J Lipid Atheroscler. 2023 May;12(2):189-200
https://doi.org/10.12997/jla.2023.12.2.189
pISSN 2287-2892·eISSN 2288-2561

JLA Journal of Lipid and Atherosclerosis

Original Article

Autophagy Enhancers Regulate Cholesterol-Induced Cytokine Secretion and Cytotoxicity in Macrophages

Su Kyoung Lee,[1,*] Eun Hee Kam,[2,*] So Yeong Cheon [3,4]

[1]Department of Neurology, Yonsei University College of Medicine, Seoul, Korea
[2]Department of Internal Medicine, Institute of Gastroenterology, Yonsei University College of Medicine, Seoul, Korea
[3]Department of Biotechnology, College of Biomedical & Health Science, Konkuk University, Chungju, Korea
[4]Research Institute for Biomedical & Health Science, Konkuk University, Chungju, Korea

Received: Feb 10, 2023
Revised: Apr 13, 2023
Accepted: May 3, 2023
Published online: May 16, 2023

Correspondence to
So Yeong Cheon
Department of Biotechnology, Research Institute for Biomedical & Health Science, College of Biomedical & Health Science, Konkuk University, 268 Chungwon-daero, Chungju 27478, Korea.
Email: sycheon14@kku.ac.kr

*These authors contributed equally to this work.

ORCID iDs
So Yeong Cheon
https://orcid.org/0000-0001-7015-4898

Funding
This article was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2020R1C1C1010613) to So Yeong Cheon.

ABSTRACT

Objective: Hypercholesterolaemia transforms macrophages into lipid-laden foam cells in circulation, which can activate the immune response. Compromised autophagy and inflammatory cytokines are involved in the pathogenesis and progression of metabolic diseases. The aim of this study was to identify the role of autophagy as a modulator of the inflammatory response and cytotoxicity in macrophages under hypercholesterolaemic conditions.
Methods: High cholesterol-induced cytokine secretion and alteration of autophagy-associated molecules were confirmed by cytokine array and western blot analysis, respectively. To confirm whether autophagic regulation affects high cholesterol-induced cytokine release and cytotoxicity, protein levels of autophagic molecules, cell viability, and cytotoxicity were measured in cultured macrophages treated autophagy enhancers.
Results: Cholesterol treatment increased cytokine secretion, cellular toxicity, and lactate dehydrogenase release in lipopolysaccharide (LPS)-primed macrophages. Concomitantly, altered levels of autophagy-related molecules were detected in LPS-primed macrophages under hypercholesterolaemic conditions. Treatment with autophagy enhancers reversed the secretion of cytokines, abnormally expressed autophagy-associated molecules, and cytotoxicity of LPS-primed macrophages.
Conclusion: Autophagy enhancers inhibit inflammatory cytokine secretion and reduce cytotoxicity under metabolic disturbances, such as hypercholesterolaemia. Modulation of autophagy may be a novel approach to control the inflammatory response observed in metabolic diseases.

Keywords: Autophagy; Inflammation; Cytokines; Cholesterol; Macrophage; Torin; Rapamycin

INTRODUCTION

Metabolic syndrome is common and highly prevalent worldwide and can lead to metabolic diseases, such as cardiovascular disease, stroke, and type 2 diabetes.[1] It is characterised by visceral obesity, hypertension, dyslipidaemia, hypertriglyceridaemia, and hyperglycaemia,[2]

Conflict of Interest
The authors have no conflicts of interest to declare.

