Effect of Lipopolysaccharide Derived from *Pantoea agglomerans* on the Phagocytic Activity of Amyloid β by Primary Murine Microglial Cells

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Abstract. *Background/Aim: Monophosphoryl lipid A, lipopolysaccharide (LPS)-derived Toll-like receptor (TLR) 4 agonist, has been shown to be effective in the prevention of Alzheimer's disease (AD) by enhancing phagocytosis of amyloid β (Aβ) by brain microglia. Our recent study demonstrated that oral administration of LPS derived from Pantoea agglomerans (LPSp) activates peritoneal macrophages and enhances the phagocytic activity via TLR4 signaling pathway; however, the effect of LPSp on Aβ phagocytosis in microglia is still unknown. Materials and Methods: Primary microglial cells were isolated from adult mouse brain by enzymatic digestion, following myelin removal and magnetic separation of cluster of differentiation (CD) 11b. Phagocytic analysis of the primary microglia was measured by using HiLyte™ Fluor 488-conjugated $Aβ_{1-42}$. Results: Using our protocols, the average yield of isolated $CD11b^+$ cells was around $2.2×10^5$ cells per brain. $CD11b^+CD45^+CD39^+$ cells were defined here as microglia. The phagocytic activity of $Aβ_{1-42}$ by the isolated microglia was confirmed. LPSp (10 ng/ml) pre-treatment for 18 h significantly increased Aβ phagocytic activity. Conclusion: The enhancement of $Aβ_{1-42}$ phagocytosis by LPSp treatment in the primary mouse microglia was demonstrated for the first time.*

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Alzheimer's disease (AD) is an age-dependent neuro-degenerative pathology characterized by a progressive and irreversible decline in cognition and behavior. The primary pathological features of AD in brain parenchyma are the progressive accumulation of amyloid β (Aβ) peptides derived from Aβ protein precursor (APP) and the aggregation of hyperphosphorylated tau protein (1). A widely accepted hypothesis of AD pathogenesis is an imbalance between production and clearance of Aβ in the central nervous system. There have been extensive studies for the development of inhibitor drugs for AD treatment. The major therapeutic strategy has been aimed at decreasing cerebral Aβ production. Many drugs have been designed and synthesized to inhibit the β- or γ-secretase that initiates Aβ production by cleaving the extracellular or intramembrane domain of APP. However, some inhibitors may cause abnormalities in the gastrointestinal tract, thymus and spleen in rodents (2, 3). An alternative promising approach is to enhance Aβ clearance by resident phagocytes of the brain. Microglia, the brain-resident macrophage, are the primary immune cells responding to injury or pathogen invasion (4). It is well-known that Aβ activates microglia to induce Aβ phagocytosis through binding to receptors on the plasma membrane of microglia (5). Microglial cells lacking Toll-like receptor (TLR) 2, TLR4, or the co-receptor cluster of differentiation (CD) 14 are not activated by Aβ treatment and do not enhance a phagocytic response compared with wild-type microglial cells (6). Kakimura *et al.* (7) demonstrated that some heat-shock proteins ((HSPs) HSP90, HSP70 and HSP32), act as TLR4 agonists, as well as increase phagocytosis and clearance of Aβ by newborn rat microglia. Furthermore, treatment of lipopolysaccharide ((LPS) *via* intracranial route) (8) or monophosphoryl lipid A derived from *Salmonella minnesota* R595 LPS ((MPL) *via* intraperitoneal route) (9)

reduce cerebral Aβ accumulation in a mouse model of AD. LPS, as well as MPL, were shown to be effective in the induction of Aβ uptake by microglial cells (9). Considering that LPS and MPL are well known as TLR4-specific agonists, these evidences prompted us to consider that TLR4 agonists may exert a beneficial effect on AD by enhancing phagocytosis/clearance of Aβ by microglia.

We recently demonstrated that oral administration of LPS derived from *Pantoea agglomerans* (LPSp), which was isolated from wheat flour (10), enhanced phagocytic activity by resident peritoneal macrophages *via* TLR4 signaling pathway in mice (11). Fukasaka *et al*. (12) demonstrated that sublingual administration of LPSp with influenza vaccine promoted anti-influenza immunoglobulin A (IgA) production on various mucosa *via* TLR4 pathway. These findings suggest that LPSp could be involved in regulating immune system and activating phagocytosis by tissue resident macrophages through TLR4 signal cascade of the peritoneum and oral mucosa. However, the activating effect of LPSp on Aβ phagocytosis by brain microglia is still unknown. In the present study, our objective was to clarify whether LPSp treatment enhances Aβ phagocytosis activity by primary mouse microglia.

