



# Moderate Dose of Lipopolysaccharide Induces Tumor Necrosis Factor-alpha and Interleukin-6 Production by Human Monocyte-derived Macrophages

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## Abstract

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**BACKGROUND:** Macrophages have been widely used for in vitro studies. Despite different types and doses of stimulatory agents that have been tested, there is no consensus for the method.

**AIM:** This study was aimed to determine a sufficient dose of lipopolysaccharide (LPS) to stimulate inflammatory response in macrophages.

**METHODS:** Whole blood was collected from four donors after written informed consent. The monocytes were isolated from peripheral blood mononuclear cells and stimulated with macrophage colony-stimulating factor, LPS, and Interferon-gamma for 6 days until differentiated into macrophages. The production of Tumor necrosis factor-alpha (TNF- $\alpha$ ) and Interleukin-6 (IL-6) were quantified after 24-h further stimulation with 100 ng/mL and 2  $\mu$ g/mL of LPS.

**RESULTS:** Both doses increased TNF- $\alpha$  production compare to their controls, but not statistically different ( $p > 0.05$ ). There were also no differences in IL-6 production between treatments,  $56.55 \pm 32.30$  pg/mL and  $70.96 \pm 65.08$  pg/mL, respectively.

**CONCLUSION:** A dose of 100 ng/mL of LPS was sufficient to stimulate inflammatory response in human monocyte-derived macrophages. A 24-h duration of macrophage stimulation was sufficient to observed the production TNF- $\alpha$ .

## Introduction

Macrophages as one of the first defense components in the immune system are found in tissues as diverse cell types [1]. Macrophage activities have been evolved by the discovery of different subtypes of macrophages in adipose tissue of obese individuals, cancerous lesions, synovial fluid of arthritis patients, or atherosclerotic plaque [2]. Thus, considering the role of inflammation in the pathophysiology of non-communicable diseases, researches on macrophages have been grown considerably.

Macrophages originated from humans and animals or macrophage cell lines such as Raw 264.7, J774.2, THP1, and U937 have been widely used [3]. Animal macrophages such as mice were also commonly used as they are more easily to collect and less expensive. However, their activities and responses may not exactly as same as what would be expressed in humans.

Another important factor to be considered is the choice of stimulatory agent for macrophage differentiation and activation. It has been established that at the presence of lipopolysaccharide (LPS), Tumor necrosis

factor-alpha (TNF- $\alpha$ ), or Interferon (IFN- $\gamma$ ) undifferentiated macrophages will be polarized into M1 macrophages. Whereas the M2 macrophages were formed when stimulated with Interleukin (IL)-4, IL-10, and IL-13 [4].

Investigations on macrophage inflammatory response were targeting M1 macrophages, to observe phagocytic function, cytokines production, migration, and nitric oxide production [5]. Various agents for stimulation have also been used in order to observe macrophage's response towards various pathogen-associated molecular patterns (PAMPs), and one of the most extensively used is LPS [3]. LPS activates inflammatory response by binding to Toll-like receptor 4 (TLR4) on the cell membrane. The TLR4/myeloid differentiation factor 2 (TLR4/MD2) receptor complex then activates downstream cascade results in the production of inflammatory cytokines [3].

*In vitro* studies have been used LPS from various bacterial species such as *Escherichia coli*, *Salmonella typhosa*, *Pseudomonas aeruginosa*, or *Klebsiella pneumoniae* [6]. However, there were no recommendations about how much and how long the treatment is should be given in order to observe the inflammatory response. Previous researches have used LPS from the lowest concentration of 10 ng/mL, moderate at 100 ng/mL, up to as high as 2  $\mu$ g/mL to

observe cytokines production such as TNF- $\alpha$ , IL, colony-stimulating factors (CSFs), and tumor growth factors [7], [8]. Hence, there was tendency to use high doses that are not likely to occur in humans.

In this study, we aimed to investigate and compare the production of pro-inflammatory cytokines, i.e., TNF- $\alpha$  and IL-6, by human monocytes-derived macrophages induced with moderate and high doses of LPS.

