released from macrophages treated with different LPS doses.

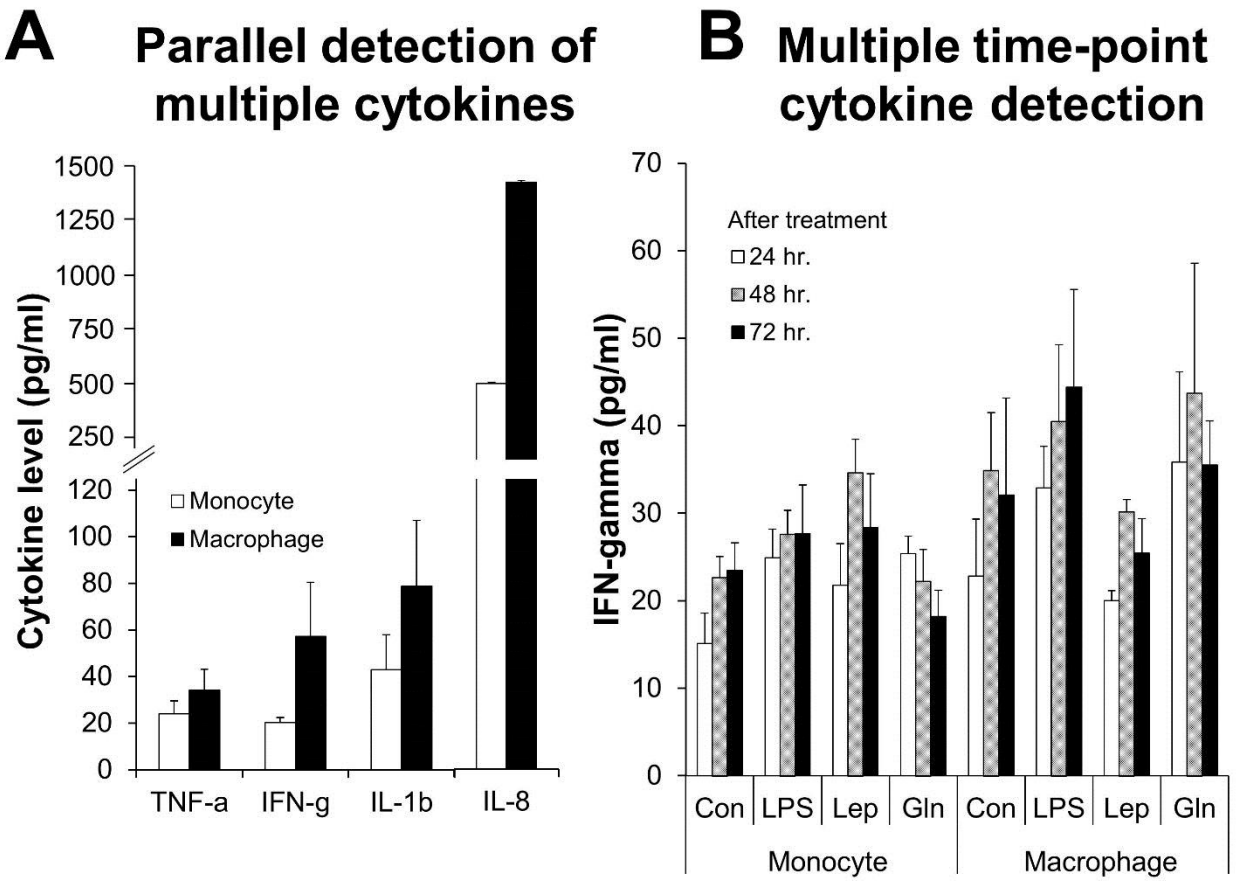

Fig 5. A) On-chip parallel detection of a panel of cytokines including TNF-α, IFN-γ, IL-1β, and IL-8 released from undifferentiated U937 monocytes and differentiated macrophages. B) Multiple time-points detection of IFN-γ released from monocytes and macrophages treated with different stimuli including no-treatment (Con), 1 μM LPS (LPS), 10 nM leptin (Lep), and 2 mM glutamine (Gln).