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ORIGINAL ARTICLE

Autophagy-related protein 5 knockdown alleviates the lipopolysaccharide-induced inflammatory response in RAW264.7 cells

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Abstract
Lipopolysaccharide (LPS) is a potent inducer of cytokine-mediated inflammatory responses. Although autophagy and LPS-induced inflammatory responses are related, it is unclear how autophagy regulates these inflammatory responses. We aimed to determine the effect of autophagy on the LPS-induced myeloid differentiation factor 88 (MyD88)/mitogen-activated protein kinase (MAPK)/nuclear transcription factor kB (NF-κB) signaling pathways in the inflammatory responses. We also determined the effect of autophagy on the MyD88-independent signaling pathway activated by LPS. To determine the effect of autophagy on LPS-induced inflammatory responses in RAW264.7 cells, we examined various in vitro assays by knockdown of autophagy-related protein 5 (ATG5), a key component of autophagy. ATG5 knockdown suppressed pro-inflammatory cytokines, including interleukin 6 and tumor necrosis factor α in LPS-stimulated RAW264.7 cells. Moreover, ATG5 knockdown also affected both MyD88-dependent (MAPK/NF-κB) and MyD88-independent (interferon regulatory factor 3) signaling pathways. This study demonstrates that dysfunctional autophagy suppresses LPS-induced inflammatory responses through both MyD88-dependent and MyD88-independent pathways in RAW264.7 cells. We propose that targeting autophagy regulation is a promising therapeutic approach for many diseases in which inflammatory responses contribute to their onset and progression of the disease.

Introduction

Autophagy is a fundamental cellular process that degrades and recycles unnecessary or damaged cellular components.[1,2] During autophagy, cytoplasmic materials, including misfolded proteins, protein aggregates, and damaged organelles are sequestered into double-membrane vesicles called autophagosomes.[1,2] These vesicles subsequently fuse

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with lysosomes, where their contents are broken down by lysosomal enzymes.[1,2] Autophagy plays a crucial role in maintaining cellular homeostasis and acts as a major quality control mechanism under stress conditions.[3,4] Autophagy protects against exogenous risk factors, including infection, as well as endogenous sources of inflammation, including molecular aggregates and damaged organelles.[5] Autophagy is tightly regulated, and its key mediators are the autophagy (ATG)-related proteins.[6,7] To date, at least 41 ATG genes have been identified.[8] Among them, ATG5 is essential for autophagosome formation[9]; its knockdown leads to a marked reduction or complete inhibition of autophagy.[10]

Lipopolysaccharide (LPS) is recognized by toll-like receptor 4, which initiates inflammatory signaling cascades through the myeloid differentiation primary response 88 (MyD88) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), as well as the MAPK pathways.[11,12] These pathways stimulate the production of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha (TNF-α), contributing to the overall inflammatory response.[13,14] In addition to this MyD88-dependent mechanism, LPS can also activate a MyD88-independent pathway involving the TNF receptor-associated factor (TRAF)-interferon regulatory factor 3 (IRF3) axis, which leads to the induction of interferons (IFNs).[15]

We previously reported that the autophagy inhibitor chloroquine (CQ) suppresses the LPS-induced increase in pro-inflammatory cytokines by inhibiting p38 and JNK MAPKs as well as NF-κB in a MyD88-dependent manner.[16] Autophagy dysfunction contributes to inflammatory diseases, as demonstrated by its genetic association with Crohn's disease.[17,18] As mentioned above, autophagy dysfunction is caused by ATG5 deficiency. However, it remains unclear whether ATG5 deficiency leads to the suppression of LPS-induced inflammatory responses. In this study, we aimed to investigate whether knockdown of ATG5, which is involved in autophagy deficiency, suppresses the expression of LPS-induced proinflammatory cytokines via inhibition of MAPKs and NF-κB. We also examined whether autophagy affects the MyD88-independent signaling pathway activated by LPS.