## Methods

### Subject

Four healthy volunteers, men, and women, aged 19–21 years old, were participated in the study. Ethical approval for blood sampling was granted by the Health Research Ethical Committee, in accordance with the declaration of Helsinki. Blood collection was performed after informed written consent and conducted on different day for each participant.

### Isolation of peripheral blood mononuclear cells (PBMCs)

Fifty milliliters of venous blood were collected in EDTA-contained vacutainer tubes. Isolation of PBMCs was performed by density gradient centrifugation on Lymphoprep™. Briefly, 50 mL of blood was mixed (1:1) with Phosphate buffered-saline (PBS) (Gibco, Waltham, MA, USA) and 8 mL of the mixture was layered carefully on 4 mL of Lymphoprep™ (Axis-Shield, Dundee, UK) in 15 mL sterile tubes (Falcon high-clarity polypropylene conical centrifuge tube, Corning, NY, USA). After centrifugation at 800 g for 20 min without break at room temperature (RT), the buffy coats were collected using sterile Pasteur pipette and pooled together in sterile tube. The buffy coats then re-suspended and washed thrice with PBS followed by centrifugation at 250 g for 10 min at RT. The supernatant was aspirated and the cell pellet was re-suspended in 2 mL of buffer (PBS, 2% FBS).

### Isolation of monocytes

Monocytes were isolated from PBMCs using EasySep monocyte isolation kit (Stemcell Technologies, Vancouver, Canada) inside an immunomagnetic cell separation tool. Briefly, 100  $\mu$ L of isolation cocktail and 100  $\mu$ L of platelet removal were added and mixed gently with 2 mL of PBMCs suspension in 5 mL polystyrene tube (Corning, NY, USA) prior to incubation for 5 min at RT. Then, 100  $\mu$ L of magnetic particle solution was added and mixed gently, and incubated for the next 5 min at RT. Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Waltham, MA, USA) was added

subsequently to make the total volume of 2.5 mL before further incubation inside the EasySep Magnet (Stemcell Technologies, USA) for 2.5 min at RT. The suspension which contained monocytes was poured off into 15 mL sterile tube and centrifuged at 400 g for 5 min at RT. The supernatant was discarded and the cell pellet was re-suspended in complete media (CM); i.e., RPMI supplemented with 10% Fetal bovine serum (FBS) (Sigma, St Louis, MO, USA), 1% penicillin-streptomycin, and 1% L-glutamine (Gibco, Waltham, MA, USA). The cells were counted and diluted with CM to make up a concentration of  $5 \times 10^4$  cells/mL.

### Culture and differentiation of macrophage

At day 0, monocytes suspended in CM were seeded on 13 mm diameter. Thermanox plastic coverslip (Nunc, Rochester, NY, USA) inside 24-well plate (Iwaki tissue culture-treated polystyrene microplate, Asahi Glass Co., Japan). A 25  $\mu$ L of 50 ng/mL M-CSF (ABclonal Technology, Woburn, MA, USA) was added into each well and the cultures were incubated at 37°C with 5% CO<sub>2</sub> in a humidified incubator (Galaxy R170, New Brunswick). On day 4, fresh media supplemented with M-CSF (50 ng/mL), 10 ng/mL of LPS from *E. coli* (O26:B6) (Sigma, St Louis, MO, USA), and 50 ng/mL IFN- $\gamma$  (Stemcell Technologies, Vancouver, Canada), was added to each well for M1 macrophages activation. On day 6, all media were removed and replaced with serum-free CM (SF-CM).

### Stimulation of macrophages with LPS

Macrophages were stimulated with two different concentrations of LPS (100 ng/mL and 2  $\mu$ g/mL) and incubated at 37°C with 5% CO<sub>2</sub>. Macrophages cultured in SF-CM only was used as control. After 24 h of incubation, all media were aspirated and centrifuged 1000 g for 5 min at 4°C. The supernatants were collected and stored at -70°C until quantification of cytokines. The cells on coverslips were washed twice with cold PBS prior to staining with Giemsa.