Data Availability Statement
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions
Conceptualization: Lee SK, Kam EH, Cheon SY; Data curation: Lee SK, Kam EH, Cheon SY; Formal analysis: Lee SK, Kam EH, Cheon SY; Funding acquisition: Cheon SY; Investigation: Lee SK, Kam EH, Cheon SY; Methodology: Lee SK, Kam EH; Supervision: Cheon SY; Visualization: Lee SK, Kam EH; Writing - original draft: Cheon SY.

and defective macrophage cholesterol metabolism causes atherosclerosis.[3] In particular, hypercholesterolaemia causes transforming circulating monocytes/macrophage into cholesterol-laden macrophages, which trigger an inflammatory immune response under a complex cytokine milieu,[4] by promoting and secretion of inflammatory mediators including interleukin (IL)-1β, IL-6, and IL-18.[5-7] The interplay between inflammation and foam cell formation is closely related to both inflammatory and metabolic diseases.[8] Therefore, the modulation of this relationship may be important for metabolic disturbances, such as hypercholesterolaemia.

Autophagy is an intracellular degradation system that removes and recycles damaged organelles or cytoplasmic accumulation.[9] Intracellular lipid droplets or lipid accumulations are metabolised by autophagy and cytoplasmic neutral hydrolases to generate energy and supply structural components to cell membranes.[10] Autophagy is involved in immune responses, and compromised autophagy can lead to inflammatory diseases.[11] Previous studies have demonstrated that increasing autophagy by rapamycin inhibits macrophage activation,[12] and that rapamycin and torin decrease inflammatory cytokine levels in macrophages.[13]

Therefore, the aim of this study was to identify autophagy as a modulator of cytokine secretion in macrophages in metabolic disorders such as high cholesterol. To do this, we examined the relationship between cholesterol loading and inflammatory cytokine in macrophages. We also confirmed the role of autophagy on inflammatory cytokine secretion in macrophages during high cholesterol conditions.

MATERIALS AND METHODS

1. Cell culture

The murine macrophage cell line, RAW 264.7, was used in this study. RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (high glucose) (WELGENE Inc., Gyeongsan, Korea), supplemented 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% antibiotic-antimycotic containing streptomycin, amphotericin B, and penicillin (Gibco). RAW 264.7 cells were incubated at 37°C in a 5% CO_2 incubator under humid conditions.

2. Lipopolysaccharide (LPS) and cholesterol treatment

RAW 264.7 cells were treated with LPS (*Escherichia coli* serotype 055:B5) and cholesterol from Sigma-Aldrich (St. Louis, MO, USA). We treated cells with 100 ng/mL LPS diluted in cell culture media with 1% FBS and 1% antibiotic-antimycotic for 2 hours and washed with phosphate-buffered saline (PBS). The cells were then treated with 25 and 50 μg/mL cholesterol, diluted in cell culture media with 1% FBS and 1% antibiotic-antimycotic, for 22 hours, respectively. At the end of incubation, the cultured cells and cell-conditioned media were collected for further experiments. To enhance autophagy, rapamycin (4 μM, 1 hour; LC Laboratories, Woburn, MA, USA) and torin-1 (1 μM, 2 hours; Tocris Bioscience, Bristol, UK) were used in this study.

3. Mouse cytokine array

The mouse cytokine array kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). LPS-primed RAW 264.7 conditioned media were collected after treatment with/without cholesterol. The mixtures containing media and antibody were added to a multi-dish containing membranes and incubated overnight at 4°C on a rocker. The next day, each membrane was incubated with streptavidin-horseradish peroxidase (HRP) for 30 minutes at

https://doi.org/10.12997/jla.2023.12.2.189

room temperature (RT). After washing, signals on the membranes were visualised by exposing the membrane to an X-ray film. Negative control spots were used as the background values.

4. Filipin III

Filipin III was purchased from Tocris Bioscience and dissolved in dimethyl sulfoxide to obtain stock solution (25 mg/mL). The stock solution was diluted in PBS to obtain a working solution (50 μg/mL). After fixation in 4% paraformaldehyde, cells were treated with Filipin III working solution for 2 hours at RT. The cells were then washed with PBS and positive signals were observed using a ZEISS LSM 980 confocal laser scanning microscope (Carl Zeiss NTS Ltd., Oberkochen, Germany).