Materials and Methods

RAW264.7 cell culture and treatment with LPS

RAW264.7 cells were purchased from the European Collection of Authenticated Cell Cultures (Salisbury, UK). Cells were grown to 80–90% confluence at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), L-glutamine (4 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL). Subsequently, the cells were passaged using trypsinization. The culture medium was replaced with FBS-free DMEM, and the cells were treated with LPS (Sigma-Aldrich, St. Louis, MO, USA).

Knockdown of ATG5 with small interfering RNA (siRNA)

Oligonucleotides targeting mouse ATG5 (BIONEER Co., YD, Republic of Korea) and control siRNA (Ctrl siRNA; BIONEER Co.) were transfected into RAW264.7 cells using Lipofectamine RNAiMAX (Invitrogen, Eugene, OR, USA) according to the manufacturer's protocol. Briefly, both ATG5 siRNA and Ctrl siRNA were diluted with Opti-MEM medium, and then, diluted Lipofectamine RNAiMAX was added. The transfection mixture was incubated at 25°C for 20 minutes. When RAW264.7 cells reached 30–50% confluence, the culture medium was replaced with DMEM (without FBS), and the transfection mixture was added to each well. The final concentration of siRNA was 100 nM.

Measurement of protein levels

ATG5, p62, IL-1, IL-6, TNF-α, phospho-p38 (p-p38), p-38, phospho-JNK (p-JNK), JNK, phospho-ERK (p-ERK), ERK, phospho-NF-κB (p-NF-κB), NF-κB, IFN-β, TRAF3, phospho-IRF3 (p-IRF3), and IRF3 protein levels were analyzed using Western blotting. RAW264.7 cells were transfected with Ctrl siRNA or ATG5 siRNA and were treated or not treated with LPS 500 ng/mL for 15 minutes to 1 hour. Following LPS exposure, the medium was removed and the cells were washed with Dulbecco's phosphate-buffered saline and lysed in lysis buffer [50 mM HEPES (pH 7.4), 5 mM EDTA, 120 mM NaCl, 1% Triton X-100, protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 10 μg/mL leupeptin), and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate)]. The lysate was centrifuged at 10,000 × g for 15 minutes, and the protein (20–100 μg) in the supernatant was resolved using 10–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were blotted onto a nitrocellulose membrane (Millipore Corp., Bedford, MA, USA). The membrane was incubated with the following primary antibodies: anti-ATG5 (#8540), p62 (#5114), anti-IL-1 (#12507), anti-IL-6 (#12912), anti-TNF-α (#11948), anti-p-p38 (#8203), anti-p-38 (#8290), anti-p-JNK (#4668), anti-JNK (#9252), anti-p-ERK (#4377), anti-ERK (#4695), anti-p-NF-κB (#3033), and anti-NF-κB (#6956) [all purchased from Cell Signaling Technology (Danvers, MA, USA)], anti-IFN-β(Sigma-Aldrich, sc-57201), anti-TRAF3 (Cell Signaling Technology, #4729), anti-p-IRF3 [Proteintech (Rosemount, IL, USA) 29528-1-AP], anti-IRF3 (Cell Signaling technology, #4302), and anti-β-actin antibody (Sigma-Aldrich, A2228). Following incubation with primary antibodies, the membrane was incubated with horseradish-peroxidase-conjugated secondary antibodies (anti-rabbit, Cell Signaling technology, #7074; or anti-mouse, Cell Signaling technology, #7076; or anti-rat, Proteintech, SA00001-15). Chemiluminescence was detected with Immobilon (Merck, Darmstadt, Germany). All experiments were performed in triplicate.

Statistical analysis

Data were combined and expressed as means ± standard deviation. Statistical significance was assessed using an unpaired two-tailed Student's *t*-test or a two-way analysis of variance (ANOVA) with Tukey's post hoc test. A p-value of < 0.05 was considered statistically significant.

Results

Effect of autophagy inhibition on LPS-induced proinflammatory cytokines of the MyD88-dependent pathway

To elucidate the impact of autophagy on the MyD88-dependent pathway activated by LPS, we examined how autophagy inhibition affects LPS-induced proinflammatory cytokines through the activation of MAPKs and NF-κB via the MyD88-dependent pathway.