Materials and Methods

Animals. Male C57BL/6 mice were obtained from Japan SLC (Shizuoka, Japan). They were housed on a-12 h light-dark cycle and allowed unrestricted access to water and standard mouse chow. Animals were used at 10-15 weeks of age. The animal experiments were approved by The Animal Care and Use Committee for Kagawa University (approval no. 15134-1).

Isolation of primary microglia from adult mouse. Primary microglial cells were isolated from adult mouse brain by enzymatic digestion, as described by Legroux *et al*. (13). Briefly, the brain was aseptically removed and kept in ice cold serum-free Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 100 units/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). The brain tissue was chopped finely with a fine sharp razor. The whole brain homogenate was then incubated in DMEM containing 1.2 units/ml dispase II, 1 mg/ml papain (Sigma-Aldrich) and 10 units/ml DNase I (Takara Bio, Shiga, Japan) for 30 min at 37˚C. The digestion was terminated by the addition of DMEM containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and the cells were centrifuged for 5 min at 300 × *g* at room temperature (RT). The pellets were re-suspended in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and centrifuged for 5 min at 300 × *g* at RT. Tissue debris was removed by passing the cell suspension through a 40-μm cell strainer (Corning, Durham, NC, USA). The cells were pelleted by centrifugation (5 min at 300 × *g* at RT) and then the supernatant was carefully poured off. The cells were re-suspended in PBS containing 0.5% BSA and 30 μl/brain magnetic myelin removal beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated for 15 min at 4˚C. After washing with PBS containing 0.5% BSA, myelin was removed by magnetic separation with autoMACS® pro separator (Miltenyi Biotec). After myelin removal, the cells were stained with anti-CD11b antibodies-conjugated magnetic beads (20 μl/brain; Miltenyi Biotec) in PBS containing 0.5% BSA and 2 mM EDTA for 15 min at 4˚C. After washing, the $CD11b^+$ cells were separated with autoMACS® pro separator. Both the $CD11b^+$ (positive) and $CD11b^-$ (negative) fractions were collected and used for further analysis. The isolated $CD11b^+$ cells were stained with Trypan blue and the number of live and dead cells was counted under the microscope. Isolated $CD11b^+$ and $CD11b^-$ cells obtained from the isolation procedure were re-suspended in PBS containing 0.5% BSA and 2 mM EDTA. The cells were immunostained with allophycocyanin (APC)-labeled anti-CD11b antibody (BioLegend, San Diego, CA, USA), phycoerythrin (PE)-labeled anti-CD45 antibody (BioLegend) and phycoerythrin with cyanine 7 (PE-Cy7)-labeled CD39 (BioLegend) for 30 min on ice. As an isotype control, APC-IgG2b, PE-IgG2b and PE-Cy7-IgG2a (BioLegend) were used. Data were acquired on a Beckman Coulter Gallios flow cytometer using the Kaluza software (Beckman Coulter, Indianapolis, IN, USA).

