Master's Thesis
석사 학위논문

Suppression of autophagy through TLR4/PI3K/ FoxO3 signaling impairs phagocytic degradation of microglia

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Advisor: Professor Seong-Woon Yu
Co-advisor: Professor Byung-Hoon Lee

By

Hyeri Nam
Department of Brain and Cognitive Sciences
DGIST

A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Master of Science in the Department of Brain and Cognitive Sciences. The study was conducted in accordance with Code of Research Ethics[1]

11. 22. 2017

Approved by

Professor Seong-Woon Yu (Advisor) _____ (signature)

Professor Byung-Hoon Lee (Co-Advisor) _____ (signature)

[1] Declaration of Ethical Conduct in Research: I, as a graduate student of DGIST, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidance of my thesis advisor.

Suppression of autophagy through TLR4/PI3K/ FoxO3 signaling impairs phagocytic degradation of microglia

Hyeri Nam

Accepted in partial fulfillment of the requirements for the degree of Master of Science.

11. 22. 2017

Head of Committee _____ (signature)
Prof. Seong-Woon Yu

Committee Member _____ (signature)
Prof. Byung-Hoon Lee

Committee Member _____ (signature)
Prof. Yong-Seok Oh

MS/BS
201625006

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ABSTRACT

Autophagy is an essential intracellular degradation process for turnover of proteins and organelles. Recently autophagy also plays important roles in immune system. Deficits of autophagy in innate immune systems are related with many inflammatory diseases including neurodegenerative disorders. Various immune triggers activate autophagy in innate immune cells including macrophages. Microglia are the resident macrophages in the central nervous system. However, the detailed mechanisms of autophagy regulation by immune triggers in microglia are not well understood. Here, we found that autophagy in microglia is suppressed by lipopolysaccharide (LPS), a prototypical inflammation inducer and toll-like receptor 4 activator. The expression levels of major autophagy related genes were significantly suppressed in LPS-treated microglia in dose- and time- dependent manner, which is contrary to the reports in macrophages. LPS-induced suppression of microglial autophagy was mainly through the activation of PI3K/AKT pathway and following inactivation of FoxO3 transcription factor, while mTOR or MAPK pathways did not play major roles. Suppression of autophagy was related to impaired phagocytic degradation as shown in LC3-associated phagocytosis (LAP) and amyloid β (Aβ) clearance in LPS-treated microglia, which were reversed by inhibition of PI3K. Taken together, our novel findings indicate the unique signaling mechanism for regulation of microglia autophagy, and point to TLR4/PI3K/FoxO3 pathway as potential therapeutic target for microglia in brain disorders.

Keywords: autophagy, microglia, PI3K, FoxO3, phagocytic degradation

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1. Introduction (Background and significance)

Autophagy is a cytosolic lysosomal degradation process that degrades aggregated, damaged, long-lived proteins and organelles, and attacking microorganisms [1, 2]. Autophagic vesicles are generated by forming autophagosomes and later autolysosomes through fusion with lysosomes [3].

Autophagy pathway is inferred to be closely related to inflammatory immune responses by eliminating toxic materials. Various immune triggers activate autophagy in innate immune cells including macrophages. Autophagy may function to regulate balance between beneficial and toxic effects of the host responses to infection and other immunological stimuli [4, 5]. Additionally, defects of autophagy are linked to pathogenesis of many neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and inflammatory syndromes such as Crohn disease, and depression [6]. Though the awareness of the interplay between autophagy and immunity was increased, the correlated signaling pathways need further investigation.

The roles and mechanisms of autophagy have been studied in several kinds of peripheral immune cells including macrophages of different organs (Fig. 1). Autophagy functions includes control of inflammation, presenting antigen recognition molecules, regulation of lymphocyte homeostasis, secretion of immune mediators, and eliminating useless organelles [7, 8]. Toll-like receptors (TLRs) are front-line of innate immunity against invading pathogens. Activation of TLRs by agonists causes immune-stimulatory responses [9, 10].

Accumulating evidence has demonstrated that TLR signaling and related pathways in immune system can induce autophagy in macrophages [11-13].

Interestingly, several regulatory pathways are involved in both autophagy and inflammatory systems. Mammalian target of rapamycin (mTOR), a well-known kinase inhibiting autophagy, need for autophagy induced by LPS and ATG7, ATG5/12 and LC3B expression in macrophages [14, 15]. Moreover, MAPK signaling and PI3K/Akt pathway which is upstream of mTOR is activated in autophagy as well as TLR-mediated proinflammatory responses [15-17]. And several transcription factors including several signaling pathways of both autophagy and inflammatory responses, such as TFEB, TFE3, FoxO3 and ZKSCAN3, were known as controlling autophagy. Transcription factor EB (TFEB) positively regulates autophagy and lysosomal related genes in macrophages. TFEB is regulated by mTOR complex 1 and MAPK signaling. In addition, forkhead box O (FoxO) also positively regulates autophagy related genes, MAP1LC3b, Gabarapl1 in macrophages and regulated by PI3K/Akt signaling pathway [18-20].

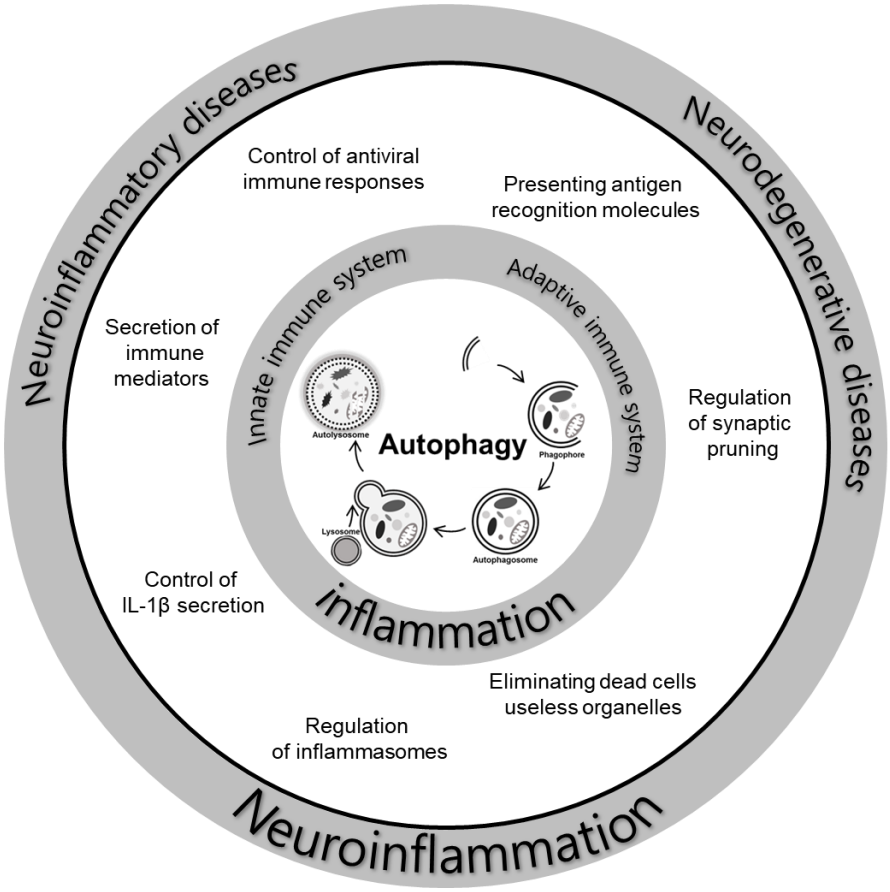


Figure 1. Function of autophagy in immunity.

Various function of autophagy well-known in innate immunity and the implication of malfunctioning autophagy to several diseases.

Microglia as the resident macrophage cells in the central nervous system (CNS) which play important roles in brain development and homeostasis by regulating inflammatory responses. Activated microglia with amoeboid morphologies release various cytokines/chemokines, and are involved in phagocytosis and synapse pruning. Microglia and macrophages exert similar cellular immune functions with many key immune pathways in common [21]. Autophagy in microglia was reported to regulate synapse pruning, degrade amyloid β, control IL-1β secretion, and clear and eliminate dead cells [22]. However, the role of autophagy and detailed mechanisms in microglia are still not well understood. Many studies have shown the results form autophagy modulation in microglia which causes the changes in functional outcome of microglia phagocytosis and inflammation.

Here, we observed that the typical agonist of TLR-4, lipopolysaccharide (LPS), suppressed the autophagy flux in microglia, which is contrary to the previous results reported in macrophages. We found the distinct mechanisms that suppression autophagy in LPS-induced microglia was through down-regulation of TLR4/PI3K/FoxO3. Furthermore, suppression of autophagy in LPS-activated microglia impaired the LC3-associated phagosomes formation and degradation of Amyloid β. Our findings of the microglial autophagy related inflammatory signaling pathway under LPS-treated conditions will provide new insight into pathogenic mechanisms of neurodegenerative diseases and their treatment.

