

Attempt to Construct an *In Vitro* Model of Enhancement of Macrophage Phagocytosis Via Continuous Administration of LPS

KAZUSHI YAMAMOTO¹, HARUKA MIZOBUCHI¹, MASASHI YAMASHITA¹, HIROYUKI INAGAWA^{1,2,3}, CHIE KOHCHI² and GEN-ICHIRO SOMA^{1,2,3}

¹Department of Control of Innate Immunity, Technology Research Association, Kagawa, Japan;

²Macrophhi Inc., Kagawa, Japan;

³Research Institute for Healthy Living, Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan

Abstract. *Background:* Continuous oral administration of lipopolysaccharide (LPS) enhances the phagocytic ability of macrophages, which is useful for preventing various diseases. Here, we attempted to create an *in vitro* model of continuous administration of LPS. *Materials and Methods:* RAW264.7 cells were stimulated with LPS three times every 24 h (repeated stimulation), and phagocytic ability and inflammatory cytokine [interleukin-6 (IL6) and tumor necrosis factor- α (TNF α)] production were measured. *Results:* The phagocytic ability was increased by a single stimulation with LPS and was maintained by repeated stimulation. IL6 production increased with a single stimulation with LPS; however, IL6 production by repeated stimulation with LPS was comparable to that of non-stimulation with LPS. On the other hand, the amount of TNF α was significantly increased by single and repeated stimulation with LPS. *Conclusion:* Repeated stimulation with LPS in RAW264.7 cells triggered a phenotype that was similar to that of macrophages after continuous oral administration of LPS. This suggests that this study model may reproduce the enhancement of macrophage phagocytosis, an effect afforded by continuous oral administration of LPS.

Lipopolysaccharide (LPS) is a glycolipid that is present in the outer membrane of Gram-negative bacteria and has long been considered harmful to health as a potent inducer of inflammation. Certainly, intravenous injection of LPS causes severe fever, diarrhea, and shock; therefore, LPS is recognized

as a dangerous substance in medicine. Conversely, we found that many edible plants, including rice, wheat, and buckwheat, had high levels of LPS (1, 2). Intravenous injection of 1 g of extracts of these edible plants into humans is equal to an LPS injection that far exceeds the lethal dose. However, despite regular oral intake of these edible plants by humans, fever, diarrhea, and shock are usually absent. In addition, rats exhibited no toxicity after oral intake of LPS at 2 g/kg of body weight (BW) (2, 3). These results indicate that completely distinct mechanisms exist regarding the control of LPS between oral intake and intravenous administration. Moreover, we showed that continuous oral administration of LPS was effective in preventing and ameliorating many diseases, such as cancer, atopic dermatitis, diabetes, arteriosclerosis, and Alzheimer's disease (4-8).

In the context of the health-maintenance effect of continuous oral administration of LPS, we are focusing on macrophages, which are major target cells of LPS. Macrophages play an important role in the phagocytosis of foreign substances, wound healing, and metabolic regulation, and their functions are activated by LPS. Recently, we showed that continuous oral administration of LPS increased the foreign-substance phagocytosis of peritoneal macrophages and enhanced the foreign-substance phagocytosis of microglia, which are macrophages of the brain, to improve Alzheimer's disease (8, 9). In addition, it was clarified that the production of inflammatory cytokines [interleukin-6 (IL6), tumor necrosis factor- α (TNF α) and interleukin-12 (IL12)] by peritoneal macrophages was not increased by continuous oral administration of LPS (9). Currently, we are conducting more-detailed studies about the enhancement of phagocytosis by macrophage resulting from continuous oral administration of LPS. However, *in vivo* studies require special techniques, such as the isolation of macrophages from living organisms, thus being complex test systems. *In vitro* models have significant advantages in the context of mechanistic analyses over *in vivo* models because they are simple test systems and can be used

Correspondence to: Dr. Kazushi Yamamoto, Control of Innate Immunity, Technology Research Association, 2217-16 Hayashi-cho, Takamatsu-shi, Kagawa 761-0301, Japan. Tel: +81 878139201, Fax: +81 878139203, e-mail: yamamoto@shizenmeneki.org

Key Words: *In vitro*, LPS, macrophage, oral administration, phagocytosis.

to evaluate the direct effect of substances on cells. In this study, we attempted to reproduce the enhancement of macrophage phagocytosis, an effect triggered by continuous oral administration of LPS, as a simple test system *in vitro*.