We first confirmed whether silencing ATG5 via siRNA effectively inhibited autophagy in RAW264.7 cells (Figure 1A-C). The expression of p62, an autophagy adaptor protein that accumulates when autophagy is impaired, was significantly increased in siATG5-treated cells. This result indicates that ATG5 knockdown successfully reduced autophagic activity in RAW264.7 cells.

We investigated the effect of autophagy inhibition by ATG5 knockdown on the MyD88-dependent pathway activated by LPS. To investigate how autophagy affects the production of these cytokines, we evaluated their expression in RAW264.7 cells stimulated with LPS following ATG5 knockdown (Figure 1D-G). In ATG5-knockdown cells, the LPS-induced increase in IL-6 was notably attenuated. Similarly, TNF-α levels were also reduced. These results suggest that autophagy positively regulates the expression of IL-6 and TNF-α in response to LPS stimulation.

Effect of autophagy inhibition on LPS-induced MAPK and NF-κB of the MyD88-dependent pathway

To elucidate the mechanism underlying the reduced cytokine production, we examined the impact of autophagy inhibition on LPS-induced MAPK and NF-κB signaling.

LPS activates the MAPK signaling cascade, including p38, JNK, and ERK, to promote pro-inflammatory cytokine expression. In our experiments, LPS stimulation increased phosphorylation levels of all three MAPKs, confirming pathway activation (Figure 2). However, ATG5 knockdown suppressed the phosphorylation of p38 and JNK, indicating selective inhibition of these MAPK branches. Contrastingly, ERK phosphorylation was unaffected by ATG5 knockdown.