2. Materials and Methods

2.1 Cell culture

All procedures for the care and use of laboratory animals were approved by the Institutional Animal Care and Use Committee of DGIST and Seoul National University. For primary microglia culture, whole brains were isolated from C57BL/6 or TLR4$^{-/-}$ mice at postnatal day 1-2. The brains were dissected in serum-free high-glucose Dulbecco's Modified Eagle Medium (DMEM, Corning 10-013-CVR) with sterile scissors and incubated with 0.25% Trypsin-ethylenediaminetetraacetic acid (EDTA) solution for 7 minutes at 37°C. An equal volume of culture medium composed of DMEM with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone) and 1% (100 U/ml) penicillin-streptomycin (Hyclone) was added to stop trypsinization and the dissected brains were dissociated with gentle pipetting. The dissociated cells were passed through a 70-μm pore mesh, and pelleted at 800 ×g for 10 minutes. Cell pellet was suspended in culture medium and seeded in a 100-mm dish per brain. Five days after plating, whole medium was changed with fresh medium. After, half of the medium for each plate was changed every other day with fresh medium. Primary microglia were confluent after 10 to 14 days and separated from mixed glial culture by tapping the plate. A highly enriched culture of primary microglia (>95%) was verified by immunostaining with anti-Iba-1 and anti-GFAP antibodies, specific microglia and astrocyte markers, respectively, as we previously reported [23, 24].

Bone marrow derived macrophages (BMDMs) were obtained from femurs and tibias from 6-7 week old C57BL/6 mice, as previously described [25]. BMDMs were grown in RPMI1640 (GIBCO) supplemented with 10% FBS and 1% penicillin-streptomycin. Raw264.7 cells and BV-2 cells were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin.

2.2 Acute isolation of primary microglia and peritoneal macrophages

For acute isolation of primary microglia, brains were removed from 7-8 weeks old C57BL/6 mice. After washing with 1X HBSS, tissue was homogenized by razor-blade chopping and transferred to two 1.5 ml tubes. Tissue was dissociated with neural dissociation kit (Miltenyi Biotec, 130-092-628) according to manufacturer's instructions. Myelin were removed using myelin removal beads (Miltenyi Biotec, 130-096-733) according to manufacturer's instructions. Suspended cells with myelin removal beads were passed through MACS separation columns (Miltenyi Biotec, 130-042-401) and centrifuge 3 min at 1200 ×g. Pellet was suspended with MACS buffer and transferred to new 1.5 ml tube. According to manufacturer's instructions, microglia were isolated using $CD11b^+$ microbeads (Miltenyi Biotec, 130-049-601).

Peritoneal macrophages were obtained from 6-8 weeks old C57BL/6 mice peritoneum, as previously described [26].

2.3 Reagents and antibodies

Following antibodies are used: β-Actin (sc-47778 HRP) was purchased from Santa Cruz Biotechnology; LC3B (NB100-2220) antibody was purchased from Novus Biologicals; phosphorylated FoxO3 (S253, ab47285) were purchased from Abcam; phosphorylated Akt (S473, 9271S; T308, 9275S), phosphorylated mTOR (S2448, 2971S), phosphorylated p44/42 MAPK (Erk 1/2, 4370), phosphorylated p70S6K (9206S) were purchased from Cell Signaling Technology. FoxO3 (648716) used for western blotting was purchased from R&D systems; Iba-1 (019-19741) was purchased from Wako pure chemical. The followings are reagents used: LPS from *Escherichia coli* 0111:B4 (L4391) was purchased from Sigma-Aldrich and diluted in PBS; Baf.A1, LY294002 (L9908) were purchased from Sigma-Aldrich. Rapamycin (BML-A275-0005) was purchased from Life Sciences Advanced Technologies, Inc. FITC-labeled amyloid β (15126) and PD98059 (9900S) were purchased from Cell Signaling Technology. Toll-like receptor ligands kit (Tlrl-kit 1mw) was purchased from Invivogen.

2.4 Plasmids and transfection

Constitutively active (CA) human HA-FoxO3 with triple mutations of T32A, S253A, and S315A (1788) were purchased from Addgene. FoxO3-CA was cloned into GFP-N1 to generate FoxO3-CA-GFP. For magnetofection experiment, DNA was mixed with Lipofectamin 2000 for 5 min. Nano-sized magnetic particles CombiMAG

(Chemicell) were then introduced to the mixture and incubated for additional 30 min at 37℃ for stabilization and efficient combination of magnetic particles with the DNA-Lipofectamin 2000 mixture. DNA-Lipofectamine 2000-magnetic particles mixture was then delivered into BV-2 cells. Transfection was performed in DMEM without FBS and antibiotics for 3 h. Then, medium was replaced with normal culture medium for 24 h and cells were re-seeded onto glass coverslips for immunocytochemistry experiments.

2.5 Immunocytochemistry

Primary microglia were fixed and permeabilized by absolute methanol. Cells were blocked with antibody diluent reagent solution (Thermo Fisher Scientific, 003218) for 30 min and incubated with the appropriate primary antibodies for 2 h at room temperature (RT), and then cells were rinsed three times with PBST (0.1% Tween-20 in PBS) and reacted with optimal fluorescent-conjugated secondary antibodies for 1 h at room temperature. Cell nuclei were stained with Hoechst 33342. Images were obtained using LSM700 confocal microscopy (Carl Zeiss).

2.6 LAP assay with zymosan

Primary mouse microglia were pretreated with LY294002 for 1 h and LPS was treated for 12 h. Cells were then incubated with zymosan (1 μg/ml for 2 h) or FITC-Aβ1-42 fibrils (0.3 μM for 6 h, 12 h and 24 h). After incubation, cells were fixed with absolute methanol at -20℃ for 20 min. The zymosan, LC3 and nucleus images were obtained

using LSM700 confocal microscopy (Carl Zeiss). Cells with LC3-positive phagosomes were counted using fluorescent microscopy (Carl Zeiss).

2.7 Proximity ligation assay (PLA)

FITC-conjugated $A\beta_{1-42}$ fibrils were prepared as previously described.[27] FITC-conjugated $A\beta_{1-42}$ fibrils were incubated with primary microglia for 2 h. After fixation and permeabilization with methanol, cells were incubated with mouse anti-amyloid-β and rabbit anti-LC3B antibodies. We then performed the incubation of the cells with a pair of PLA probes (Sigma, DUO92002 and DUO92004), probe ligation, amplification of signals (Sigma, DUO92007) and mounting (Sigma, DUO82040) according to manufacturer's instructions. Images of FITC-$A\beta_{1-42}$ fibrils and PLA were obtained by LSM700 confocal microscopy (Carl Zeiss). The intensity of FITC was analyzed using 'Coloc' of Zen software (Carl Zeiss). The number of PLA puncta were assessed using fluorescent microscopy (Carl Zeiss).

2.8 Western blotting

Cells were harvested and lysed in 1% Triton X-100 lysis buffer containing 250 mM sucrose, 50 mM NaCL, 20 mM Tris-HCl, 1mM EDTA with 1× protease and phosphate inhibitor cocktail (Thermo Fisher Scientific, 78440),

1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride for 15 min on ice. After incubation, centrifugation (16,100 × g, 10 min) was performed and each sample's lysate was collected. BCA protein assay reagents (Thermo Fisher Scientific, 23225) were used to measure protein concentration in the lysate. Prepared samples were loaded into the gel and electro-transferred to polyvinylidene difluoride membrane with a semi-dry electrophoretic transfer cell (Bio-Rad). Membranes were blocked with 5% non-fat dry milk powder dissolved in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature. The membranes were incubated with primary antibodies for overnight in 4℃ on a shaking incubator. The membranes were washed with TBST for 3 times, 10 min each. Peroxidase-conjugated secondary antibodies diluted in blocking solution was applied for 1 h at room temperature. After washing, proteins of interest were detected using either Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, 32106) or Western Bright ECL (Advansta, K-12045-D50).

2.9 Quantitative real-time polymerase chain reaction (qRT-PCR)

To assess change in the expression levels of autophagy genes after stimulation with LPS, cells were lysed using the QIAzol Lysis Reagent (Qiagen) and RNA was isolated following manufacturer's instructions (Qiagen). cDNA was synthesized using the ImProm-II Reverse Transcriptase kit (Promega) and oligo dT primers. qRT-PCR was performed on the RT product using Taq Polymerase (Invitrogen) and the primers of the examined genes were

listed in Table 1. Primers were mixed with TOPreal™ qPCR 2× PreMIX (SYBR Green with low ROX) (Enzynomics, Daejeon, Republic of Korea) and mRNA was quantified by qRT-PCR using the CFX96 Real-Time System (Bio-Rad). β-actin was used as the reference gene for normalization.