Materials and Methods

Cell culture and LPS treatment. The murine macrophage-like cell line RAW264.7 was obtained from the American Type Culture Collection (Manassas, VA, USA). The experimental design is depicted in Figure 1. RAW264.7 cells (2×10^5 cells/ml) were seeded in 12-well tissue culture plates ($n=3$) and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich) at 37°C in 5% CO₂. RAW264.7 cells were treated with or without purified LPS derived from *Pantoea agglomerans* (Macrophix Inc., Kagawa, Japan). The concentration of LPS used was set to 100 pg/ml and 1 ng/ml as a low dose and 100 ng/ml as a high-dose control, as it has been reported that phagocytosis was induced by 100 pg/ml and 1 ng/ml of LPS (10), while LPS was generally used at 100 ng/ml to activate macrophages *in vitro* (11, 12). For repetitive treatment with LPS, cells received fresh medium containing LPS three times a day. For single treatment with LPS, cells received fresh medium without LPS for the first 48 h, and then received fresh medium containing LPS once. Samples were collected at the time points indicated in Figure 1 and analyzed for phagocytic activity/cytokines.

Phagocytosis assay. Phagocytic activity was measured by flow cytometry as described previously, with minor modifications (10). In brief, 24 h after the last treatment of RAW264.7 cells with LPS, cells were incubated with fluorescent latex beads (Fluoresbrite® YG Microspheres 2.0 µm; Polysciences, Warrington, PA, USA) at a cell:bead ratio of 1:5 for 3 h. Cells were washed, to remove non-internalized particles, and then detached by 0.25% trypsin treatment (Life Technologies, Carlsbad, CA, USA). The phagocytosis ratio and phagocytosis intensity of beads in the cells were measured using a Beckman Coulter Gallios flow cytometer and Kaluza software (Beckman Coulter, Indianapolis, IN, USA). The phagocytosis ratio reflects the proportion of cells that engulfed beads and the phagocytosis intensity reflects the number of engulfed beads.

Determination of cytokines (ELISA). The culture supernatants were collected 24 h after the last treatment of RAW264.7 cells with LPS. The levels of IL6 and TNFα in culture supernatants were measured using a commercial sandwich ELISA kit (BioLegend, San Diego, CA, USA), according to the manufacturers' instructions.

Statistical analysis. Statistical analysis was performed using Ekuseru-Toukei 2012 (SSRI, Tokyo, Japan). The results are presented as the mean±standard deviation. The differences between the groups were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. Significance was set at $p < 0.05$.

Results

Phagocytic evaluation in RAW264.7 cells after repeated stimulation with LPS. The effect of repeated stimulation with LPS on the phagocytic ability of RAW264.7 cells was examined. There was a significant increase in the phagocytic

ratio after a single stimulation with 100 pg/ml of LPS compared with no stimulation. This increase was maintained by repeated stimulation with LPS. In contrast, there was no significant difference in phagocytic intensity after single stimulation with 100 pg/ml of LPS compared with no stimulation, whereas a significant increase was observed after repeated stimulation with LPS (Figure 2A). Moreover, there was a significant increase in phagocytic ratio after single stimulation with 1 ng/ml of LPS compared with non-stimulation with LPS. This increase was maintained by repeated stimulation with LPS. There was also a significant increase in phagocytic intensity after single stimulation with 1 ng/ml of LPS compared with no stimulation. This increase was maintained by repeated stimulation with LPS (Figure 2B). In addition, there was a significant increase in phagocytic ratio after single stimulation with 100 ng/ml of LPS compared with non-stimulation with LPS. This increase was maintained by repeated stimulation with LPS. There was also a significant increase in phagocytic intensity after single stimulation with 100 ng/ml of LPS compared with no stimulation and this increase was maintained by repeated stimulation with LPS (Figure 2C). Finally, examination of phagocytic ratio and phagocytic intensity as the ratio of their values after LPS stimulation to those before LPS stimulation revealed that both increased dependent on the LPS concentration (Figure 2D). This suggests that phagocytic ability was increased by single stimulation with LPS and that this effect was maintained by repeated stimulation with LPS.