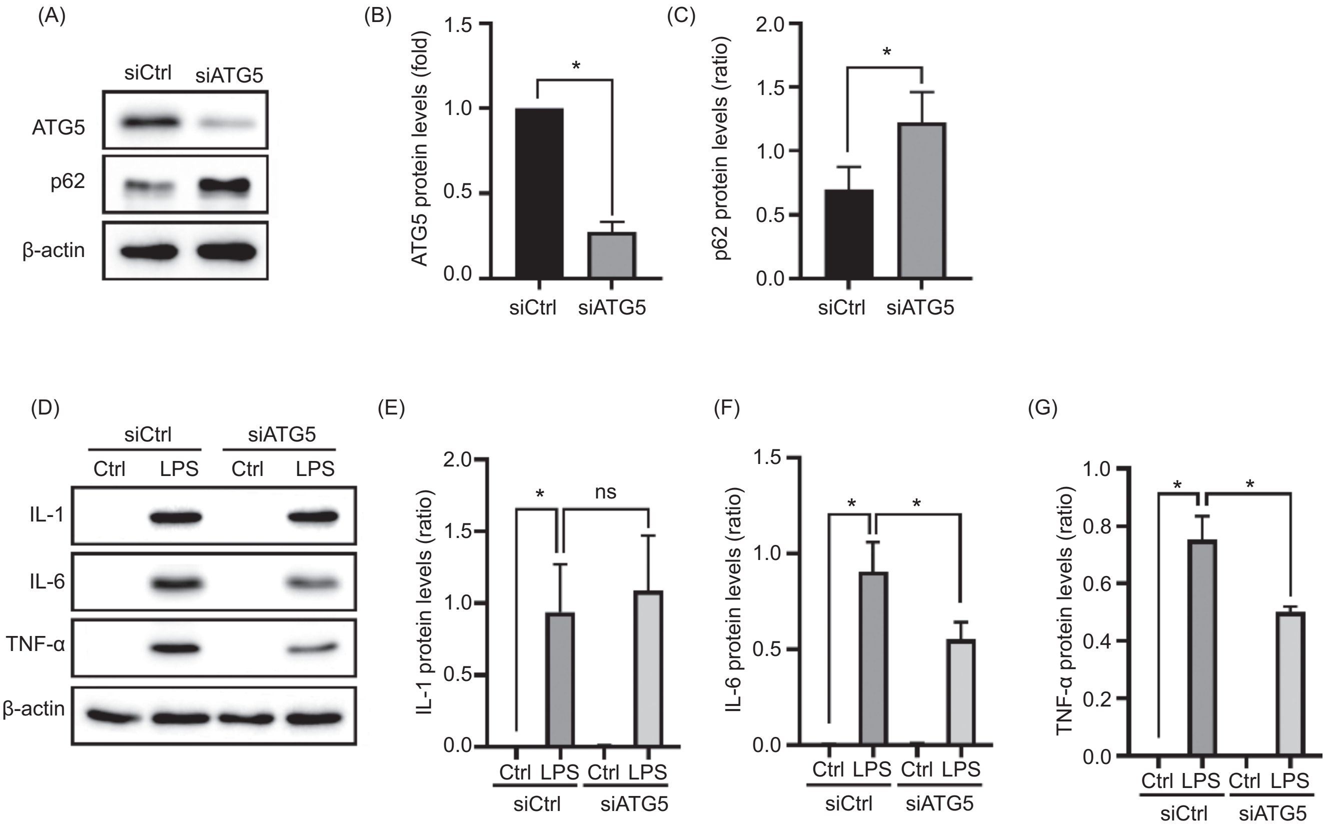

Figure 1 Effect of autophagy inhibition on LPS-induced proinflammatory cytokines of the MyD88-dependent pathway. RAW264.7 cells were transfected with control siRNA (Ctrl siRNA) or ATG5 siRNA and subsequently treated with or without LPS (500 ng/mL) for 1 hour. (A) Representative immunoblots of ATG5 and p62. (B, C) Densitometric quantification of ATG5 (B) and p62 (C) protein levels normalized to β-actin. (D) Representative immunoblots of IL-1, IL-6, and TNF-α. (e-g) Densitometric quantification of IL-1 (E), IL-6 (F), and TNF-α (G) normalized to β-actin. Band intensities were analyzed using ImageJ. Data are presented as means ± SD from three independent experiments. Statistical significance was assessed using an unpaired two-tailed Student's *t*-test or a two-way analysis of variance (ANOVA) with Tukey's post hoc test. *Significant difference ($p < 0.05$). LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; siRNA, small interfering RNA; Ctrl, control; ATG5, autophagy-related protein 5; IL, interleukin; TNF-α, tumor necrosis factor α; SD, standard deviation.