Gene	Gene reference number	5`-Sense primer sequence-3`	5`-Antisense primer sequence-3`
Map1LC3B	026160.4	GATAATCAGACGGCGCTT	ACTTCGGAGATGGGAGTG
β-actin	007393.5	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT
Atg3	026402.3	ACACGGTGAAGGGAAAGGC	TGGTGGACTAAGTGATCTCCAG
Atg4a	017318603.1	CGTGGTATGGATTCTGGGGAA	TGGGTTGTTCTTTTTGTCTCTCC
Atg4b	006529793.2	CGGCACTTAGGTCGAGATTGG	ACTCCCATTTGCGCTATCTGA
Atg4c	006503073.3	GATGAAAGCAAGATGTTGCCTG	TCTTCCCTGTAGGTCAGCCAT
Atg4d	017313301.1	AGGGGACAAACCCGTATCC	CCATACTTGACGTTGTTCCAGG
Atg5	017313783.1	TGTGCTTCGAGATGTGTGGTT	ACCAACGTCAAATAGCTGACTC
Beclin1	019584.3	ATGGAGGGGTCTAAGGCGTC	TGGGCTGTGGTAAGTAATGGA
Atg7	006506710.3	TCTGGGAAGCCATAAAGTCAGG	GCGAAGGTCAGGAGCAGAA
Atg9a	011238690.2	TTCTGAAGTGACGAGACCTGC	AGCCTCGGCTTATAGCACTCA
Atg10	011244552.2	TTCTGAAGTACGAGACCTGC	AGCCTCGGCTTATAGCACTCA
Atg12	026217.3	TGAATCAGTCCTTTGCCCCT	CATGCCTGGGATTTGCAGT
Atg13	017319122.2	CCAGGCTCGACTTGGAGAAAA	AGATTTCCACACACATAGATCGC
Atg14	021181632.1	GAGGGCCTTTACGTGGCTG	AATAGACGAAATCACCGCTCTG
Atg16l1	021155819.1	GCCCAGTTGAGGATCAAACAC	CTGCTGCATTTGGTTGTTCAG

Table 1. Primer sequences for qRT-PCR

2.10 Statistical analysis

At least three independent experiments were performed for each condition of experiments and data were presented as mean ± standard deviation (SD) values. Statistical analysis was performed by either unpaired Student's t-test or one-way analysis of variance (ANOVA) and the statistical significance was obtained using Graphpad Prism (GraphPad Software, San Diego, CA, USA)

3. Results

3.1 Autophagy is suppressed by LPS in microglia

Inducing autophagy by inflammation, especially TLRs' activation, is well known in macrophages [28]. Therefore, we asked whether stimulation by TLRs can also affect autophagy activity in microglia. We treated in primary microglia Pam3CSK4 (Pam N-palmitoyl-S-dipalmitoylglyceryl Cys-Ser-$(Lys)_4$), poly I:C (polyinosinic-polycytidylic acid), LPS (Lipopolysaccharide), and ssRNA (viral single-stranded RNA), the ligands to TLR1/2, 3, 4 and 7, respectively. Autophagy flux was generally measured using levels of microtubule-associated protein 1 light chain 3 (MAP1LC3/LC3), marker for autophagosome formation. Interestingly, Ligands of TLRs significantly decreased autophagy flux in primary microglia. And blocking autolysome formation using Bafilomycin A1 (Baf.A1), a selective inhibitor of vacuolar H^+-ATPase, showed suppression of autophagy flux (Fig.2A). To focus studying the detailed mechanisms, we selected LPS, a TLR4 ligand and prototypical potent immune stimulus. Then, microglia and macrophages were treated with LPS in dose and time-dependent manners and lysosomal inhibitor Baf.A1 was added 2 h before sampling to block autophagy flux. LPS suppressed autophagy flux in both primary mouse microglia and BV-2 in time- and dose-dependent manner (Fig. 2B, C and F). Conversely, LPS induced autophagy flux in macrophages, BMDM and Raw264.7 cells (Fig. 2D and E). To confirm whether the suppression of autophagy flux in microglia was specific to LPS treatment, we gave nutrient starvation to microglia.

Microglia was starved with DMEM media without serum during 2, 4, 16 hours and treated with Baf.A1 2 h before sampling. Autophagy flux following nutrient starvation was not impaired in microglia (Fig. 2G), indicating that autophagy suppression was specific to LPS treatment in microglia.

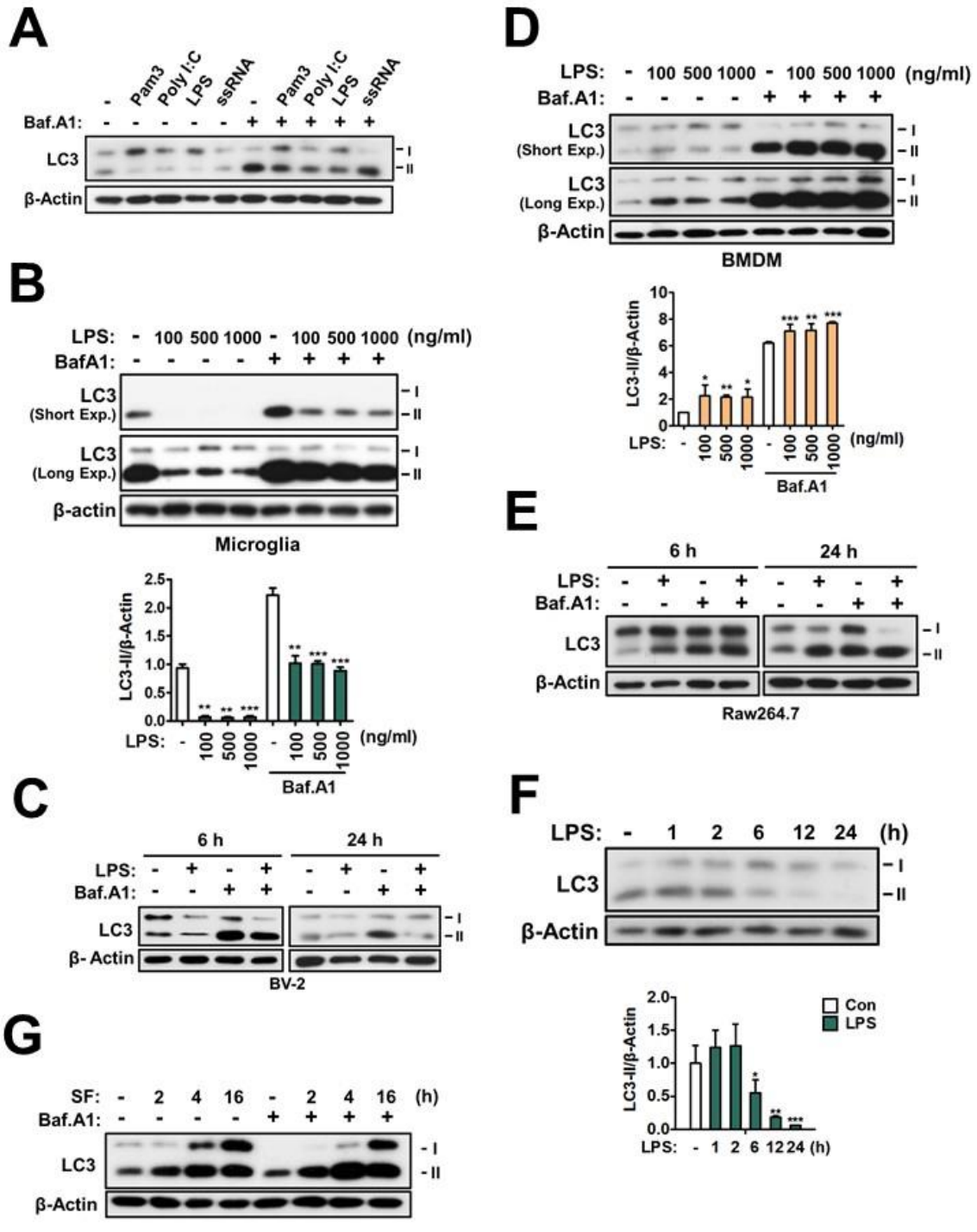

A
- Pam3 Poly I:C LPS ssRNA - Pam3 Poly I:C LPS ssRNA
Baf.A1: - - - - - + + + + +
LC3
β-Actin
I
II
B
LPS: - 100 500 1000 - 100 500 1000 (ng/ml)
BafA1: - - - - + + + +
LC3 (Short Exp.)
LC3 (Long Exp.)
β-actin
Microglia
LC3-II/β-Actin
LPS: - 100 500 1000 - 100 500 1000 (ng/ml)
Baf.A1
C
6 h
24 h
LPS: - + - + - + - +
Baf.A1: - - + + - - + +
LC3
β- Actin
BV-2
D
LPS: - 100 500 1000 - 100 500 1000 (ng/ml)
Baf.A1: - - - - + + + +
LC3 (Short Exp.)
LC3 (Long Exp.)
β-Actin
BMDM
LC3-II/β-Actin
LPS: - 100 500 1000 - 100 500 1000 (ng/ml)
Baf.A1
E
6 h
24 h
LPS: - + - + - + - +
Baf.A1: - - + + - - + +
LC3
β-Actin
Raw264.7
F
LPS: - 1 2 6 12 24 (h)
LC3
β-Actin
Con
LPS
LC3-II/β-Actin
LPS: - 1 2 6 12 24 (h)
G
SF: - 2 4 16 - 2 4 16 (h)
Baf.A1: - - - - + + + +
LC3
β-Actin

Figure 2. Autophagy is suppressed by LPS in microglia.

(A) Representative Western blots of primary microglia treated with various TLR ligands; Pam3CSK4 (100 ng/ml) for TLR1/2, Poly I:C (HMW) (1 μg/ml) for TLR3, LPS (1 μg/ml) for TLR4, and ssRNA (1 μg/ml) for TLR7. (B) Primary microglia were treated with different dosages of LPS (100, 500, 1000 ng/ml) for 24 h. Baf.A1 treatment confirmed LPS-induced autophagy suppression. Graph, quantification of LC3-II after normalization to β-actin (n=3). (C) BV-2 cells treated with LPS (1 μg/mL) for 6 and 24 h. (D) BMDM were treated with different dosages of LPS (100, 500, 1000 ng/ml) for 24 h. Graph, quantification of LC3-II after normalization to β-actin (n=3). (E) Raw264.7 macrophages treated with LPS (1 μg/mL) for 6 and 24 h. (F) Primary microglia were treated with LPS (1 μg/mL) for 1, 2, 6, 12, and 24 h. Graph, quantification of LC3-II after normalization to β-actin (n=4). (G) Cells were starved with DMEM without serum (SF) for 2, 4, 16 hours. In all experiments, Baf.A1 (20 nM) was added 2 h before sampling. All data are presented as mean±SEM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to the LPS-untreated control.