5. Total cholesterol assay (cholesterol influx or uptake)

The total cholesterol assay (DoGenBio Co., Ltd., Seoul, Korea) was performed after treatment with LPS with or without cholesterol. According to the manufacturer's protocol, cells were homogenised in 200 μL of solution of chloroform, isopropanol, and NP-40 on ice. After centrifugation at 15,000 × g for 10 minutes, the samples were dried at 50°C. Cholesterol assay buffer was added to each dried sample and the reaction mixture was then added to each well. Samples were incubated at 37°C for 30 minutes, and absorbance was measured at 570 nm using a VERSA max microplate reader (Molecular Devices, Sunnyvale, CA, USA).

6. Western blot analysis

Cells were lysed with lysis buffer containing protease and phosphatase single-use inhibitor cocktail (1:100; Thermo Fisher Scientific, Waltham, MA, USA), and incubated for 20 minutes on ice. After incubation, the lysates were centrifuged at 8,000 rpm at 4°C for 10 minutes. The protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific), and the samples were denatured at 97°C for 5 minutes. The samples were then loaded, separated on precast 12-well gradient (4%–15%) polyacrylamide gels (SMOBIO Technology, Inc., Hsinchu, Taiwan), and transferred onto an Immobilon-P PVDF membrane (Millipore, Burlington, MA, USA). The membranes were blocked with 5% skim milk and incubated overnight on a rocker with a primary antibody at 4°C. The primary antibodies used were mouse anti-autophagy-related gene (ATG)5 (B-9), mouse anti-ATG16 (E-10), mouse anti-ATG7 (B-9), and mouse anti-cathepsin B from Santa Cruz Biotechnology (Dallas, TX, USA), and rabbit anti-LC3B, rabbit anti-p62, rabbit anti-cathepsin D, and rabbit anti-lysosomal-associated membrane protein 1 (LAMP-1) (C54H11) from Cell Signaling Technology (Danvers, MA, USA). After washing with tris-buffered saline with Tween20, the membranes were incubated with HRP conjugated secondary anti-mouse and anti-rabbit IgG antibodies from Santa Cruz Biotechnology for 1 hour at RT. HRP-conjugated β-actin antibody was used as an internal control. Membranes were incubated with western blotting detection reagent (Cytiva, Marborough, MA, USA), and signals were visualised using ImgeQuant LAS 4000 (GE Healthcare, Chicago, IL, USA), and measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

7. Enzyme-linked immunosorbent assay (ELISA)

Secreted IL-1β and IL-27 (Invitrogen, Carlsbad, CA, USA), IL-1α and IL-6 (Abbkine Scientific Co., Ltd., Wuhan, China), and RANTES (R&D Systems, Inc.) levels in conditioned media were evaluated using ELISA. All procedures were performed according to the manufacturer's instructions. Briefly, each sample and standard were loaded onto each well and incubated overnight at 4°C for maximum sensitivity. After all reactions, the plates were read at 450 nm using a VERSA max microplate reader (Molecular Devices).

https://doi.org/10.12997/jla.2023.12.2.189

JLA Journal of Lipid and Atherosclerosis

8. Lysotracker

Lysotracker probes (Invitrogen) were used to track acidic organelles in the RAW 264.7. Probe stock solution (1 mM) was diluted in conditioned media to prepare a working solution (75 nM), and cells were treated with this working solution for 30 minutes. Subsequently, the cells were observed under ZEISS LSM 980 confocal laser scanning (Carl Zeiss NTS Ltd.).

9. Lactate dehydrogenase (LDH) assay

Cell conditioned media were transferred to a multi-well plate to detect released LDH levels due to cell membrane damage and cytotoxicity using an LDH assay kit (Invitrogen). The reaction mixture was added to each sample well, mixed well by tapping, and incubated at RT for 30 minutes. The stop solution was then added to each well, and the plates were read at 490/680 nm using a VERSA max microplate reader (Molecular Devices).

10. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). Statistical data were obtained by performing 2-way analysis of variances followed by a Tukey's *post hoc* tests. Results are presented as the mean ± standard error of the mean (SEM) of at least 3 independent experiments. A p-value <0.05 was considered as statistically significant in this study.

RESULTS

1. High cholesterol triggers cell cytotoxicity and secretion of cytokines in LPS-primed macrophages

By using Filipin III, which binds cholesterol, we confirmed the increased cholesterol uptake in cholesterol treated LPS-primed RAW 264.7 cells, which showed increased expression of Filipin III fluorescence, compared to the untreated control (**Fig. 1A**). Treatment with cholesterol (25 and 50 μg/mL) showed efficient cholesterol influx in LPS-primed RAW 264.7 cells (**Fig. 1B**). To detect cell cytotoxicity, we conducted a cell viability assay and an LDH assay in LPS-primed macrophages after treatment with cholesterol (**Fig. 1C and D**). The graphs show that treatment with cholesterol (50 μg/mL) significantly decreased cell viability and increased LDH release compared to the control or LPS-primed macrophage. A cytokine array was performed to determine whether cholesterol affected cytokine secretion in immune cells. Blots from the cytokine array kit were incubated with LPS-primed RAW 264.7 conditioned medium after treatment with cholesterol (50 μg/mL). Seven cytokines with strong immuno-positive spots were detected after cholesterol treatment (**Fig. 1E**). Cytokines including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1α, IL-1β, IL-6, IL-27, and RANTES were upregulated in LPS-primed RAW 264.7 conditioned medium after treatment with cholesterol compared to the control (**Fig. 1E**).

2. High cholesterol loading impairs autophagy in LPS-primed macrophages

To confirm whether cholesterol could impair the autophagy system in macrophages, we measured the protein levels of autophagy components in LPS-primed RAW 264.7 cells after treatment with cholesterol using western blot analysis (**Fig. 2A and B**). The levels of LC3-II, a marker of autophagosomes, and p62, a marker of autophagic cargo receptors, were increased after treatment with cholesterol, compared to the untreated control. In addition, the protein levels of ATG16 and ATG5, which are essential factors for autophagosome formation, were decreased in both LPS-primed and non-primed macrophages after cholesterol treatment.