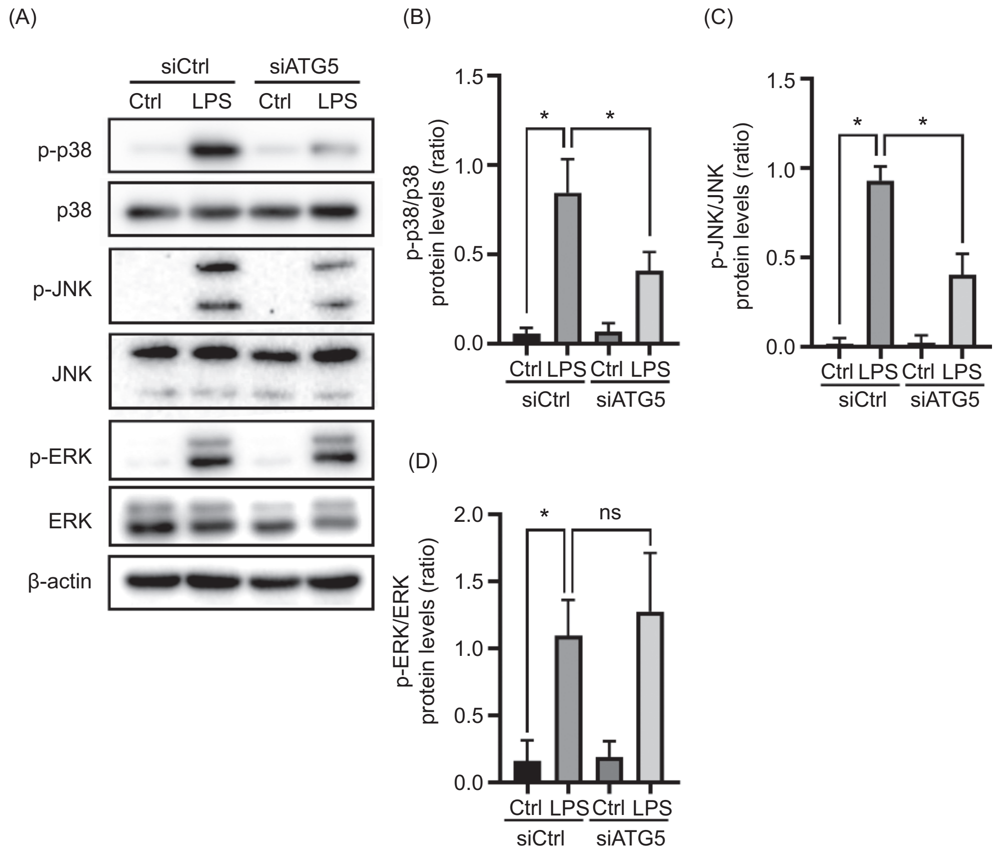

Figure 2 Effect of autophagy inhibition on the LPS-activated MAPK pathway of the MyD88-dependent pathway. RAW264.7 cells were transfected with Ctrl siRNA or ATG5 siRNA and subsequently treated with or without LPS (500 ng/mL) for 15 minutes. (A) Representative immunoblots of p38, JNK, and ERK. (B–D) Densitometric quantification of p38 (B), JNK (C), and ERK (D) protein levels normalized to β-actin. Band intensities were analyzed using ImageJ. Data are presented as means ± SD from three independent experiments. Statistical significance was assessed using a two-way analysis of variance (ANOVA) with Tukey's post hoc test. *Significant difference ($p < 0.05$). LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; siRNA, small interfering RNA; Ctrl, control; ATG5, autophagy-related protein 5; SD, standard deviation; MAPK, mitogen-activated protein kinase.

We also evaluated the NF-κB signaling pathway, which is another major mediator of LPS-induced inflammation (Figure 3). ATG5 knockdown reduced the phosphorylation of NF-κB in response to LPS, suggesting that autophagy is involved in NF-κB activation under inflammatory conditions. Furthermore, we examined the effect of ATG5 knockdown on MyD88 (Figure 4). Knockdown of ATG5 resulted in a decrease in MyD88 protein levels.

These results suggest that inhibition of autophagy through ATG5 knockdown suppresses LPS-induced expression of proinflammatory cytokines by interfering with the activation of the MyD88/MAPKs/NF-κB signaling pathway.

Effect of autophagy inhibition by ATG5 knockdown on the MyD88-independent pathway

We examined the effect of ATG5 knockdown on type I interferon production mediated by the TRAF–IRF3 pathway, a MyD88-independent signaling cascade activated by LPS (Figures 5 and 6). In ATG5-knockdown cells, LPS-induced production of IFN-β, as a type I interferon, was significantly suppressed. Consistently, phosphorylation of IRF3 was also decreased, suggesting impaired activation of the TRAF–IRF3 signaling axis.

These findings indicate that autophagy contributes to LPS-induced IFN-β production, likely by supporting efficient TRAF–IRF3 pathway activation.