3.2 LPS decreases the mRNA expression of autophagy-related genes (*Atg*) in microglia

To determine whether autophagy suppression is due to a decrease in the expression levels of autophagy-related genes *(Atg*) in LPS-treated microglia, we measured the mRNA expression levels of LC3 and various *Atg* genes in microglia and macrophages. *Atg* gene series including LC3b, *Atg*3, *Atg*4a-d, *Atg*5, Beclin1, *Atg*7, *Atg*12, *Atg*13, *Atg*14, and *Atg*16l1 were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). LPS significantly decreased representative *Atg* genes at 24 hours after LPS treatment (Fig. 3A). Next, we compared the changes in the expression of *Atg* genes between microglia and macrophages following LPS treatment. In contrast to microglia, *Atg* genes were not reduced in BMDMs for 24 hours (Fig. 3B). To confirm whether these result in primary mouse cell culture was also observed in immune cell types under more physiological condition, we performed acute isolation of microglia and peritoneal macrophages from LPS-i.p.-injected mice. mRNA expression level of cytokines indicated successful stimulation following LPS in both acutely isolated microglia and peritoneal macrophages (data is not shown). Acutely isolated microglia suppressed in all *Atg* genes, while acutely isolated peritoneal macrophages showed overall upregulation of *Atg* genes (Fig. 3C). These all results indicate LPS also transcriptionally suppresses autophagy in microglia but induces autophagy in their macrophage following same conditions.

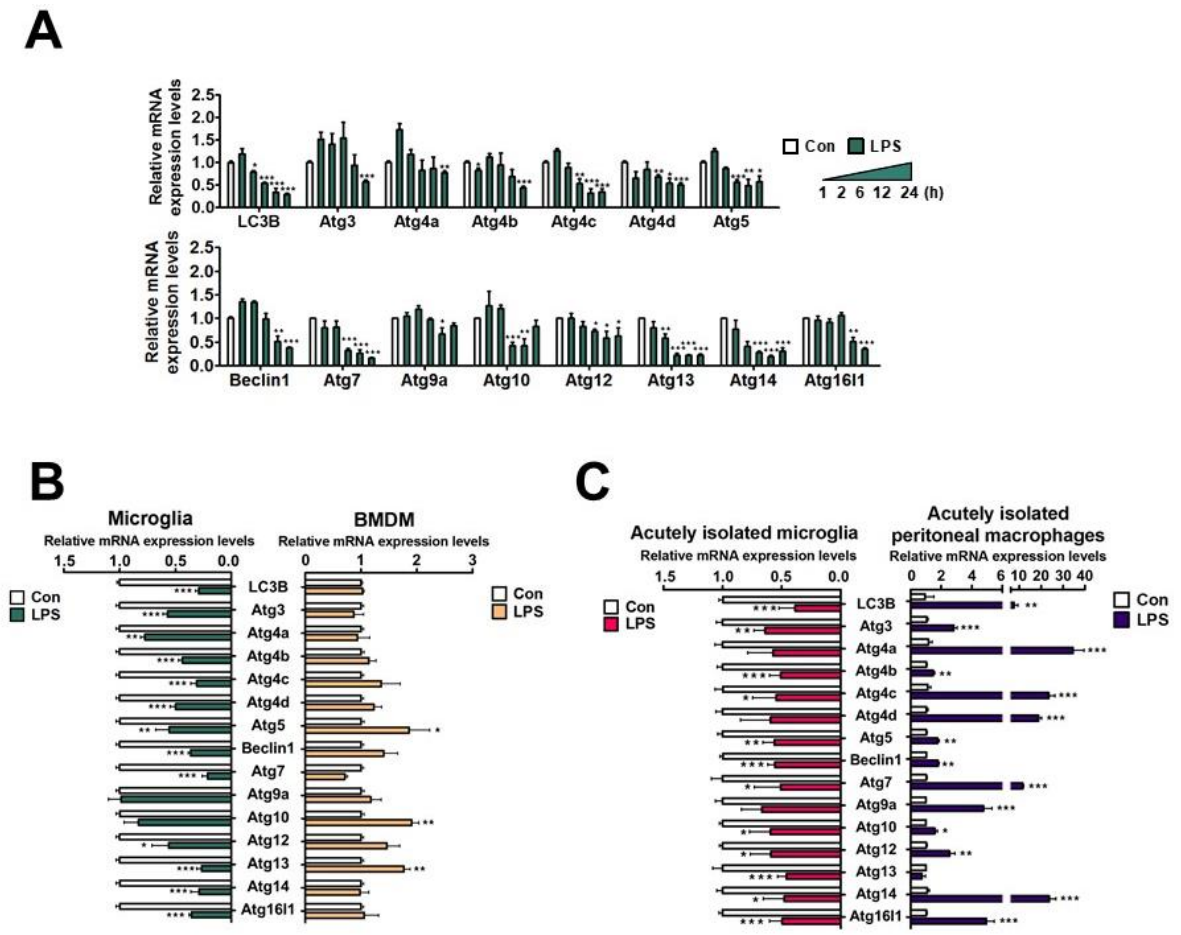


Figure 3. LPS decreases the expression of *Atg* genes in microglia.

(A) Time course analysis of the mRNA levels of LC3B and other *Atg* genes were performed by qRT-PCR following LPS treatment (n=3). (B) Comparison of the relative mRNA expression levels of the *Atg* genes between primary microglia (n=5) and BMDM (n=4) 24 h after LPS (1 μg/mL) treatment. (C) Comparison of relative mRNA expression levels of *Atg* genes between microglia and peritoneal macrophages acutely isolated from the same LPS-injected mice (5 μg/kg, i.p.). Microglia and peritoneal macrophages were acutely isolated 24 h post-injection of LPS (n=3). In all experiments, mRNA levels were normalized to β-Actin. All data are presented as mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 compared to the control (Con).

3.3 LPS-induced autophagic suppression is mediated by TLR4-dependent signaling

We wonder whether the autophagic suppression by LPS in microglia was mediated TLR4-dependent immune response. Microglia from TLR4$^{-/-}$ mice were stimulated by LPS. *Tlr4* was successfully knocked-out as shown in Figure 4A (data from Seoul National University of Dentistry). TLR4 deficient microglia preserved a level of autophagy flux despite LPS treatment (Fig. 4B). In addition, LPS treatment did not affect the expression level of all *Atg* genes in TLR4$^{-/-}$ microglia (Fig. 4C). Thus, LPS-induced autophagic suppression was obviously dependent TLR4-mediated inflammatory signaling.