Evaluation of inflammatory cytokine production in RAW264.7 cells after repeated stimulation with LPS. The effect of repeated stimulation with LPS on inflammatory cytokine production in RAW264.7 cells was examined. IL6 was not detected in culture supernatant after stimulation with 100 pg/ml of LPS. There was, however, a significant increase in TNFα after single stimulation with 100 pg/ml of LPS compared with no stimulation. Moreover, TNFα was significantly lower after repeated stimulation with LPS compared with single stimulation with LPS. In contrast, there was a significant increase in TNFα after repeated stimulation with LPS compared with no stimulation (Figure 3A). Although IL6 was not detected after no stimulation and repeated stimulation with 1 ng/ml of LPS, there was a great increase in the level of this molecule after single stimulation with LPS. There was also a significant increase in TNFα after single stimulation with 1 ng/ml of LPS compared with non-stimulation with LPS. In contrast, there was a significant decrease in TNFα after repeated stimulation with LPS compared with single stimulation with LPS, and a significant increase in TNFα after repeated stimulation with LPS compared with no stimulation (Figure 3B). The pattern was the same for RAW264.7 cells treated with 100 ng/ml of LPS (Figure 3C). The examination of the increment in these

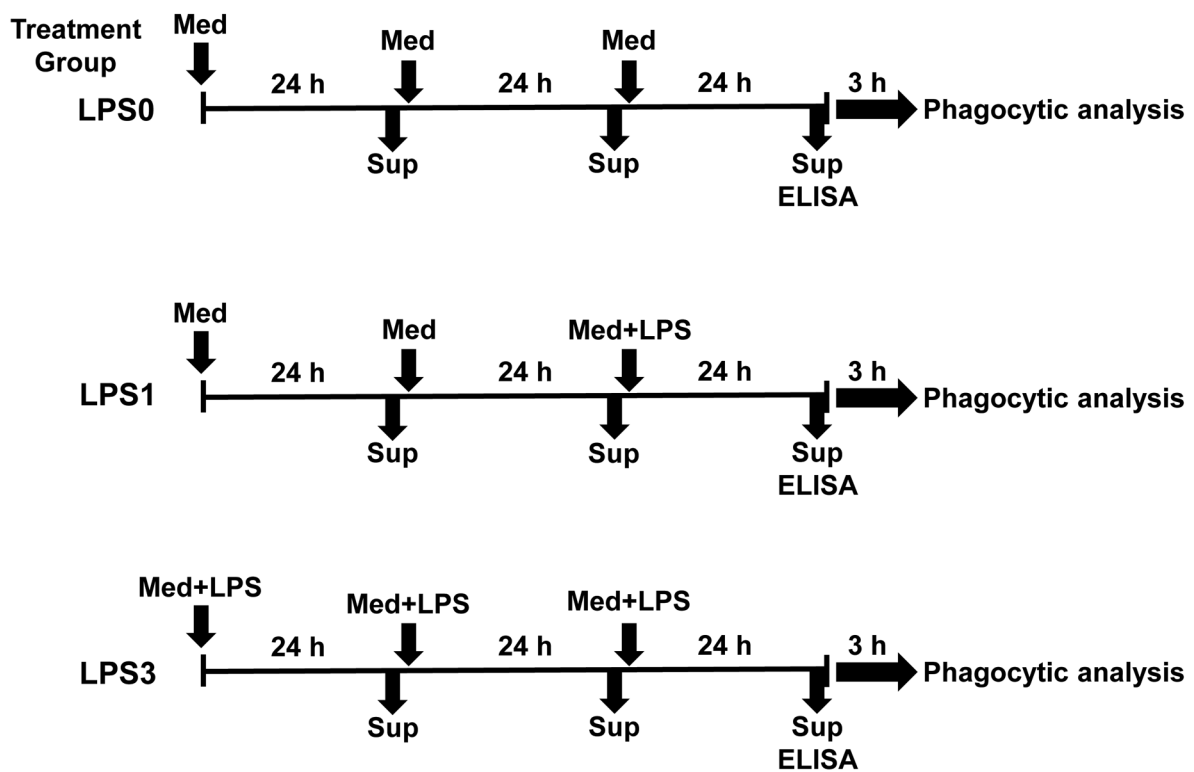


Figure 1. Experimental design. ELISA: Enzyme-linked immunosorbent assay; LPS: lipopolysaccharide, Med: Medium, Sup: culture supernatant.

cytokines after LPS stimulation revealed that IL6 production was greatly increased after single stimulation with high-dose LPS, but was not detected after repeated stimulation, regardless of LPS concentration. Finally, TNF α production dose-dependently increased after stimulation with up to 1 ng/ml of LPS, but remained practically unchanged after stimulation with >1 ng/ml of LPS (Figure 3D). This suggests that a single stimulation with LPS increases the production of inflammatory cytokines, whereas repeated stimulation with LPS yields almost no IL6, similar to that observed for untreated RAW264.7 cells. Conversely, TNF α production was increased after repeated stimulation with LPS compared with non-stimulation.