Phagocytosis assay. A flow cytometry method was used for the phagocytosis assay. Isolated $CD11b^+$ cells obtained from the isolation procedure described above were seeded into 96-well tissue culture plate (1×10^5 cells/well) and allowed to adhere for 2 h. The medium was replaced with DMEM in the presence or absence of LPSp (Macrophi, Kagawa, Japan) and the cells were incubated for an additional 18 h at 37˚C. After washing twice with DMEM, cells were incubated with HiLyte™ Fluor 488-$A\beta_{1\text{-}42}$ (HF488-$A\beta_{1\text{-}42}$; Anaspec, Fremont, CA, USA) at the concentration of 0.5 μg/well for 3 h at 37˚C. Negative control cells were treated with DMEM without HF488-$A\beta_{1\text{-}42}$. Cytochalasin D (CyD; Wako, Osaka, Japan), which inhibits actin polymerization, was used at 20 μM and cells were pretreated for 30 min before adding the HF488-$A\beta_{1\text{-}42}$. To remove non-internalized HF488-$A\beta_{1\text{-}42}$, cells were washed twice with ice-cold PBS. Cells were detached by 0.25% trypsin treatment and collected by centrifugation for 5 min at 300 × *g*. Cell pellets were re-suspended in PBS containing 0.5% BSA and 2 mM EDTA and immunostained with APC-labeled anti-CD11b antibody, PE-labeled anti-CD45 antibody and PE-Cy7-labeled CD39 for 30 min on ice. The geometric mean fluorescence intensity (MFI) of HF488 in the $CD11b^+CD45^+CD39^+$ cells was measured using a Beckman Coulter Gallios flow cytometer using the Kaluza software. In the experiments of LPSp treatment, the internalized fluorescence of HF488-$A\beta_{1\text{-}42}$ was evaluated as follows; (MFI of the sample without CyD treatment) – (MFI of the sample with CyD treatment), whereas the relative phagocytic activity was calculated by dividing the internalized fluorescence of HF488-$A\beta_{1\text{-}42}$ of the sample by that of the sample without the LPSp treatment.

Statistical analysis. Statistical comparisons of the differences between treatments for other parameters were analyzed by one-way ANOVA combined with the Tukey-Kramer *post-hoc* test. A *p*-value <0.05 was considered statistically significant. All results are expressed as the mean±SEM. All analyses were performed with StatMate V (ATMS, Tokyo, Japan).

Results

Viability and yield of $CD11b^+$ cells. The viability and yield of isolated $CD11b^+$ cells were analyzed by using Trypan blue exclusion assay. Using our present protocols, we have been

Table I. *Average yield of live cells and brain weight of adult mice used in the present study.*

	Live cells (%)	Live cells/brain ($\times 10^5$ cells/brain)	Tissue weight (g)
$CD11b^+$ cells	90.0±3.7	2.2±0.3	0.38±0.06

The live cells in the $CD11b^+$ fraction obtained by enzymatic digestion and magnetic separation, as described in the Materials and Methods section, were counted by Trypan blue staining.

able to isolate approximately 2.2×10^5 cells $CD11b^+$ cells from mouse brains weighing about 0.38 *g* (Table I).