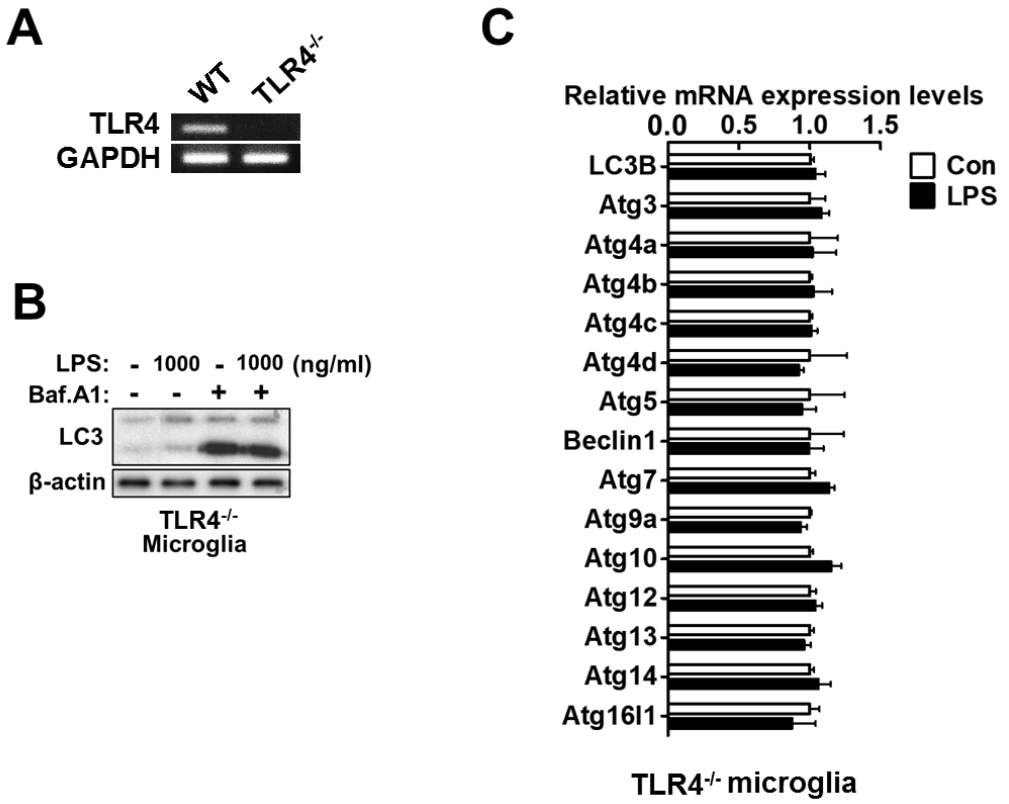
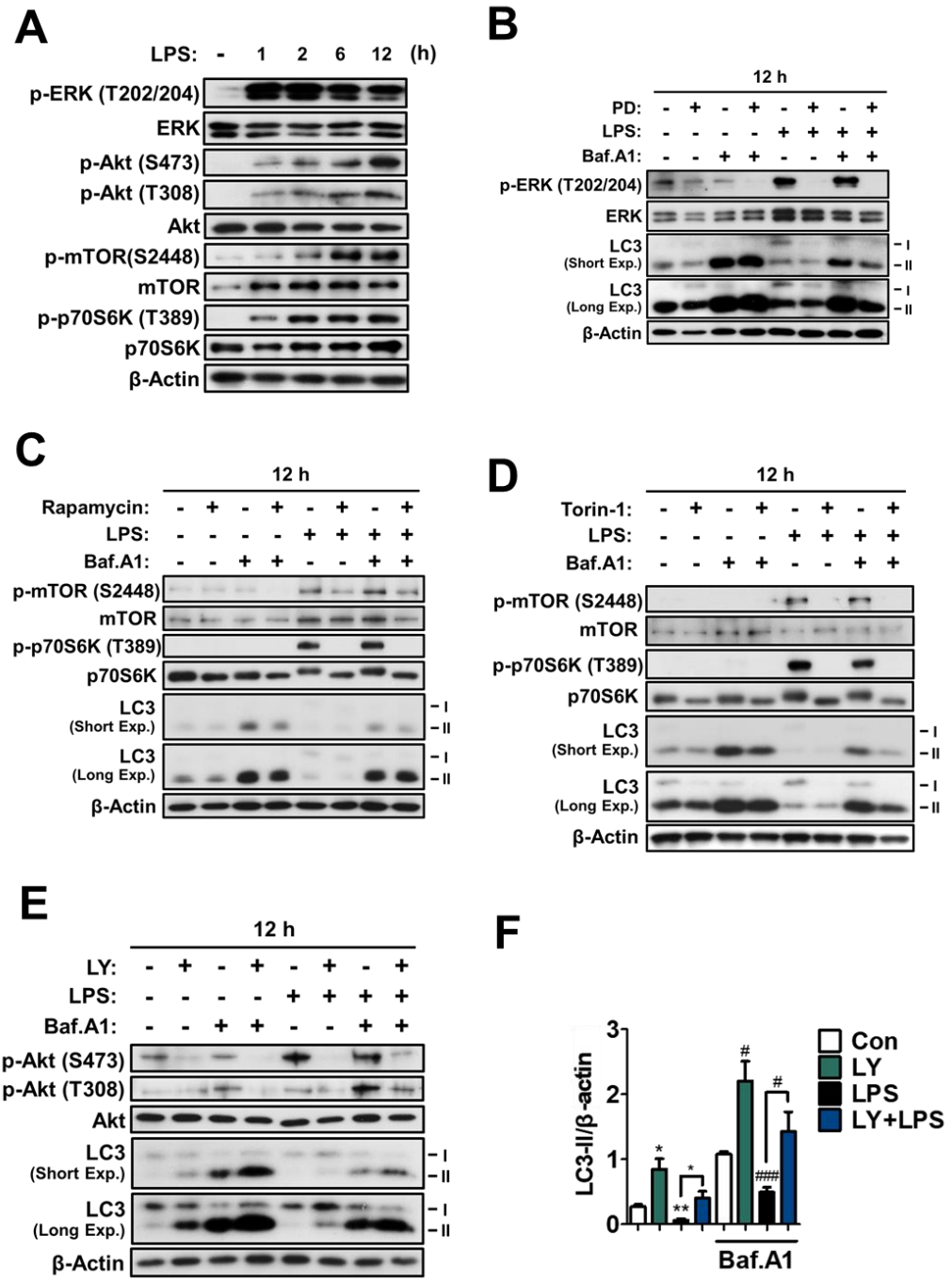


Figure 4. LPS-induced autophagic suppression is mediated by TLR4-dependent signaling in microglia.

(A) PCR using Tlr4 primers in *Tlr4*$^{-/-}$ mice compared with Wild Type (WT). (B) Treatment of LPS (1 μg/mL) for 24 in microglia isolated from *Tlr4*$^{-/-}$ *mice.* Baf.A1 (20 nM) was added 2 h before sampling. (C) mRNA expression levels of *Atg* genes after treatment of LPS (1 μg/mL) for 24 h compared to vehicle-treated wild-type microglia.

3.4 LPS down-regulates autophagy via PI3K/Akt signaling pathway

We monitored well-known upstream signaling kinases of autophagy, which are involved in inflammatory signaling, to examine whether down-regulation of autophagy genes is due to a decrease in autophagy initiation signaling. We focused on the PI3K/Akt, mTOR/p70S6K and MEK/EKR as these kinases were well known as major negative regulator of autophagy and they were activated by LPS treated microglia. LPS increased the phosphorylation of Akt (S473, T308), ERK (T202/204), mTOR (S2448) and p70S6K (T389) time-dependently (Fig. 5A). Thus, we tested whether these kinases are affected autophagic suppression in LPS-treated microglia using inhibitor of each kinase. Treatment of PD98059 (PD) as an inhibitor of MEK did not recover the suppression of autophagy by LPS (Fig. 5B). We also treated Rapamycin and Torin-1 as well-known inhibitors of mTOR to LPS-induced microglia. mTOR inhibitors could reduce the level of mTOR and p70S6K but failed to reverse the suppression of autophagy flux by LPS (Fig. 5C and D). Therefore, we examined mTOR signaling is not a key regulator of autophagy following our experimental conditions. Conversely, PI3K inhibitor, LY294002 (LY), could dephosphorylate Akt at both serine 473 and threonine 308 and remarkably reversed the LC3-II level as similar as control group (Fig. 5D). Collectively, these results suggest that PI3K/Akt signaling pathway is major player in LPS-induced suppression of microglia autophagy.


A
LPS: - 1 2 6 12 (h)
p-ERK (T202/204)
ERK
p-Akt (S473)
p-Akt (T308)
Akt
p-mTOR(S2448)
mTOR
p-p70S6K (T389)
p70S6K
β-Actin
B
12 h
PD:
LPS:
Baf.A1:
p-ERK (T202/204)
ERK
LC3 (Short Exp.)
LC3 (Long Exp.)
β-Actin
I
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12 h
Rapamycin:
LPS:
Baf.A1:
p-mTOR (S2448)
mTOR
p-p70S6K (T389)
p70S6K
LC3 (Short Exp.)
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LPS:
Baf.A1:
p-mTOR (S2448)
mTOR
p-p70S6K (T389)
p70S6K
LC3 (Short Exp.)
LC3 (Long Exp.)
β-Actin
E
12 h
LY:
LPS:
Baf.A1:
p-Akt (S473)
p-Akt (T308)
Akt
LC3 (Short Exp.)
LC3 (Long Exp.)
β-Actin
F
LC3-II/β-actin
Con
LY
LPS
LY+LPS
Baf.A1

Figure 5. LPS suppresses autophagy via PI3K/Akt signaling pathway in primary microglia.

(A) Phosphorylation of Akt (S308, S473), mTOR (S2448), ERK (T202/204) and p70S6K (T389) in primary microglia time-dependently following LPS treatment (1 μg/mL). (B, C and D) Inhibition of MEK with PD98059 (PD, 50 μM) (B) or mTOR with rapamycin (1 μM). (C) or Torin-1 (500 nM). (D) in primary microglia treated with LPS (1 μg/mL) for 12 h. (E) Inhibition of PI3K with LY294002 (LY, 20 μM) in primary microglia treated with LPS (1 μg/mL). (F) Graph, quantification of LC3-II after normalization to β-actin (n=4). In all experiments, blots shown are representative of at least three experiments with similar results. All inhibitors were pretreated 1 h prior to LPS treatment. All data are presented as mean± SEM. *$p<0.05$, **$p<0.01$, #$p<0.05$ and ###$p<0.001$ compared to control.

3.5 Phosphorylation of FoxO3 by PI3K/Akt suppresses autophagy in microglia

We examined which key autophagy transcription factors are related to downregulation of autophagy related genes and are associated with PI3K/Akt signaling pathway that suppressed autophagy flux in LPS-induced microglia. We chose one of Akt downstream factors, FoxO3, is a good target for autophagic suppression by TLR4/PI3K/Akt since FoxO3 transcription factor is already well-known for positively regulating LC3b and several Atg genes under various conditions in some cell types [29]. Akt negatively regulated FoxO3 activation and nuclear translocation of FoxO3 is induced [30]. Unsurprisingly, LPS induced FoxO3 phosphorylation at serine 253 in a time-dependent manner (Fig. 6A). In addition, LY treatment dephosphorylated FoxO3 for 6, 12 hours (Fig. 6B). Next, we observed the nuclear translocation of FoxO3 in LPS-treated microglia in contrast control group following LY treatment conditions using an antibody specific to FoxO3. 4 different layers in a single cell were taken and intensity of FoxO3 were measured using Zen software (Fig. 6C and D). In microglia, FoxO3 was encouraged to export to cytosol by LPS-induced FoxO3 phosphorylation. However, PI3K inhibition by LY treatment could recover FoxO3 nuclear retention in LPS-treated microglia (Fig. 6C) and could recover the expression levels of several autophagy related genes sufficiently (Fig. 6E). To examine how the activation of PI3K/Akt signaling pathway and subsequent FoxO3 phosphorylation caused suppression of autophagy in only microglia, not in macrophages, we observed different pattern of nuclear translocation of FoxO3 in macrophages. In macrophages, FoxO3 was largely localized in nuclear in basal state and LPS treatment increased nuclear FoxO3 conversely (Fig. 6D).