Discussion

In our previous study, oral administration of 0.01 to 1 mg/kg BW/day of LPS to C3H/HeN mice for 1 week led to an increase in the phagocytic ability of peritoneal macrophages (9). As phagocytosis is a fundamental function of macrophages and is involved in the maintenance of homeostasis, it can be an important factor in the health-maintenance effect of the oral administration of LPS shown in previous studies (4-8). However, the underlying

mechanism is unknown; thus, further analyses are required. Here, the *in vitro* study used a simpler system compared with *in vivo* ones and is able to evaluate the direct effect of substances on cells; thus, it is applicable to the analysis of the mechanisms underlying the physiological effects of various substances (13-15). Therefore, it was thought that the research into the effect of the continuous administration of LPS on macrophage phagocytic ability would be further advanced if this effect could be reproduced *in vitro*. In this study, we attempted to create an *in vitro* model of the enhancement effect on macrophage phagocytosis of continuous administration of LPS, as reported previously using our *in vivo* oral study (9).

Here we focused on the cell line, LPS concentration, and method for LPS stimulations. RAW264.7 cells were selected as an *in vivo* model of macrophages. RAW264.7 cells are monocyte/macrophage-like cells originating from Abelson leukemia virus-transformed cell line derived from BALB/c mice. They exhibit phagocytosis and common macrophage phenotypes, such as increased phagocytosis and nitric oxide and inflammatory cytokine production due to a single stimulation of LPS; therefore, these cells have been widely used as a model of macrophages for over 40 years (16). RAW264.7 cells are an optimal *in vitro* model of macrophages.