Discussion

In this study, we demonstrate that knockdown of the autophagy-related gene, ATG5, reduces LPS-induced production of pro-inflammatory cytokines in RAW264.7 macrophages. These results suggest that autophagy plays an important role in LPS-induced inflammatory responses. In fact, there are several reports on the role of autophagy in LPS-induced inflammatory responses. Autophagy suppresses cytokine production via inflammasome degradation.[19] Inhibition of autophagy enhances IL-1β secretion.[20] Based on these findings, we initially hypothesized that autophagy inhibitors would enhance the LPS-induced upregulation

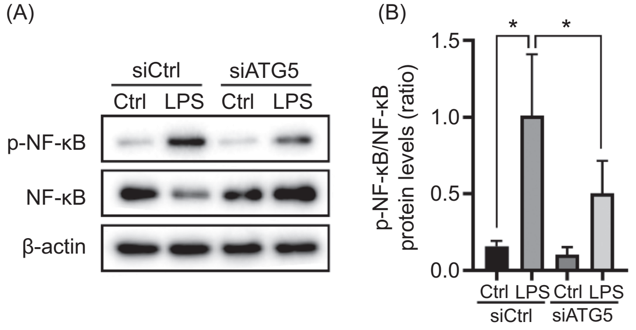

Figure 3 Effect of autophagy inhibition on the LPS-activated NF-κB pathway of the MyD88-dependent pathway. RAW264.7 cells were transfected with Ctrl siRNA or ATG5 siRNA and subsequently treated with or without LPS (500 ng/mL) for 15 minutes. (A) Representative immunoblots of NF-κB. (B) Densitometric quantification of NF-κB protein levels normalized to β-actin. Band intensities were analyzed using ImageJ. Data are presented as means ± SD from three independent experiments. Statistical significance was assessed using a two-way analysis of variance (ANOVA) with Tukey's post hoc test. *Significant difference ($p < 0.05$). LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; siRNA, small interfering RNA; Ctrl, control; ATG5, autophagy-related protein 5; SD, standard deviation; NF-κB, nuclear transcription factor κB.

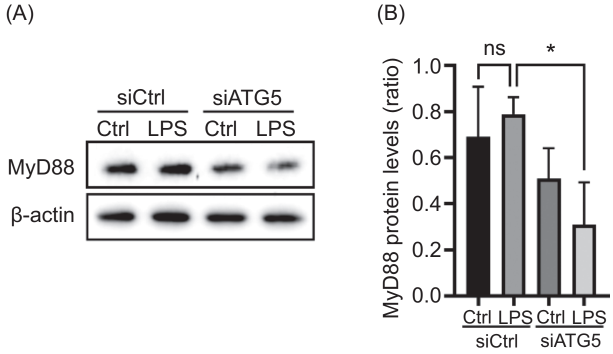

Figure 4 Effect of autophagy inhibition on MyD88. RAW264.7 cells were transfected with Ctrl siRNA or ATG5 siRNA and subsequently treated with or without LPS (500 ng/mL) for 15 minutes. (A) Representative immunoblots of MyD88. (B) Densitometric quantification of MyD88 protein levels normalized to β-actin. Band intensities were analyzed using ImageJ. Data are presented as means ± SD from three independent experiments. Statistical significance was assessed using a two-way analysis of variance (ANOVA) with Tukey's post hoc test. *Significant difference ($p < 0.05$). LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; siRNA, small interfering RNA; Ctrl, control; ATG5, autophagy-related protein 5; SD, standard deviation.

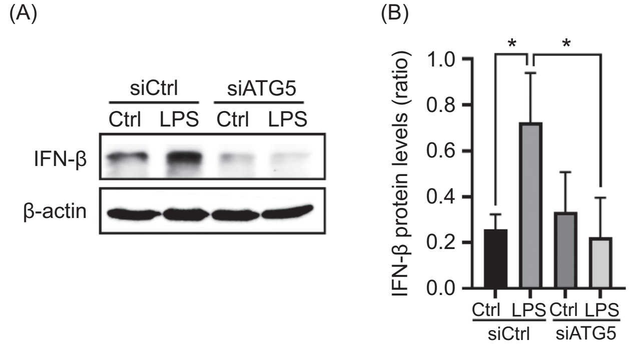

Figure 5 Effect of autophagy inhibition on LPS-activated type I interferons of the MyD88-independent pathway. RAW264.7 cells were transfected with Ctrl siRNA or ATG5 siRNA and subsequently treated with or without LPS (500 ng/mL) for 30 minutes. (A) Representative immunoblots of IFN-β. (B) Densitometric quantification of IFN-β protein levels normalized to β-actin. Band intensities were analyzed using ImageJ. Data are presented as means ± SD from three independent experiments. Statistical significance was assessed using a two-way analysis of variance (ANOVA) with Tukey's post hoc test. *Significant difference ($p < 0.05$). LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; siRNA, small interfering RNA; Ctrl, control; ATG5, autophagy-related protein 5; SD, standard deviation; IFN-β, Interferon β.