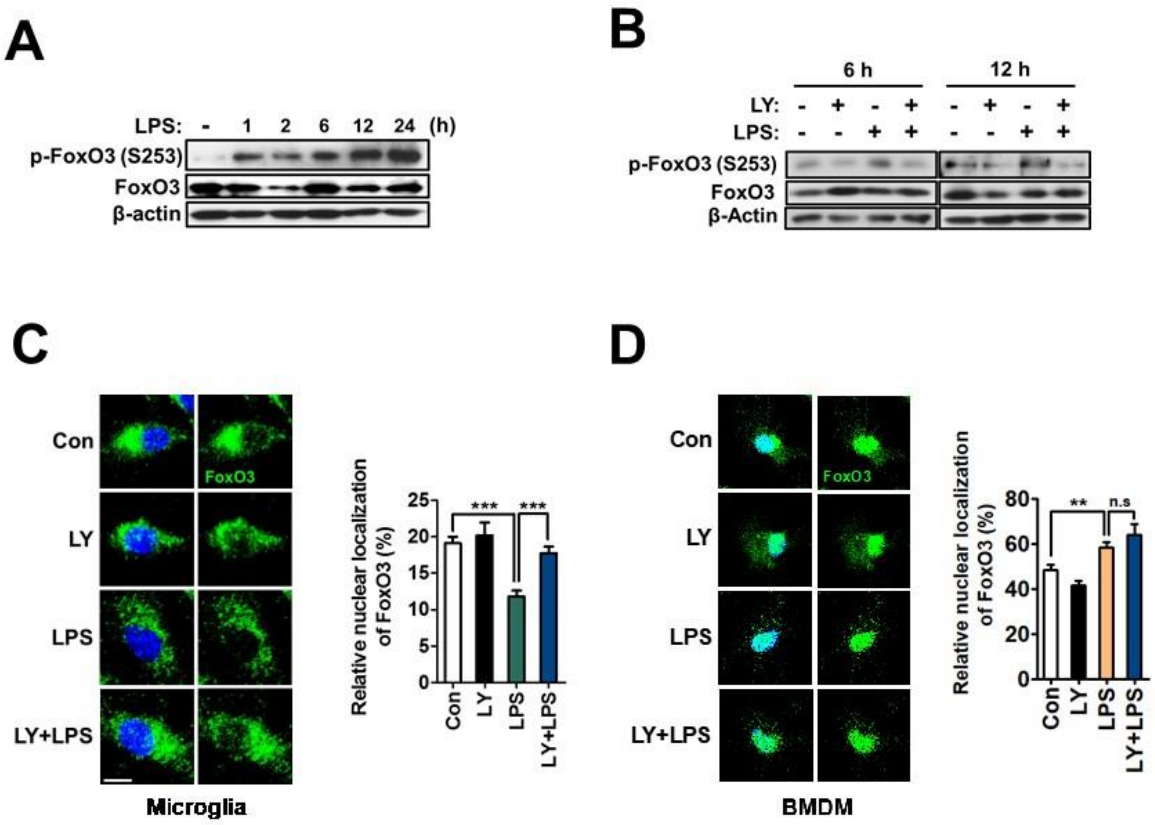
A
LPS: - 1 2 6 12 24 (h)
p-FoxO3 (S253)
FoxO3
β-actin
B
6 h
12 h
LY: - + - + - + - +
LPS: - - + + - - + +
p-FoxO3 (S253)
FoxO3
β-Actin
C
Con
LY
LPS
LY+LPS
FoxO3
Microglia
Relative nuclear localization of FoxO3 (%)
***
***
Con
LY
LPS
LY+LPS
D
Con
LY
LPS
LY+LPS
FoxO3
BMDM
Relative nuclear localization of FoxO3 (%)
**
n.s
Con
LY
LPS
LY+LPS
E
Relative mRNA expression levels
LC3B
Atg3
Atg4a
Atg4b
Atg4c
Atg4d
Atg5
Beclin1
Atg7
Atg9a
Atg10
Atg12
Atg13
Atg14
Atg16l1
Con
LY
LPS
LY+LPS

Figure 6. Phosphorylation of FoxO3 by PI3K/Akt suppresses autophagy in microglia.

(A) Phosphorylation of FoxO3 (S253) in primary microglia following LPS treatment (1 μg/mL). (B) Phosphorylation of FoxO3 (S253) by PI3K inhibitors, LY294002 (LY, 20 μM) for 6 and 12 h. Representative blots are shown with similar results of three experiments. (C and D) Nuclear translocation of FoxO3 by LPS and inhibition of PI3K in microglia and BMDM respectively. Endogenous FoxO3 (green) protein was visualized by immunocytochemisty using LSM700 confocal microscopy (Carl Zeiss). Scale bars, 10 μm. Graph, quantitative analysis of FoxO3 nuclear localization (n=3). (D) Expressions of Atg genes during PI3K inhibition by LY (20 μM) for 12 h in LPS-treated microglia. Relative mRNA expression levels of Atg genes were quantified by qRT-PCR after normalization to β-Actin (n=3). All data are presented as mean± SEM. ns, non-significant. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to control.

To establish the certain role of FoxO3 in microglial autophagy, we overexpressed the constitutively active form (CA) of mutant FoxO3 in microglia. CA form of FoxO3 has 3 mutated sites at T32A, S253A and S315A and these mutated sites blocked the phosphorylation of FoxO3 by Akt. FoxO3-CA-GFP expression showed largely localization to nucleus in microglia (Fig. 7A). Endogenous LC3 puncta number were increased in FoxO3-CA-expressing microglia that examined autophagy flux recovery in the presence or absence of LPS (Fig. 7B). Taken together, these results suggest that PI3K/Akt activation by TLR triggers and subsequent inhibition of FoxO3 are distinct major mechanisms for suppression of autophagy under inflammatory-induced microglia.

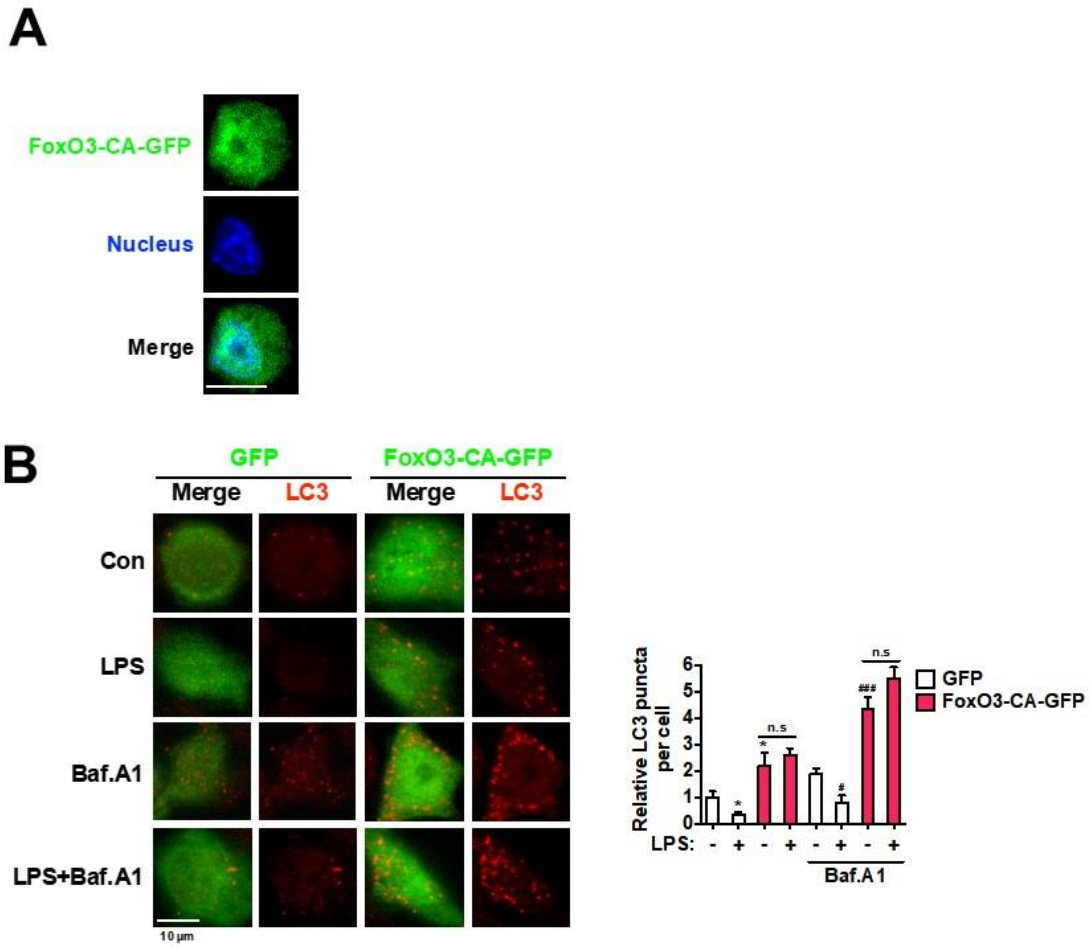

Figure 7. Overexpression of FoxO3-CA increases LC3 puncta in the presence or absence of LPS.