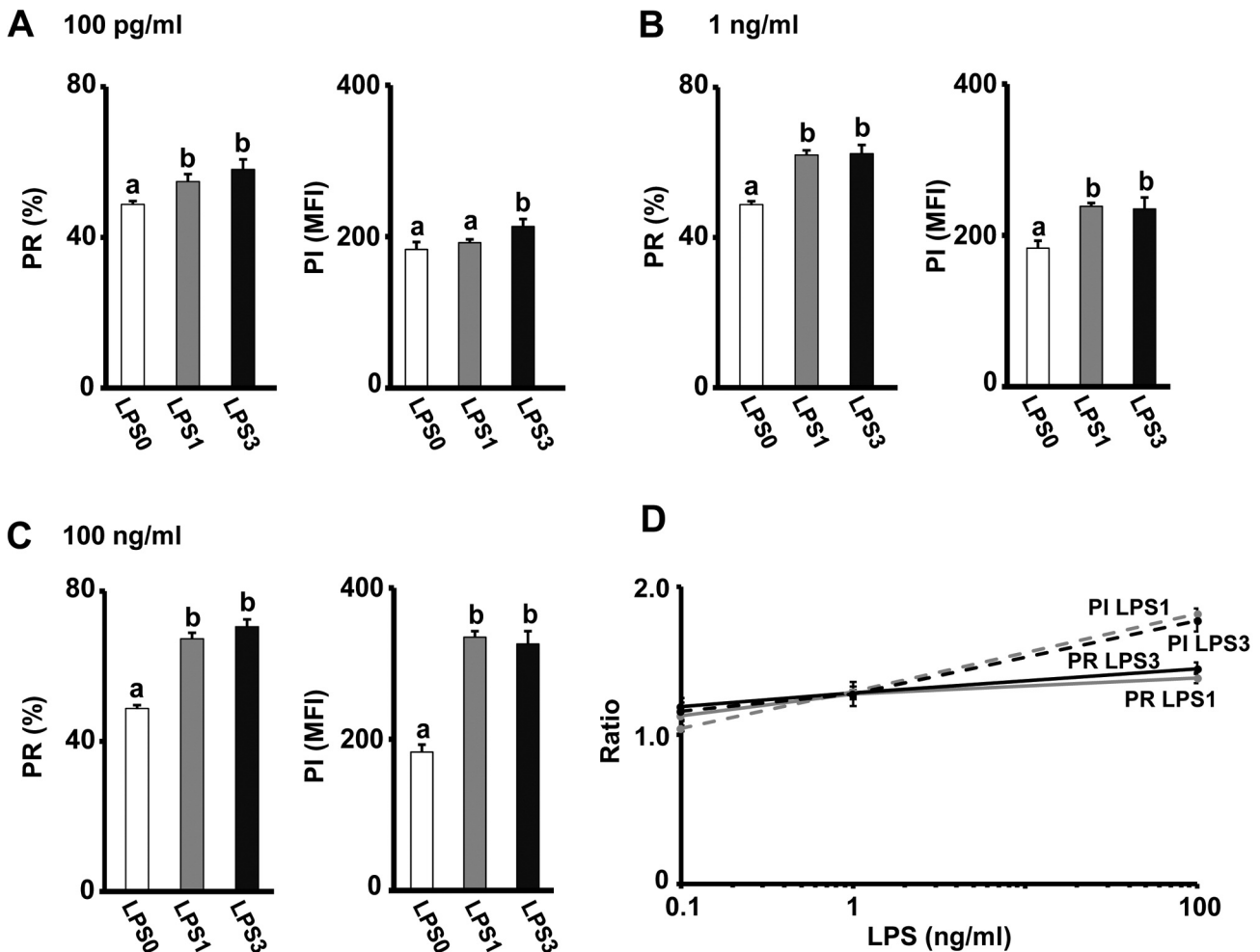


Figure 2. Analysis of phagocytic ratio (PR) and phagocytic intensity (PI) of fluorescent latex beads engulfed by RAW264.7 cells treated without (LPS0) or with 100 pg/ml (A), 1 ng/ml (B), or 100 ng/ml (C) of lipopolysaccharide one (LPS1) or three (LPS3) times every 24 h. D: The ratio of PR and PI by LPS1 or LPS3 to that by LPS0 (D). MFI: Mean fluorescent intensity. Values are presented as the mean±SD, n=3. Different letters indicate significant differences at p<0.05.

In this study, repeated stimulation with low-dose LPS was performed to establish the *in vitro* model of continuous administration of LPS. When creating an *in vitro* model of the administration of LPS, we must focus on the concentration of LPS and the number of LPS stimulations. An *in vitro* test usually adopts a single stimulation with LPS of 10-100 ng/ml. However, this stimulation method is used for models of sepsis or endotoxin shock caused by a single administration of LPS to the vein or peritoneal cavity, which is completely different from the stimulation that arises from the oral administration of LPS, which does not lead to toxicity (2, 3). Firstly, the concentration of LPS *in vivo* when taken orally is several thousand times lower than that of LPS *via* injection to a vein or the peritoneal cavity. In fact, when LPS is administered to mice at 0.3-1 mg/kg BW/day, its concentration in blood and

tissues is reported to be approximately 0.03-1 ng/ml (7, 8, 17). Secondly, the effects of oral administration of LPS are demonstrated by its long-term administration *via* routes such as diet or water, *i.e.* continuous administration. Therefore, it is considered that stimulation by a super low-dose of LPS occurs repeatedly in the living body. Therefore, to model this phenomenon *in vitro*, it is necessary to repeatedly administer a super-low dose of LPS. Therefore, to reproduce the stimulatory condition of oral administration of LPS using an *in vitro* test, repeated stimulation with low-dose LPS was set as an experimental condition. In this study, compared with the conventional *in vitro* test, repeated stimulation was performed with low-dose LPS (100 pg/ml and 1 ng/ml).

On comparing this study with a previous *in vivo* study (9), we found common results in terms of increased phagocytosis