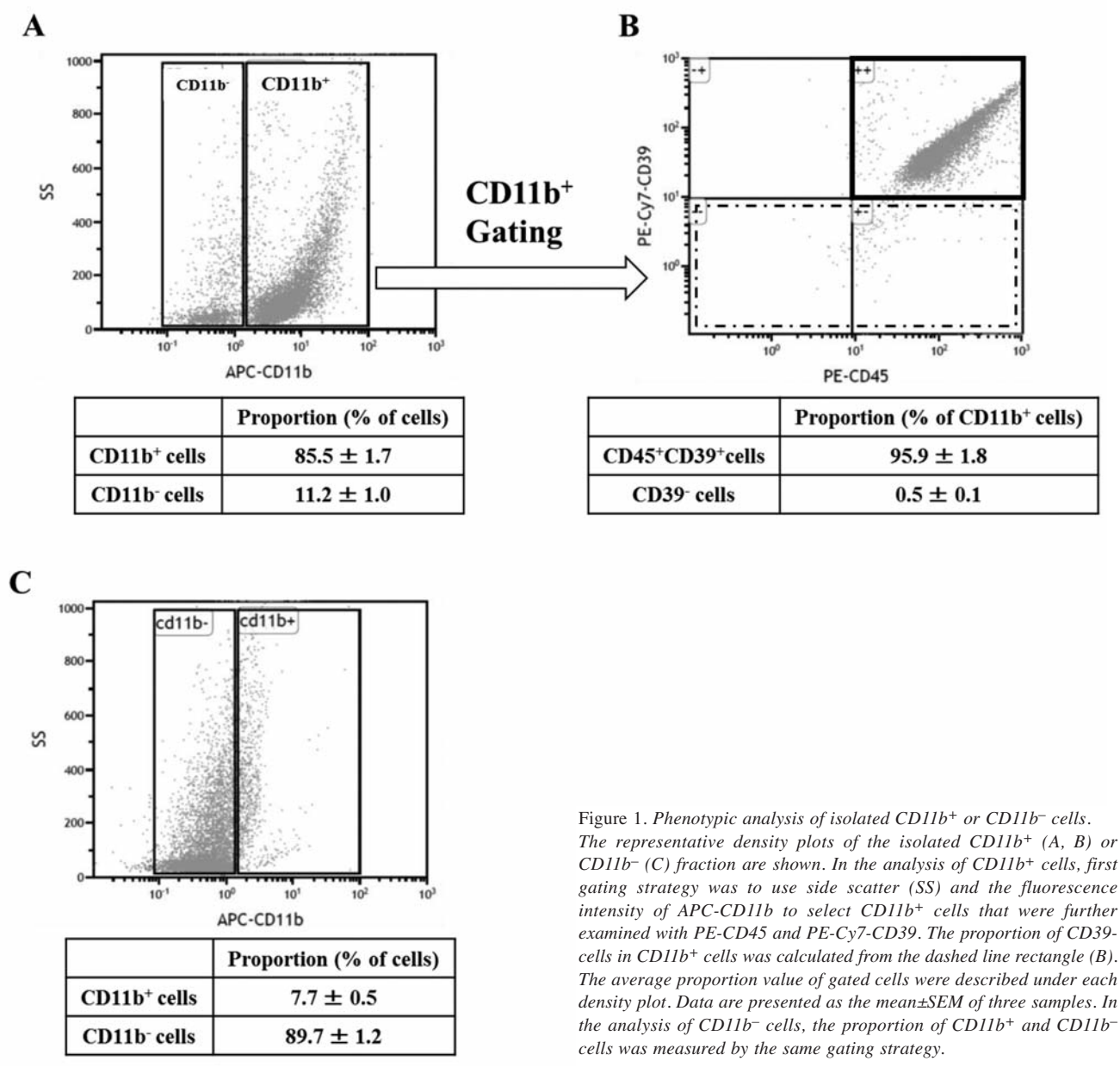
Phenotypic analysis of isolated $CD11b^+$ cells. Phenotypic analysis of the $CD11b^+$ cells was performed by flow cytometry. CD11b, CD45 and CD39 were used as microglial cell markers. CD11b, an integrin family member, is expressed by myeloid cells, including microglia, monocytes and macrophages (14). CD45, a transmembrane glycoprotein, is expressed on hematopoietic cells except erythrocytes (15). $CD11b^+$ $CD45^+$ cells isolated from mouse brain by enzymatic digestion are confirmed as microglia (13). In addition, surface expression of CD39 (ecto-nucleoside triphosphate diphosphohydrolase 1) in adult microglia can be used to distinguish recruited monocytes from peripheral blood (16). As shown in Figure 1, $CD11b^+$ cells were predominant (85.5±1.7%) in the isolated $CD11b^+$ fraction. Further phenotypic analysis of $CD11b^+$ cells indicated that microglia, defined as $CD11b^+CD45^+CD39^+$, constituted the highest proportion (95.9±1.8%), while CD39- cells, containing infiltrated blood monocytes, were less than 1% (0.5±0.1%) of the $CD11b^+$ cells. The $CD11b^-$ fraction obtained as effluent in $CD11b^+$ separation step was also analyzed for evaluating the separation ability of $CD11b^+$ cells by the magnetic columns and anti-CD11b magnetic beads. As expected, the proportion of remaining $CD11b^+$ cells was as low as 7.7±0.5%.

Phagocytosis analysis of Aβ by $CD11b^+CD45^+CD39^+$ primary mouse microglial cells. To confirm whether the isolated primary microglia cells possess phagocytic activity of Aβ peptides, phagocytosis assay was performed by using the isolated microglia cells treated with fluorophore (HF488)-conjugated $A\beta_{1\text{-}42}$. As shown in Figure 2, the HF488-$A\beta_{1\text{-}42}$ treatment significantly increased the MFI of HF488 in the $CD11b^+CD45^+CD39^+$ cells (microglia) compared to negative control, whereas co-incubation with CyD significantly decreased the MFI in the cells compared to the treatment HF488-$A\beta_{1\text{-}42}$ alone. Mandrekar *et al*. (17) reported that CyD treatment decreased the uptake of $A\beta_{1\text{-}42}$ by microglial cells through inhibition of phagocytic responses. These results suggest that the primary mouse microglial cells obtained here possess an uptake activity of $A\beta_{1\text{-}42}$ *via* phagocytic responses.