(A) Overexpression of FoxO3-CA-GFP in the nucleus in BV-2 cells. (B) LPS (1 μg/mL) was treated for 12 h and visualization of LC3 puncta (red) was carried out by immunocytochemistry with an antibody against the endogenous LC3. Scale bar, 10 μm. Graph, quantification of the puncta from at least 30 cells per condition, using LSM780 confocal microscopy (Carl Zeiss). Baf.A1 (20 nM) was added 2 h before sampling. All data are presented as mean±SEM. ns, non-significant. *$p<0.05$, #$p<0.05$ and ###$p<0.001$ compared to the control group.

3.6 LPS suppresses LC3-associated phagocytosis (LAP) and Aβ degradation

Phagocytosis is the process which internalize and degrade particles such as microorganisms. As important role of microglia in brain is these phagocytic function, we estimated the phagocytic degradation in LPS-treated microglia for physiological function of autophagic suppression. Autophagy of immune cells was significantly associated with phagocytic degradation following TLR stimulated conditions [31, 32]. We analyzed LC3-associated phagocytosis (LAP) in microglia using zymosan. Zymosan is a glucan particle from microorganisms and generally used for studying phagocytosis in immune cells [33, 34]. Interestingly, 15 % of zymosan entered LC3-positive phagosomes by LPS compared with basal control group. Subsequently, LY treatment enlarged the rates of zymosan surrounded by LC3-positive phagosomes in LPS-treated microglia (Fig. 8A). Furthermore, we confirmed whether the reduction of the number of LC3-positve phagosomes was by autophagic suppression. We demonstrate knockdown of Atg7 in microglia did not affect any changes of the number of LC3-positve phagosomes even LPS and LY treatment (Fig. 8B).

Next, we investigate pathological meaning in our experimental conditions, we measured the association between LC3 and fibrillary Amyloid beta (Aβ) using proximity ligation assay (PLA). PLA was directly showed the association of LC3 and ingested FITC-tagged fibrillary Aβ which indicated in figure 8C with red fluorescent signal. LPS treatment significantly decreased the red PLA signals, but LY treatment could increase the amount of LC3

associated to fibrillary Aβ (Fig. 8C). Thus, we hypothesized that LPS treatment suppressed LC3 association and caused accumulation of fibrillary Aβ after any time. As predictably, fibrillary Aβ were accumulated in LPS-induced microglia for 24 hours, while degradation of fibrillary Aβ were normal in control group and LY treated group in LPS-treated microglia (Fig. 8D). Collectively, PI3K/Akt signaling pathway activated by LPS suppressed LC3-associated phagocytic degradation via decrease of autophagy flux and transcriptional levels.

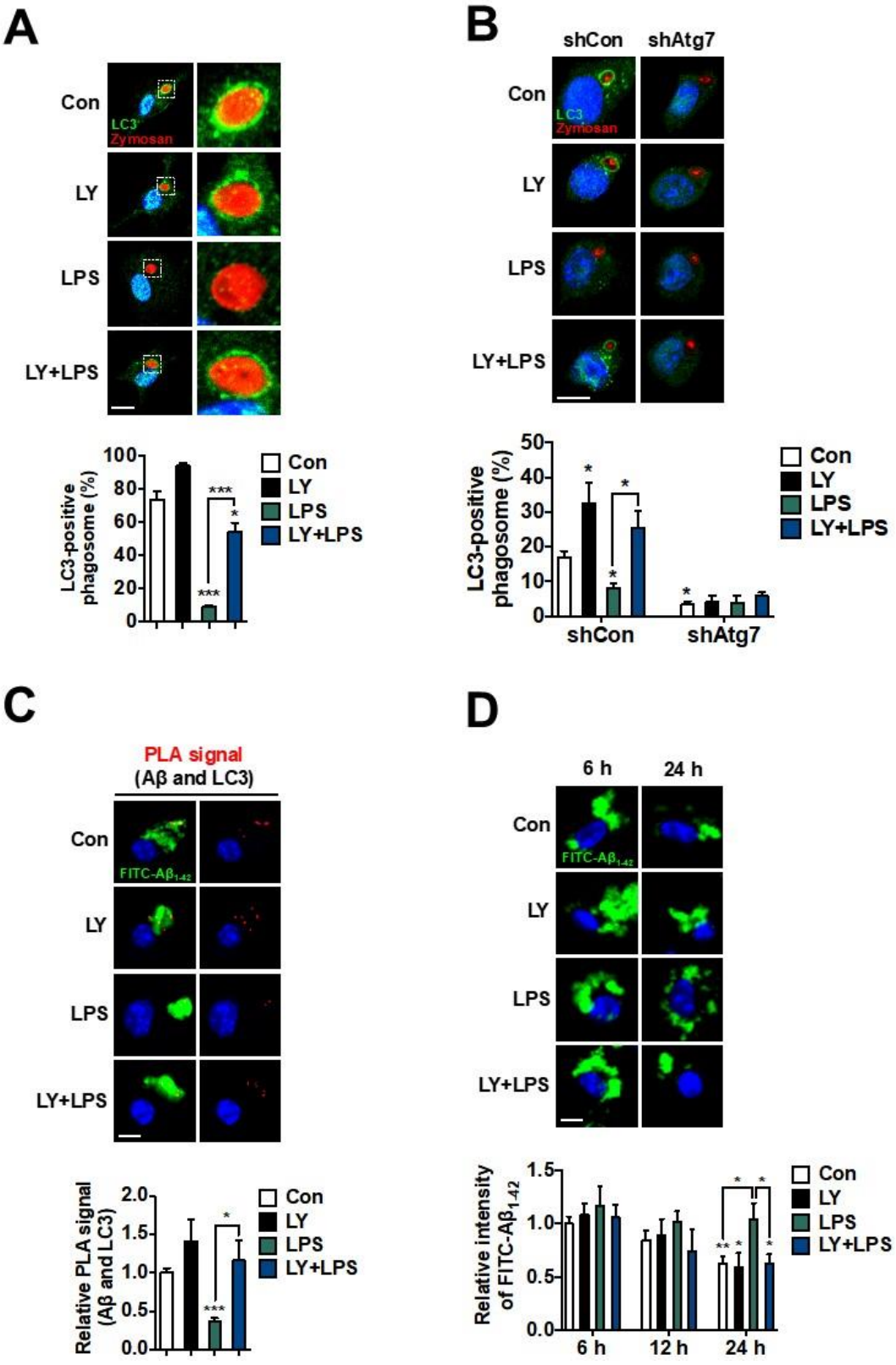
A
Con
LY
LPS
LY+LPS
LC3
Zymosan
LC3-positive phagosome (%)
Con
LY
LPS
LY+LPS
B
shCon
shAtg7
C
PLA signal
(Aβ and LC3)
FITC-Aβ1-42
Relative PLA signal (Aβ and LC3)
D
6 h
24 h
Relative intensity of FITC-Aβ1-42
12 h

Figure 8. LPS suppresses LC3-associated phagocytosis (LAP) and Aβ degradation via PI3K/Akt signaling pathway in primary microglia.

(A and B) Primary microglia and Atg7-knockdown BV-2 were stimulated with LPS for 12 h and then incubated with zymosan for another 2 h. LY was pretreated 1 h prior to LPS. Immunocytochemistry images show anti-LC3 (green), zymosan (red) and nucleus (blue). Graph, quantitative analysis of LC3-positive phagosomes from three independent experiments (n=45~113). (C) PLA signal between LC3 and Aβ in primary microglia treated with LPS (1 μg/mL) for 12 h and pretreatment with LY (20 μM) for 1 h. (D) For this experiment, FITC-conjugated fibril $A\beta_{1-42}$ was added to the cells for 6, 12 or 24 h and the fluorescent intensity of the remaining FITC- $A\beta_{1-42}$ was measured using fluorescence microscope (Carl Zeiss). Graph, quantification of Aβ fibril intensities from three independent experiments (n=82~106). All data are presented as mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 compared to control.

4. Discussion

In this study, we delineated that autophagy in microglia is suppressed by pro-inflammatory triggers. More importantly, we found distinct mechanisms of microglial autophagy suppression by LPS, in opposite of macrophages. Our novel finding was that LPS-induced suppression of microglial autophagy was mainly through the activation of PI3K/Akt pathway and following inactivation of FoxO3 transcription factor. Furthermore, suppression of LPS-treated microglial autophagy was related to impaired phagocytic degradation as shown in LC3-associated phagocytosis (LAP) and amyloid β (Aβ) degradation (Fig. 9).

Accumulating evidence indicates that TLR signaling and related pathways in immune system can induced autophagy in macrophages. Autophagy functions have been well studied in peripheral immune cells including control of inflammation and degrading useless organelles [7]. Corresponding with macrophages evidences, the role of autophagy in microglia have been studied in neuroinflammatory diseases [8]. However, detailed mechanisms between microglial autophagy and neuroinflammation of microglia is still less understood.