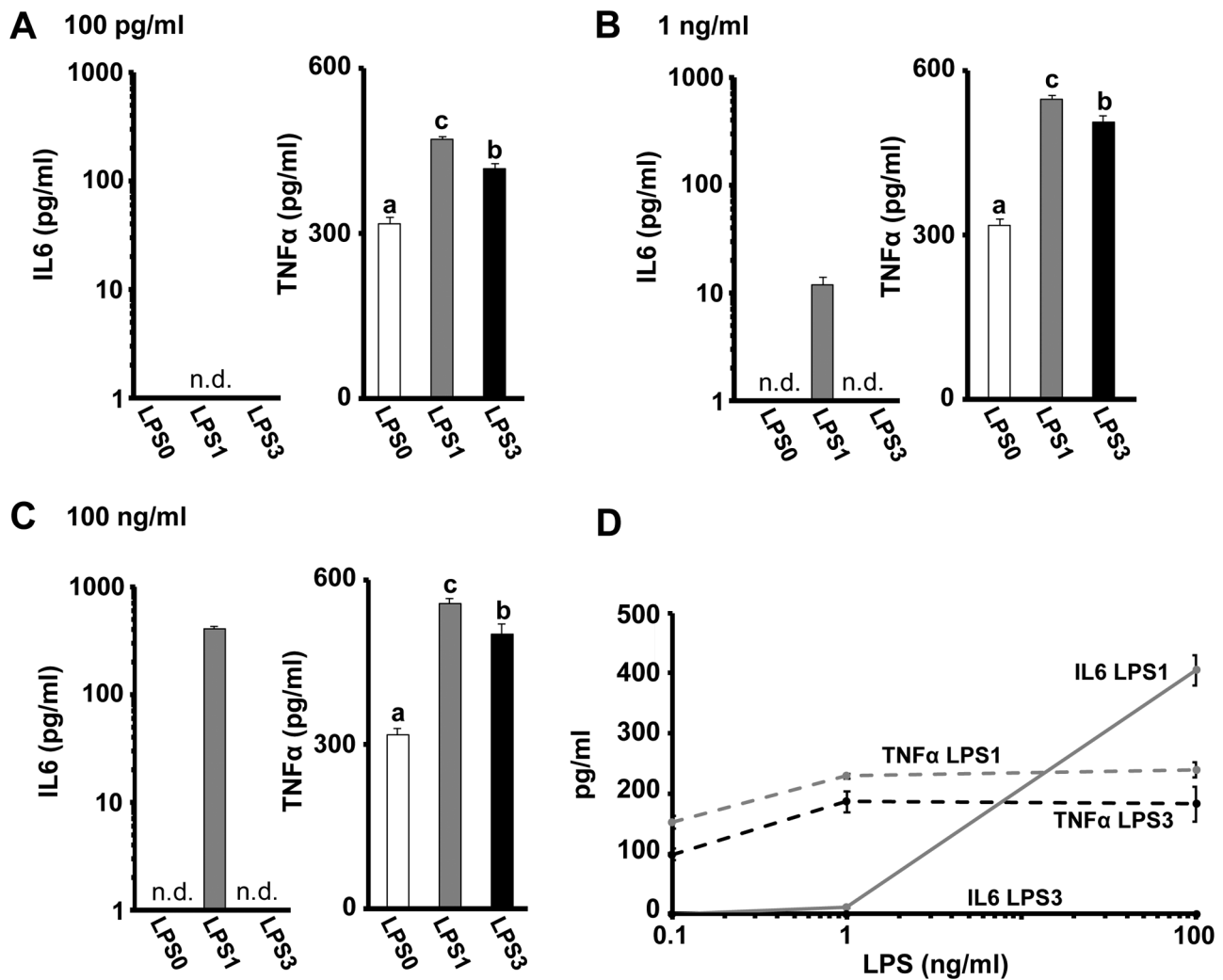


Figure 3. Analysis of cytokines culture supernatant of RAW264.7 cells treated without (LPS0) or with 100 pg/ml (A), 1 ng/ml (B), or 100 ng/ml (C) of lipopolysaccharide one (LPS1) or three (LPS3) times every 24 h. Interleukin-6 (IL6) and tumor necrosis factor- α (TNF α) were measured by enzyme-linked immunosorbent assay. D: Increase in cytokine levels by LPS1 and LPS3 relative to that by LPS0. n.d.: Not detected. Values are presented as the mean \pm SD, n=3. Different letters indicate significant differences at $p < 0.05$.

due to administration of LPS (Figure 2). In addition, a previous *in vivo* study also demonstrated that phagocytosis increased depending on the LPS dose (9). Similarly, the present study also showed that the increase in phagocytosis was dependent on the LPS concentration, in this respect, thereby reproducing the results of the previous *in vivo* study. From the above, it was considered that this test system can be an *in vitro* model that reproduces the macrophage phagocytic-enhancing effect achieved by continuous oral administration of LPS.

In this study, we also focused on inflammatory cytokine production by LPS stimulation (Figure 3). A single stimulation of LPS is known to enhance both phagocytosis and inflammatory cytokine production (18, 19) but in a

previous *in vivo* study, continuous oral administration of LPS did not increase production of inflammatory cytokines such as IL6, TNF α , and IL12 (9). In this study, we found that the production of these cytokines was increased by a single stimulation of LPS, as shown in other reports (18, 19). However, the amount produced was reduced with repeated stimulation, and in particular, IL6 was hardly produced, similar to that in the unstimulated group. Taken together, repeated stimulation with LPS might reproduce the state of macrophages induced by continuous oral administration of LPS, or the phagocytic ability increased but the inflammatory cytokine production did not increase. Conversely, there was no change in the amount of TNF α produced due to continuous administration of LPS in the previous *in vivo*

study (9) but there was a significant increase in the amount of TNF α produced by repeated stimulation with LPS compared with unstimulated cells in this study. In addition, IL12 production was not detected in the culture supernatant of RAW264.7 cells (data not shown). Furthermore, unlike the linear increase shown in phagocytosis on stimulation with LPS, the amount of IL6 production varied greatly in an LPS concentration-dependent manner, whilst TNF α production did not. The cytokine production pattern by LPS stimulation concentration was different depending on the type of cytokine. Detailed analysis, including gene analysis, is needed to determine whether repeated LPS stimulation reproduces the state of inflammatory cytokines induced in macrophages by continuous oral administration of LPS.