of proinflammatory cytokines. However, contrary to this expectation, our results demonstrated that autophagy inhibitors suppressed the LPS-induced increase in proinflammatory cytokine levels. Specifically, the expression of IL-6 and TNF-α was markedly decreased, accompanied by suppression of MAPK (p38 and JNK) and NF-κB activation. ATG5 knockdown also led to a reduction in MyD88 protein levels, suggesting that autophagy inhibited LPS-induced inflammatory responses by regulating the MyD88-dependent signaling pathway. Furthermore, autophagy inhibition also impaired the MyD88-independent TRAF–IRF3 axis, resulting in attenuated phosphorylation of IRF3 and reduced production of IFN-β. These findings collectively indicate that autophagy facilitates both MyD88-dependent and MyD88-independent signaling cascades activated by LPS.

Our earlier work demonstrated that pharmacological inhibition of autophagy by CQ suppressed LPS-induced cytokine expression primarily via p38, JNK, and NF-κB pathways.[16] The present study uses the genetic inhibition of ATG5 to provide complementary evidence, thereby strengthening the hypothesis that autophagy inhibition suppresses inflammatory responses through multiple signaling mediators. However, IL1, which is increased by LPS, was suppressed by CQ but not by ATG5 knockdown. This is different from the results for IL6 and TNFα. Although IL-6 and TNF-α are known to be induced primarily through MyD88-dependent pathways (through MAPK and NF-κB pathways),[21,22] IL-1 is also regulated by pathways distinct from MyD88 dependence, particularly the NLRP3 inflammasome-mediated pathway.[23] CQ is known to suppress the activation of NLRP3 inflammasome, which may contribute to the suppression of IL-1.[24] Conversely, the effect of ATG5 knockdown was mainly limited to the MyD88-dependent pathway, and it is possible that it did not have a sufficient effect on the control of IL-1. The mechanisms by which autophagy is inhibited using siATG5 and CQ are fundamentally different, targeting distinct stages of the autophagic process. siATG5-mediated knockdown suppresses the expression of the ATG5 gene, an essential component of the ATG12–ATG5–ATG16L1