Our study revealed that suppression of autophagy flux or transcriptional levels in LPS-induced microglia was caused by activation of TLR4, subsequent PI3K/Akt pathway and transcriptional suppressor of FoxO3. Autophagy suppression dependent TLR4 activation impaired LC3-associated phagocytic functions and Amyloid beta clearance in microglia. The impaired autophagy deeply is associated with neurodegenerative diseases.

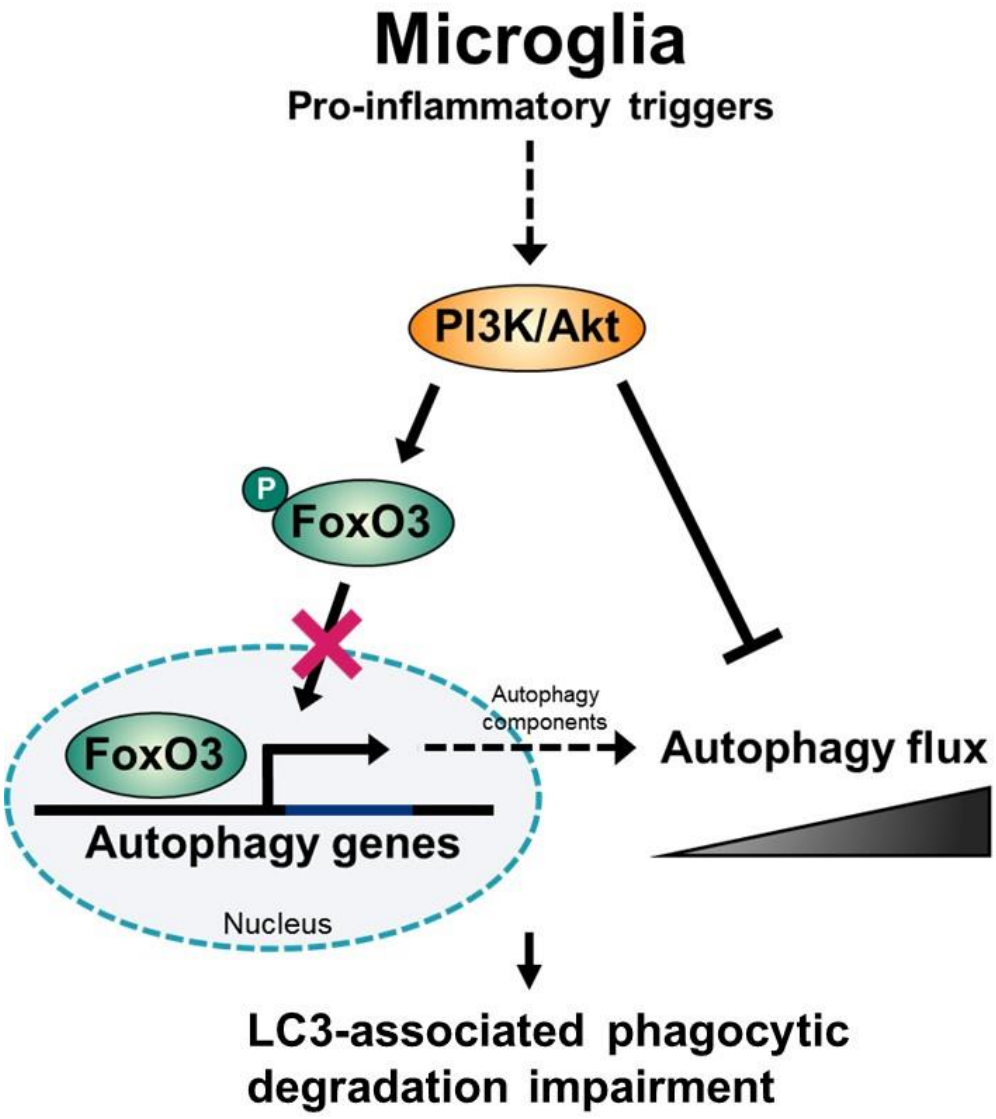

Figure 9. Schematic diagram of distinct microglial autophagy related inflammatory signaling pathway under pro-inflammatory conditions.

Distinct mechanisms that suppression autophagy in LPS-induced microglia was through down-regulation of TLR4/PI3K/FoxO3 and impaired the LC3-associated phagosomes formation and degradation of Amyloid β. Furthermore, it is well-known that autolysosome impairment in autophagy flux in Alzheimer's disease brain induced amyloid beta aggregation and phagocytic function in microglia impaired in AD model for amyloid beta clearance [35, 36]. Thus, we examined that activation of TLR4/PI3K signaling leads to impairment of amyloid beta degradation through LAP in microglia. This evidence has important implication for treatment neurodegenerative disease such as Alzheimer's disease.

Phosphoinositide 3-kinase (PI3K) is a lipid kinase that phosphorylates phosphatidylinositol (PI). There are three classes in mammalian cells; class I PI3K produces $PI(3,4,5)P_3$, class III PI3K/Vps34 produces PI3P. Class I PI3K is mainly activated by growth factors, leading to Akt phosphorylation. The activated Akt stimulates mTORC1 and subsequently suppression of autophagy. Whereas the class III PI3K/Vps34 is well-known as a positive regulator of autophagy and it plays role in vesicular trafficking in the endosomal/lysosomal system [37-39]. Therefore, it is necessary to investigate how the difference between the two classes influence autophagy suppression by LPS. This point makes the mechanism of PI3K by LPS stimulation more detailed. In our study, we observed suppression of microglial autophagy by LPS is through class I PI3K/Akt/FoxO3. And we would confirm the effect of class III PI3K by measuring the level of PtdIns, which is substrate of class III PI3K using

lipid mass technologies. Additionally, we could observe the different mechanisms of PI3K with other macrophages.

Besides, it is still remains whether our findings *in vitro* experimental conditions are consistent *in vivo* microglial autophagy regulations. *In vivo* experiment, we would observe that autophagy suppression in microglia from inflammation-induced mice and whether phagocytic degrading function is also impaired. We could perform direct fibrillary amyloid beta injection to hippocampus of mouse brain and confirm the impairment of clearance function of them from suppression of autophagy flux or transcriptional levels by TLR4/PI3K/FoxO3 axis.

To sum up, our interest findings of distinct mechanisms that suppression autophagy in LPS-induced microglia was through down-regulation of TLR4/PI3K/FoxO3 and impaired the LC3-associated phagosomes formation and degradation of Amyloid β. Collectively, our findings of the microglial autophagy related inflammatory signaling pathway under LPS-treated conditions will provide new insight into pathogenic mechanisms of neurodegenerative diseases and their treatment.

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요 약 문

미세아교세포에서 TLR4/PI3K/Fox03 기전을 통한 자가포식작용 억제와 식작용을 통한 분해 능력 저해

미세아교세포는 중추신경계에서 면역을 담당하는 세포이며, 말초신경계의 대식세포와 비슷한 역할을 수행한다. 대식세포와 마찬가지로 미세아교세포에서도 톨유사수용체의 리간드 등에 의해서 신경염증반응이 수반된다. 기존 연구에서 다양한 신경염증반응 유도는 대식세포에서 자가포식작용을 활성화한다고 알려져 있다.

자가포식작용은 세포 내에 불필요한 단백질이나 세포소기관을 분해하는 체내 항상성 유지를 위한 필수적인 과정이다. 최근 많은 연구에서는 자가포식작용은 선천적 면역 체계에서 사이토카인 분비, 염증조절복합체 조절, 항원제시작용, 병원균 제거 등의 중요한 역할을 하는 것으로 알려졌다. 또한, 선천적 면역 체계에서 자가포식작용에 결함이 생기면 다양한 염증성 질환과 퇴행성 질병에 연관되어 있다고 알려져 있어 자가포식작용과 면역체계 사이의 중요성은 대식세포를 이용한 연구가 활발히 이루어지고 있다. 하지만 미세아교세포에서 면역반응과 자가포식작용 사이의 자세한 기전과 역할은 명확히 밝혀지지 않고 있다.

본 연구는 미세아교세포가 말초신경계의 대식세포와 반대로 신경염증반응 유도 시 자가포식작용이 억제되는 것을 발견하였고, 그에 따른 자세한 기전과 역할을 밝히고 있다. 신경염증반응을 유도하는 톨유사수용체 4 의 리간드인 지질다당류 (LPS)는 PI3K/Akt 인산화효소를 활성화하고 Fox03 전사인자를 조절하여 자가포식작용을 억제함을 증명하였다. 이어서 자가포식작용의 억제는 미세아교세포의 식작용을 통한 분해능력을 저해시킴을 아밀로이드 베타를 이용해 증명하고 있다. 따라서, 본 연구는 미세아교세포의 자가포식작용 조절에 대한 기전을 밝히고, 증명한 미세아교세포 특이적인 기전과 역할은 알츠하이머의 발병 원인을 찾고 표적 치료를 하는 데 도움이 될 수 있으며 나아가 향후 다양한 신경염증성 질환 또는 신경 퇴행성 질병의 치료법에 기여할 가능성을 보여주고 있다.

핵심어: 미세아교세포, 자가포식작용, 신경염증반응, PI3K/Fox03, 식작용