Effect of LPS derived from Pantoea agglomerans (LPSp) on the phagocytic activity of Aβ by primary mouse microglial cells. To assess whether LPSp affects the HF488-$A\beta_{1\text{-}42}$ phagocytic activity in the isolated primary microglia, the phagocytic assay was performed by using microglia that were pre-incubated with LPSp for 18 h. As shown in Figure 3, LPSp treatment (10 ng/ml) significantly enhanced the relative phagocytic activity of HF488-$A\beta_{1\text{-}42}$ in the $CD11b^+CD45^+CD39^+$ microglia.

Discussion

The most widely accepted hypothesis of AD pathogenesis is widely known as "amyloid cascade hypothesis". This theory proposes that certain gene mutations or cell injury is involved in imbalance of Aβ production and clearance in the central nervous system, resulting in abnormal Aβ accumulation in the form of senile plaques. Recent studies have demonstrated that TLR4 signaling plays a crucial role in Aβ phagocytosis/clearance by brain resident phagocyte microglia (7-9). In the present study, we demonstrated that pre-treatment of LPSp (a TLR4-specific agonist) enhanced the phagocytosis of HF488-$A\beta_{1\text{-}42}$ by the primary mouse microglia. Primary microglial cells were isolated by enzymatic degradation and magnetic separation of CD11b-positive cells. We herein obtained approximately 2×10^5 $CD11b^+$ cells per brain (Table I). This result is consistent with recent studies reporting that the yield of $CD11b^+$ microglia is within a range between 2×10^5 and 8×10^5 cells per mouse brain isolated by magnetic or Percoll gradient methods (13, 18, 19). Further phenotype analysis of the isolated cells (Figure 1) indicated that $CD11b^+$ $CD45^+$ $CD39^+$ microglial cells were the predominant type (around 96% of $CD11b^+$ cells), while the presence of CD39-negative cells was quite low (approximately 0.5% of $CD11b^+$ cells), indicating a negligible contamination by monocytes from peripheral blood. The phagocytosis assay confirmed that primary microglia possessed the phagocytic activity of HF488-$A\beta_{1\text{-}42}$ (Figure 2), thus suggesting that the isolation procedure itself may not alter physiological properties of microglia. Further studies are required for analyzing the expression of receptors, such as TLR4 and Aβ binding receptors. The isolated primary microglia were used to evaluate whether the LPSp treatment



	Proportion (% of cells)
CD11b$^+$ cells	85.5 ± 1.7
CD11b$^-$ cells	11.2 ± 1.0

	Proportion (% of CD11b$^+$ cells)
CD45$^+$CD39$^+$cells	95.9 ± 1.8
CD39$^-$ cells	0.5 ± 0.1

	Proportion (% of cells)
CD11b$^+$ cells	7.7 ± 0.5
CD11b$^-$ cells	89.7 ± 1.2

Figure 1. *Phenotypic analysis of isolated CD11b$^+$ or CD11b$^-$ cells. The representative density plots of the isolated CD11b$^+$ (A, B) or CD11b$^-$ (C) fraction are shown. In the analysis of CD11b$^+$ cells, first gating strategy was to use side scatter (SS) and the fluorescence intensity of APC-CD11b to select CD11b$^+$ cells that were further examined with PE-CD45 and PE-Cy7-CD39. The proportion of CD39- cells in CD11b$^+$ cells was calculated from the dashed line rectangle (B). The average proportion value of gated cells were described under each density plot. Data are presented as the mean±SEM of three samples. In the analysis of CD11b$^-$ cells, the proportion of CD11b$^+$ and CD11b$^-$ cells was measured by the same gating strategy.*

affected HF488-A$\beta_{1\text{-}42}$ phagocytosis. As shown in Figure 2, 10 ng/ml of LPSp enhanced the HF488-A$\beta_{1\text{-}42}$ phagocytosis. Recent studies have indicated that Juzen-taiho-to (a Chinese herbal medicine used for boosting human immune functions) activates microglial phagocytosis to A$\beta_{1\text{-}42}$ *in vivo* (20), whereas oral administration of Juzen-taiho-to decreases Aβ accumulation in the brain of a mouse model of AD (21). Furthermore, Montenegro *et al*. reported the crucial role of LPS as macrophage-activating substance in Juzen-taiho-to experimentation, implying that the activation of tissue resident microphages by oral administration of LPS might be involved in the prevention of AD progression (22). Our recent study demonstrated that oral administration of LPSp (1 mg/kg for 7 days) activates resident peritoneal macrophages and enhances the phagocytic activity *via* the TLR4 signaling pathway (11), thus suggesting a potential *in vivo* preventive effect on AD that is currently under investigation. We also reported that oral administration of LPS derived from the subaleurone layer of rice (23) and acetic acid bacteria (24) increases the macrophage activity and protects pollen allergy in a mouse model of cedar pollen allergy, suggesting that LPS derived from dietary foods and bacteria might be of benefit