### Cell staining

The coverslips inside wells were left to dry in RT prior to fixation with 98% methanol for 1 min. The methanol was discarded and the coverslips were soaked in Giemsa (diluted with distilled water, 1:1) for 5 min. After the last wash with distilled water, the coverslips were taken out of the wells and left to dry before mounted onto slides.

### Cells evaluation

Observation was performed using Zeiss Primo Star microscope (Zeiss, Germany). All visible

macrophages with good morphology were counted using 10 × magnification in the adjacent area but not overlapped, from left to right side and from upper to lower side. Each field of view was 0.2 mm wide.

### Analysis of cytokines production

The concentration of TNF- $\alpha$  and IL-6 were quantified using Human Pico Kine ELISA kits (My BioSource Inc., San Diego, CA, USA) according to manufacturer's protocols. Assays were performed in duplicate. The intra assay coefficient of variation was <11%.

### Statistic

Four independent experiments were performed, each was run in duplicate. Results are expressed as mean  $\pm$  SD, otherwise stated. Statistical analyses were performed using SPSS version 21 (IBM Corporation, Armonk, NY, USA). Comparison between groups was carried out with one-way ANOVA and Tukey HSD for post-hoc analyses. A value of  $p < 0.05$  is considered significant.

## Results

Differentiation of monocytes into macrophages was observed after 7 days of culture, despite visible damage in a few cells (Figure 1).

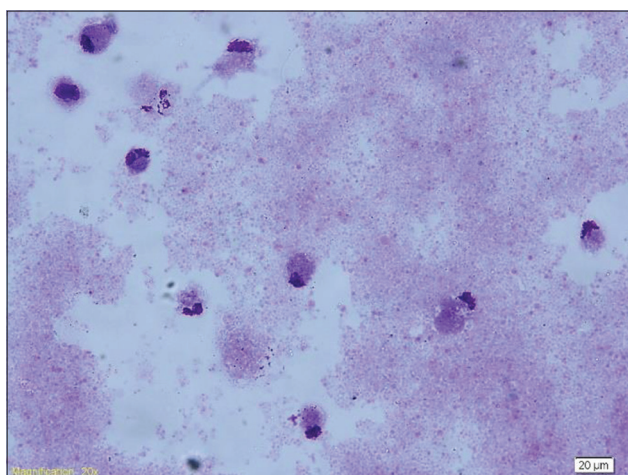


Figure 1. Monocyte-derived macrophages stained with Giemsa after 6-days activation with 50 ng/ml of M-colony stimulating factors including 2-days treatment of 10 ng/ml of lipopolysaccharide (LPS) + 50 ng/ml of IFN- $\gamma$ , followed by 24-h stimulation with 100 ng/ml of LPS. Images were captured using Olympus BX51 microscope and Olympus DP20 digital camera with  $\times 40$  magnification, scale marker 20  $\mu$ m.

The TNF- $\alpha$  levels in control wells were ranged between 21 and 71.75 pg/mL. The productions of TNF- $\alpha$  after LPS stimulation were tend to be higher compare to control, but not statistically significant (Figure 2).

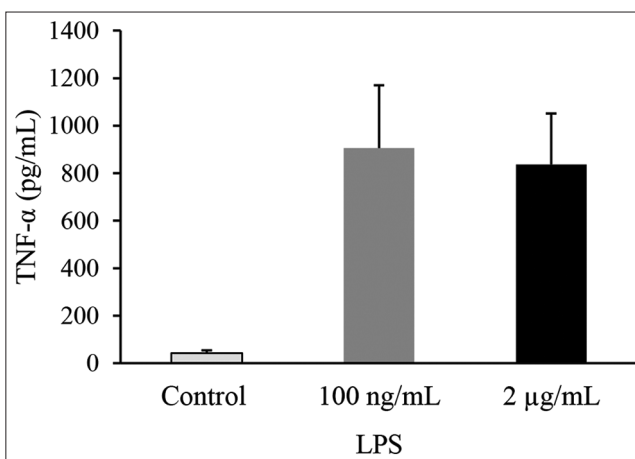


Figure 2. Concentrations of tumor necrosis factor alpha measured in supernatant of macrophage cell culture after 24-h incubation without lipopolysaccharide (LPS) (as control) and with 100 ng/ml and 2  $\mu$ g/ml of LPS. Each column represents the mean concentration with standard error bar ( $n = 4$ ).