To the best of our knowledge, it was shown in this study for first time that the phagocytosis-enhancement effect in macrophages induced by continuous oral administration of LPS can be reproduced *in vitro*. Further analysis is warranted to confirm the usefulness of this model. Because phagocytosis is a fundamental function of macrophages involved in maintaining health, the mechanism by which continuous (oral) administration of LPS enhances macrophage phagocytosis by can be analyzed using this *in vitro* model. We hope that additional information is accumulated on the health benefits of oral administration of LPS.

Conflicts of Interest

KY, HM, MY, HI and GS are employed by the Control of Innate Immunity, Technology Research Association. HI, CK, and GS are employed by Macrophix Inc. This does not alter our adherence to journal policies on sharing data and materials.

Authors' Contributions

KY, HI, CK and GS conceptualized the study and coordinated the experiments. KY, HM and MY performed the experiments and KY performed data curation and formal analysis. HI and GS acquired the funding and supervised the project.

References

- Inagawa H, Nishizawa T, Tsukioka D, Suda T, Chiba Y, Okutomi T, Morikawa A, Soma GI and Mizuno D: Homeostasis as regulated by activated macrophage. II. LPS of plant origin other than wheat flour and their concomitant bacteria. *Chem Pharm Bull (Tokyo)* 40(4): 994-997, 1992. PMID: 1525958. DOI: 10.1248/cpb.40.994.
- Taniguchi Y, Yoshioka N, Nishizawa T, Inagawa H, Kohchi C and Soma G: Utility and safety of LPS-based fermented flour extract as a macrophage activator. *Anticancer Res* 29(3): 859-864, 2009. PMID: 19414320.
- Inagawa H, Kohchi C and Soma G: Oral administration of lipopolysaccharides for the prevention of various diseases: benefit and usefulness. *Anticancer Res* 31(7): 2431-2436, 2011. PMID: 21873155.
- Hebushima T, Matsumoto Y, Watanabe G, Soma G, Kohchi C, Taya K, Hayashi Y and Hirota Y: Oral administration of immunopotentiator from *Pantoea agglomerans* 1 (IP-PA1) improves the survival of B16 melanoma-inoculated model mice. *Exp Anim* 60(2): 101-109, 2011. PMID: 21512265. DOI: 10.1538/expanim.60.101
- Inagawa H, Saitoh F, Iguchi M, Nishizawa T, Okutomi T, Morikawa A, Soma GI and Mizuno D: Homeostasis as regulated by activated macrophage. III. Protective effect of LPSw (lipopolysaccharide (LPS) of wheat flour) on gastric ulcer in mice as compared with those of other LPS from various sources. *Chem Pharm Bull (Tokyo)* 40(4): 998-1000, 1992. PMID: 1525959. DOI: 10.1248/cpb.40.998
- Wakame K, Komatsu K, Inagawa H and Nishizawa T: Immunopotentiator from *Pantoea agglomerans* prevents atopic dermatitis induced by dermatophagoides farina extract in NC/Nga mouse. *Anticancer Res* 35(8): 4501-4508, 2015. PMID: 26168493.
- Kobayashi Y, Inagawa H, Kohchi C, Kazumura K, Tsuchiya H, Miwa T, Okazaki K and Soma GI: Oral administration of *Pantoea agglomerans*-derived lipopolysaccharide prevents development of atherosclerosis in high-fat diet-fed apoE-deficient mice *via* ameliorating hyperlipidemia, pro-inflammatory mediators and oxidative responses. *PLoS One* 13(3): e0195008, 2018. PMID: 29584779. DOI: 10.1371/journal.pone.0195008
- Kobayashi Y, Inagawa H, Kohchi C, Kazumura K, Tsuchiya H, Miwa T, Okazaki K and Soma GI: Oral administration of *Pantoea agglomerans*-derived lipopolysaccharide prevents metabolic dysfunction and Alzheimer's disease-related memory loss in senescence-accelerated prone 8 (SAMP8) mice fed a high-fat diet. *PLoS One* 13(6): e0198493, 2018. PMID: 29856882. DOI: 10.1371/journal.pone.0198493
- Inagawa H, Kobayashi Y, Kohchi C, Zhang R, Shibasaki Y and Soma G: Primed activation of macrophages by oral administration of lipopolysaccharide derived from *Pantoea agglomerans*. *In Vivo* 30(3): 205-211, 2016. PMID: 27107076.
- Kobayashi Y, Inagawa H, Kohchi C, Okazaki K, Zhang R, Kobara H, Masaki T and Soma GI: Lipopolysaccharides derived from *Pantoea agglomerans* can promote the phagocytic activity of amyloid β in mouse microglial cells. *Anticancer Res* 37(7): 3917-3920, 2017. PMID: 28668895. DOI: 10.21873/anticancer.11774
- Islam SU, Lee JH, Shehzad A, Ahn EM, Lee YM and Lee YS: Decursinol angelate inhibits LPS-induced macrophage polarization through modulation of the NF κ B and MAPK signaling pathways. *Molecules* 23(8): 1880, 2018. PMID: 30060484. DOI: 10.3390/molecules23081880
- Liu Y, Verma VK, Malhi H, Gores GJ, Kamath PS, Sanyal A, Chalasani N, Gao B and Shah VH: Lipopolysaccharide downregulates macrophage-derived IL-22 to modulate alcohol-induced hepatocyte cell death. *Am J Physiol Cell Physiol* 313(3): C305-C313, 2017. PMID: 28637673. DOI: 10.1152/ajpcell.00005.2017
- Lee H, Lee DS, Chang KJ, Kim SH and Cheong SH: Anti-inflammatory action of glucose-aurine reduced by inhibiting NF- κ B activation in LPS-activated RAW264.7 macrophages. *Adv Exp Med Biol* 1155: 989-999, 2019. PMID: 31468462. DOI: 10.1007/978-981-13-8023-5_82
- Chen L, Chen P, Liu J, Hu C, Yang S, He D, Yu P, Wu M and Zhang X: *Sargassum fusiforme* polysaccharide SFP-F2 Activates