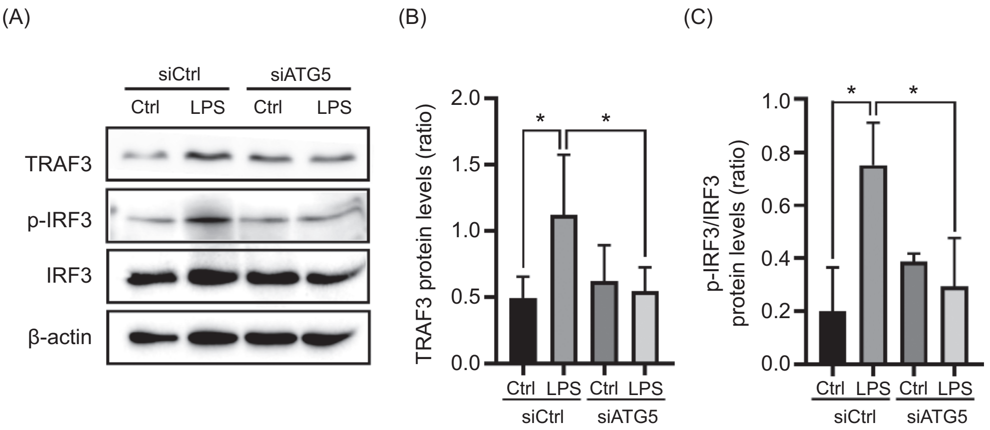

Figure 6 Effect of autophagy inhibition on LPS-activated TRAF–IRF3 pathway of MyD88-independent pathway. RAW264.7 cells were transfected with Ctrl siRNA or ATG5 siRNA and subsequently treated with or without LPS (500 ng/mL) for 15 minutes. (A) Representative immunoblots of TRAF3 and IRF3. (B, C) Densitometric quantification of TRAF3 (b) and IRF3 (C) protein levels normalized to β-actin. Band intensities were analyzed using ImageJ. Data are presented as means ± SD from three independent experiments. Statistical significance was assessed using a two-way analysis of variance (ANOVA) with Tukey's post hoc test. *Significant difference ($p < 0.05$). LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; siRNA, small interfering RNA; Ctrl, control; ATG5, autophagy-related protein 5; SD, standard deviation; TRAF, tumor necrosis factor receptor-associated factor; IRF3, interferon regulatory factor 3.

complex required for autophagosome formation. Hence, autophagy is inhibited at an early stage, preventing autophagosome biogenesis. This genetic approach allows for a more specific and sustained inhibition of autophagy, making it suitable for investigating the structural and functional roles of autophagy-related genes.[25,26] Our findings in ATG5 knockdown cells are consistent with findings from a report demonstrating that autophagy regulates IL-1β signaling via the p62 degradation pathway. Specifically, it shows that disruption of autophagy alters p62 turnover, thereby affecting inflammatory responses.[27] Contrastingly, CQ inhibits autophagy at a late stage by accumulating in lysosomes and increasing their intraluminal pH, which impairs lysosomal enzyme activity and disrupts both autophagosome–lysosome fusion and degradation. Unlike siATG5, CQ does not prevent autophagosome formation; rather, it leads to the accumulation of autophagosomes because of impaired degradation. However, CQ is less specific and may affect other lysosomal and endo-lysosomal processes unrelated to autophagy.[28,29] Thus, while both approaches are commonly used to block autophagy, they differ substantially in their specificity, duration of action, and the stage of autophagy they target. Indeed, comparative studies on ATG5 knockdown and CQ addition have reported distinct intracellular events, further supporting that genetic and pharmacological autophagy inhibition are not phenotypically equivalent.[30] These may account for the differing responses to LPS-induced IL-1β increases in CQ-treated cells and ATG5 knockdown cells. Autophagy plays a critical role in maintaining various aspects of cellular homeostasis, including the regulation of inflammatory responses[31, 32] Autophagy principally serves an adaptive role in protecting organisms against diverse pathologies, including infections, cancer, neurodegeneration, aging, and heart disease[33,34] Our findings demonstrate that inhibiting autophagy suppresses both the LPS-induced MyD88/MAPK/inflammatory cytokine pathway and the LPS-induced TRAF–IRF3/IFN-β signaling pathway. These results suggest that suppressing inflammatory pathways may lead to improved treatments for many diseases in which inflammatory responses contribute to their onset and progression. Our result indicates that ATG5 knockdown reduces MyD88 expression levels. This result suggests that autophagy is involved in maintaining the stability or availability of upstream adaptor proteins, not merely supporting downstream kinase activation.

This study has some limitations. Most notably, all experiments were performed exclusively using the RAW264.7 murine macrophage cell line. Relying on a single in vitro model significantly limits the generalizability of the findings to other cell types or physiological contexts. Therefore, future studies should incorporate additional cell lines and in vivo models to validate the observed effects. Furthermore, although ATG5 knockdown reduced the expression of pro-inflammatory cytokines, the physiological relevance and functional implications of this effect in vivo remain unclear and warrant further investigation.

Conclusion

Our findings reveal a previously unrecognized role of autophagy dysfunction against LPS-induced pro-inflammatory signaling through both MyD88-dependent and MyD88-independent pathways. These results suggest that autophagy modulation could serve as a potential strategy for disease treatment by fine-tuning immune responses in inflammatory conditions.

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Author's Contribution

All authors contributed equally to this article.

Conflict of interest

The authors declare no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

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