There was a variability of TNF- $\alpha$  production between experiments. In two of the four experiments, LPS treatment resulted in up to 50-fold higher of TNF- $\alpha$  level compare to their controls. In which 100 ng/mL of LPS resulted in 1543.95  $\pm$  501.61 pg/mL and 2  $\mu$ g/mL of LPS resulted in 1295.65  $\pm$  204.48 pg/mL compare to 35.65  $\pm$  20.72 pg/mL in control ( $p = 0.03$  and  $p = 0.05$ , respectively). Whereas in two other experiments only resulted in 2–5-fold higher of TNF- $\alpha$  levels compare to their controls. In which 100 ng/mL of LPS resulted in 267.14  $\pm$  271.11 pg/mL and 2  $\mu$ g/mL of LPS resulted in 377.77  $\pm$  446.30 pg/mL compare to 50.1  $\pm$  30.47 pg/mL in control ( $p = 0.77$  and  $p = 0.58$ , respectively).

There were no differences in IL-6 levels between treatments after 24-h of LPS stimulation (Figure 3). In which 100 ng/mL and 2  $\mu$ g/mL of LPS stimulation resulted in 56.55  $\pm$  32.30 pg/mL and 70.96  $\pm$  65.08 pg/mL, respectively, compare to 55.16  $\pm$  23.19 pg/mL in control. Different responses were also observed in IL-6 production between experiments. The two which have higher levels of TNF- $\alpha$  also have higher levels of IL-6, 78.53  $\pm$  22.74 pg/mL compare to 27.25  $\pm$  10.02 pg/mL in the two others, after 100 ng/mL of LPS stimulation.

## Discussion

Observation on lean and obese humans found a concentration of 3–6 EU/mL of LPS in the blood, which is equal to approximately 0.5–1 ng/mL of LPS [9]. Whereas in septic condition, the level may raise up to 3-fold [10]. However, *in vitro* studies often used supraphysiological concentration of LPS to stimulate inflammatory responses in macrophage. In this study, we compared two supraphysiological doses, i.e., 100 ng/mL and 2  $\mu$ g/mL of LPS. Those doses were

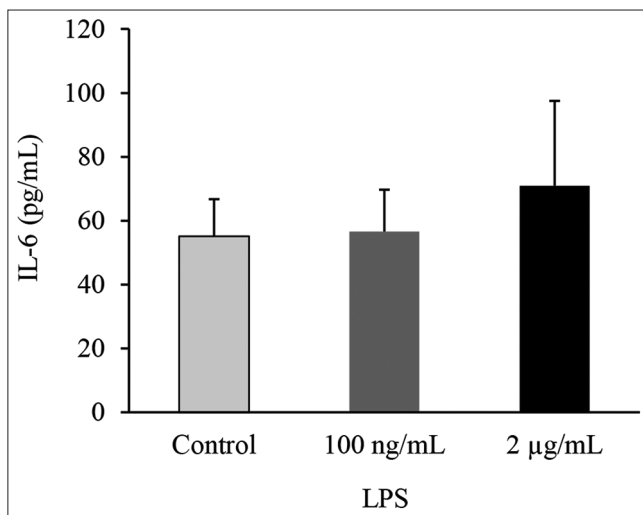


Figure 3. Concentrations of interleukin-6 measured in supernatant of macrophages cell culture after 24-h incubation without lipopolysaccharide (LPS) (as control) and with 100 ng/ml and 2 µg/ml of LPS. Each column represents the mean concentration with standard error bar (n = 4).

chosen according to previous studies on macrophages [11]. We hypothesized that the lower dose is sufficient to eliciting inflammatory responses from human monocyte-derived macrophages.