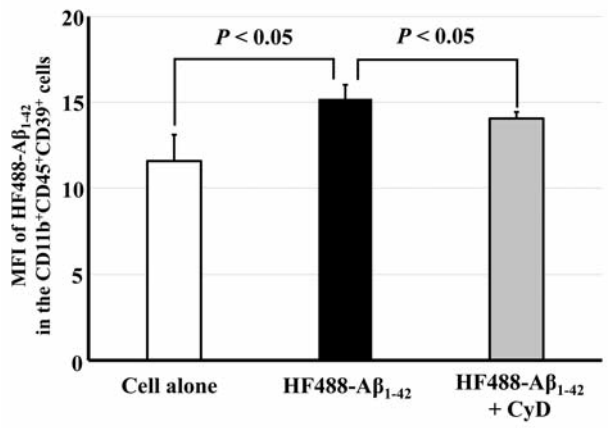


Figure 2. *Phagocytic analysis of the isolated primary microglia. Primary microglia (1×10^5 cells/well) were incubated with HF488-Aβ_{1-42} for 3 h at 37˚C. Negative control cells were treated with DMEM without HF488-Aβ_{1-42}. CyD was used at 20 μM and cells were pretreated for 30 min before adding HF488-Aβ_{1-42}. The MFI of HF488 in the CD11b$^+$CD45$^+$CD39$^+$ cells was measured using a Beckman Coulter Gallios flow cytometer using the Kaluza software. Data are presented as the mean±SEM of three samples (one-way ANOVA with Tukey-Kramer post-hoc test).*

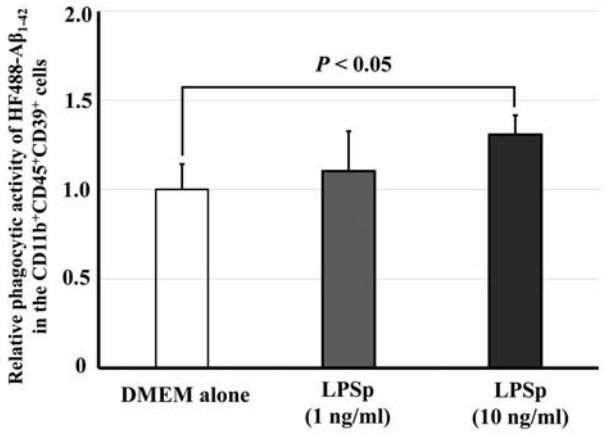


Figure 3. *Phagocytic analysis of primary microglia treated with LPSp. Primary microglia (1×10^5 cells/well) were pretreated with DMEM alone or LPSp (1, 10 ng/ml) for 18 h at 37˚C and then incubated with HF488-Aβ_{1-42} for 3 h at 37˚C. CyD was used at 20 μM and cells were pre-treated for 30 min before adding HF488-Aβ_{1-42}. The MFI of HF488 in the CD11b$^+$CD45$^+$CD39$^+$ cells was measured using a Beckman Coulter Gallios flow cytometer using the Kaluza software. The internalized fluorescence of HF488-Aβ_{1-42} was evaluated as follows: (MFI of the sample without CyD treatment) – (MFI of the sample with CyD treatment), whereas the relative phagocytic activity was calculated by dividing the internalized fluorescence of HF488-Aβ_{1-42} of the sample by that of the negative control sample. Data are presented as the mean±SEM of three samples (one-way ANOVA with Tukey-Kramer post-hoc test).*

in AD. In conclusion, we demonstrated for the first time that LPSp treatment enhances Aβ_{1-42} phagocytosis by primary microglia isolated from adult mice.

Conflicts of Interest

The Authors have no financial conflicts of interest.

Acknowledgements

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