- the NF- κ B signaling pathway *via* CD14/IKK and P38 axes in RAW264.7 cells. *Mar Drugs* *16*(8): 264, 2018. PMID: 30071655. DOI: 10.3390/md16080264
- 15 Wang J, Fang X, Wu T, Fang L, Liu C and Min W: In vitro immunomodulatory effects of acidic exopolysaccharide produced by *Lactobacillus planetarium* JLAU103 on RAW264.7 macrophages. *Int J Biol Macromol* *156*: 1308-1315, 2020. PMID: 31760015. DOI: 10.1016/j.ijbiomac.2019.11.169
- 16 Taciak B, Białasek M, Braniewska A, Sas Z, Sawicka P, Kiraga Ł, Rygiel T and Król M: Evaluation of phenotypic and functional stability of RAW264.7 cell line through serial passages. *PLoS One* *13*(6): e0198943, 2018. PMID: 29889899. DOI: 10.1371/journal.pone.0198943
- 17 Wendeln AC, Degenhardt K, Kaurani L, Gertig M, Ulas T, Jain G, Wagner J, Häsler LM, Wild K, Skodras A, Blank T, Staszewski O, Datta M, Centeno TP, Capece V, Islam MR, Kerimoglu C, Staufenbiel M, Schultze JL, Beyer M, Prinz M, Jucker M, Fischer A and Neher JJ: Innate immune memory in the brain shapes neurological disease hallmarks. *Nature* *556*(7701): 332-338, 2018. PMID: 29643512. DOI: 10.1038/s41586-018-0023-4
- 18 Islam MA, Pröll M, Hölker M, Tholen E, Tesfaye D, Looft C, Schellander K and Cinar MU: Alveolar macrophage phagocytic activity is enhanced with LPS priming, and combined stimulation of LPS and lipoteichoic acid synergistically induce pro-inflammatory cytokines in pigs. *Innate Immun* *19*(6): 631-643, 2013. PMID: 23608822. DOI: 10.1177/1753425913477166
- 19 Wu TT, Chen TL and Chen RM: Lipopolysaccharide triggers macrophage activation of inflammatory cytokine expression, chemotaxis, phagocytosis, and oxidative ability *via* a toll-like receptor 4-dependent pathway: validated by RNA interference. *Toxicol Lett* *191*(2-3): 195-202, 2009. PMID: 19735705. DOI: 10.1016/j.toxlet.2009.08.025

Received May 23, 2020

Revised June 19, 2020

Accepted June 23, 2020