Statistically, there were no significant differences in TNF- $\alpha$  productions between both doses, treatments, and control group. These results indeed in contrary to what has been shown previously [7], [8]. According to our data, this probably due to differences in response between subjects. There were only small increases in two experiments compare to significant increases in the two others. Whereas, the concentrations of TNF- $\alpha$  in control wells of all experiments were almost similar. This suggests that macrophages from donors were response in similar manner to produced TNF- $\alpha$  when they were not stimulated with LPS. Therefore, although our results failed to show significant differences after stimulations, this may be due to different responses elicited in each experiment. Indeed, a previous study on macrophages has suggested that variability between individuals is common when working with human primary cells [5].

Between the two doses, our results showed that a dose of 100 ng/mL of LPS was sufficient to stimulate higher TNF- $\alpha$  production from human monocyte-derived macrophages for *in vitro* experiment. This finding corresponds to previous report that showed no differences in TNF- $\alpha$  production by RAW264 macrophage cell line stimulated with either 100 or 1000 ng/mL of LPS [12]. This suggests that it is not necessary to use much higher doses which are also not applicable physiologically.

However, a lower dose than 100 ng/mL should also be considered in future experiments, in order to mimic physiological conditions in the human body. Treatment with 10 ng/mL of LPS was

resulted in cytokines production by human PBMCs and monocytes [7]. Yet, caution should also be practiced when using low doses, as recent findings have revealed lowering effect of LPS on cytokine production when administered in very low dose; in which 50 to 100 pg/mL of LPS suppressed IL and TNF- $\alpha$  productions by human and mouse macrophages [13].

Our data showed that LPS treatment was not significantly increased IL-6 production compare to control. Between the two doses, there was a slight increase in 2 µg/mL group. Whereas, IL-6 levels at the baseline of treatment (i.e., after 6 days of monocytes activation and differentiation) were undetected.

Those results showed that there was a significant IL-6 production, although did not influenced by LPS stimulation. Our result is different with previous studies that generally reported increase production of IL-6 from various types of LPS-induced macrophages [3]. Together with other cytokines and chemokines, IL-6 is a pro-inflammatory cytokine which produced by LPS-induced primary cells and cell lines. Both types of macrophage can produce IL-6, in which the ratio was 7-fold higher in M1 compared to M2. However, there were also different properties of cytokine secretion according to cell types, such as monocytes which produce 6-fold higher of IL-6 than macrophages [14].

In this study, we assumed that we managed to differentiate monocytes into M1 macrophages by using CSF-1 along with specific stimulation using LPS and IFN- $\gamma$  [15]. However, we did not perform the immunophenotyped assay to ensure the transformation. This was due to the lack of equipment we had in our laboratory. This limitation may confound our results and may related to the low increase of IL-6 production. Other than that, in this study, we only measured two cytokines that are commonly assayed. It would be more evident if we could measure another pro-inflammatory cytokine such as IL-1 $\beta$ , IL-8 or IL-Ra [3].

In addition, to determine the dose of LPS stimulation, we were also evaluated the duration of treatment. In this study, we incubated the macrophages with LPS for 24 h before collecting the culture media and cells for assessments. Durations of 24 and 48 h have been performed in many experiments elsewhere [11]. Nevertheless, a phenomenon of peak cytokine production apparently has been observed in previous research. Schildberger *et al.* found that a peak of TNF- $\alpha$  production was observed at 4 h and decline afterward, which was different from the IL-6 level that still on increment until 24 h [7]. These findings were supported by another report that observed significant TNF- $\alpha$  production only after 2 h and still on increment up to 12 h but decrease afterward [8]. The long period of

treatment may have contributed for the low levels of TNF- $\alpha$  in some of our samples. It should be worth to consider taking sample every hour to optimizing the duration of observation.

## Conclusion

Our results suggest that 100 ng/mL of LPS was sufficient to induce inflammatory response in human monocyte-derived macrophages. A 24-h duration of stimulation was sufficient to observed the production TNF- $\alpha$ . But a shorter time and consecutive time point for taking samples are also worth to consider to assess cytokine production.

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