https://doi.org/10.12997/jla.2023.12.2.189

JLA Journal of Lipid and Atherosclerosis

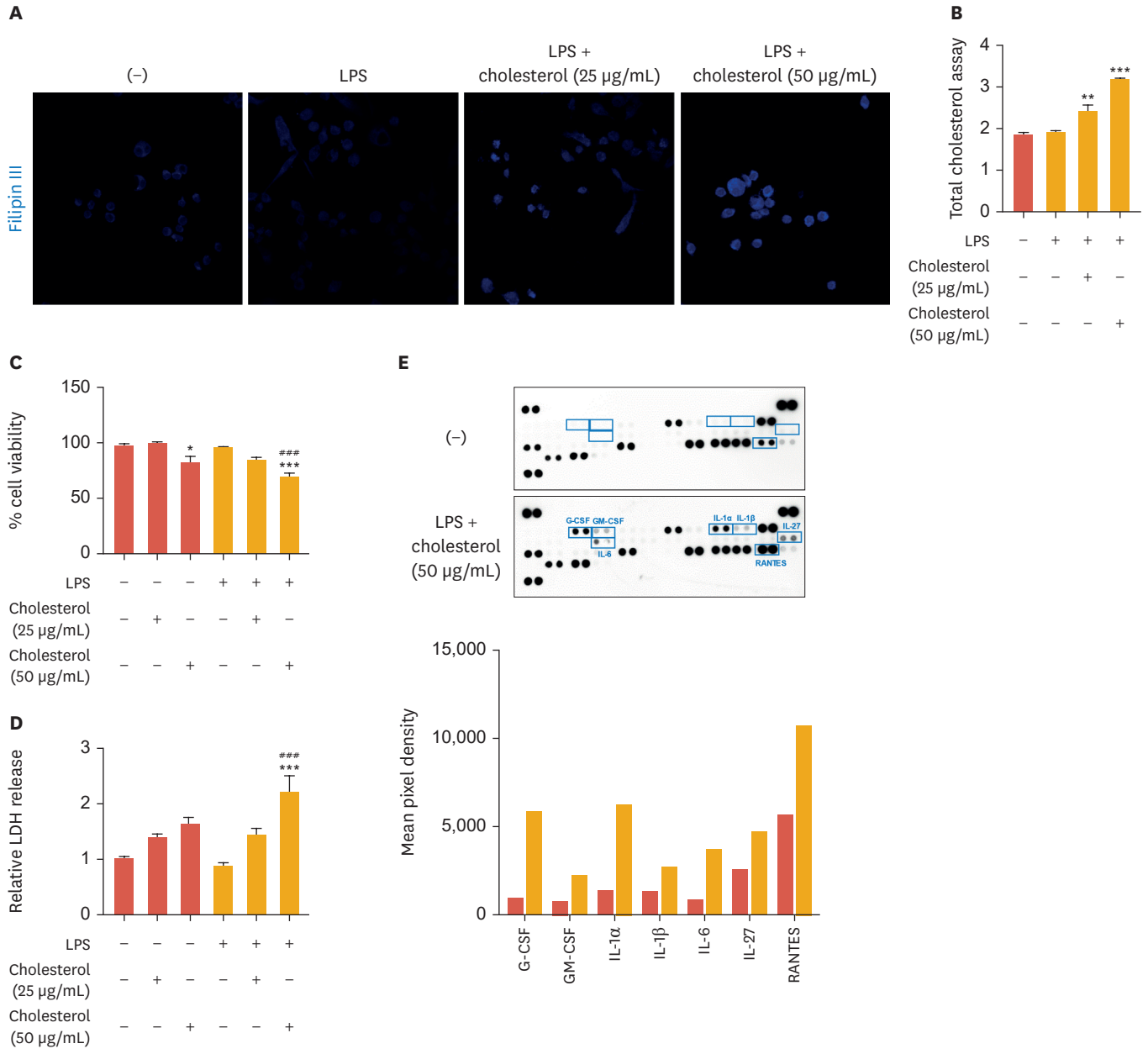

Fig. 1. High cholesterol loading induces cellular cytotoxicity and cytokine hypersecretion in LPS-primed macrophages. (A) High Filipin III fluorescence in LPS-primed macrophages under high cholesterol (25 and 50 μg/mL) conditions. (B) Measurement of cholesterol uptake by total cholesterol assay in LPS-primed macrophages under high cholesterol (25 and 50 μg/mL) conditions. (C, D) Assessment of cell viability using WST assay, and cell cytotoxicity using LDH release assay after priming with LPS for 2 hours, followed by stimulation with high cholesterol concentration for 22 hours. (E) Increased cytokine expressions, including G-CSF, GM-CSF, IL-1α, IL-1β, IL-6, IL-27, and RANTES, in LPS-primed macrophages under high cholesterol (50 μg/mL) conditions. Values are presented as mean ± standard error of the mean (3–4 repeats).
LPS, lipopolysaccharide; LDH, lactate dehydrogenase; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin.
*p<0.05, **p<0.01, ***p<0.001 vs. untreated RAW 264.7 macrophages (without both treatments with LPS and cholesterol); ###p<0.001 vs. LPS-primed macrophages.

Defective lysosomes are the major cause of impaired autophagy flux as a result of the abnormal fusion of autophagosomes and lysosomes.[14] Lysotracker probes were used to label lysosomes in RAW 264.7 macrophages (**Fig. 2C**). Strong fluorescence of lysotracker probes was observed under untreated conditions. This fluorescence was decreased in LPS-primed macrophages after treatment with cholesterol, suggesting impairment of lysosomes. Lysosome-related

https://doi.org/10.12997/jla.2023.12.2.189

JLA Journal of Lipid and Atherosclerosis

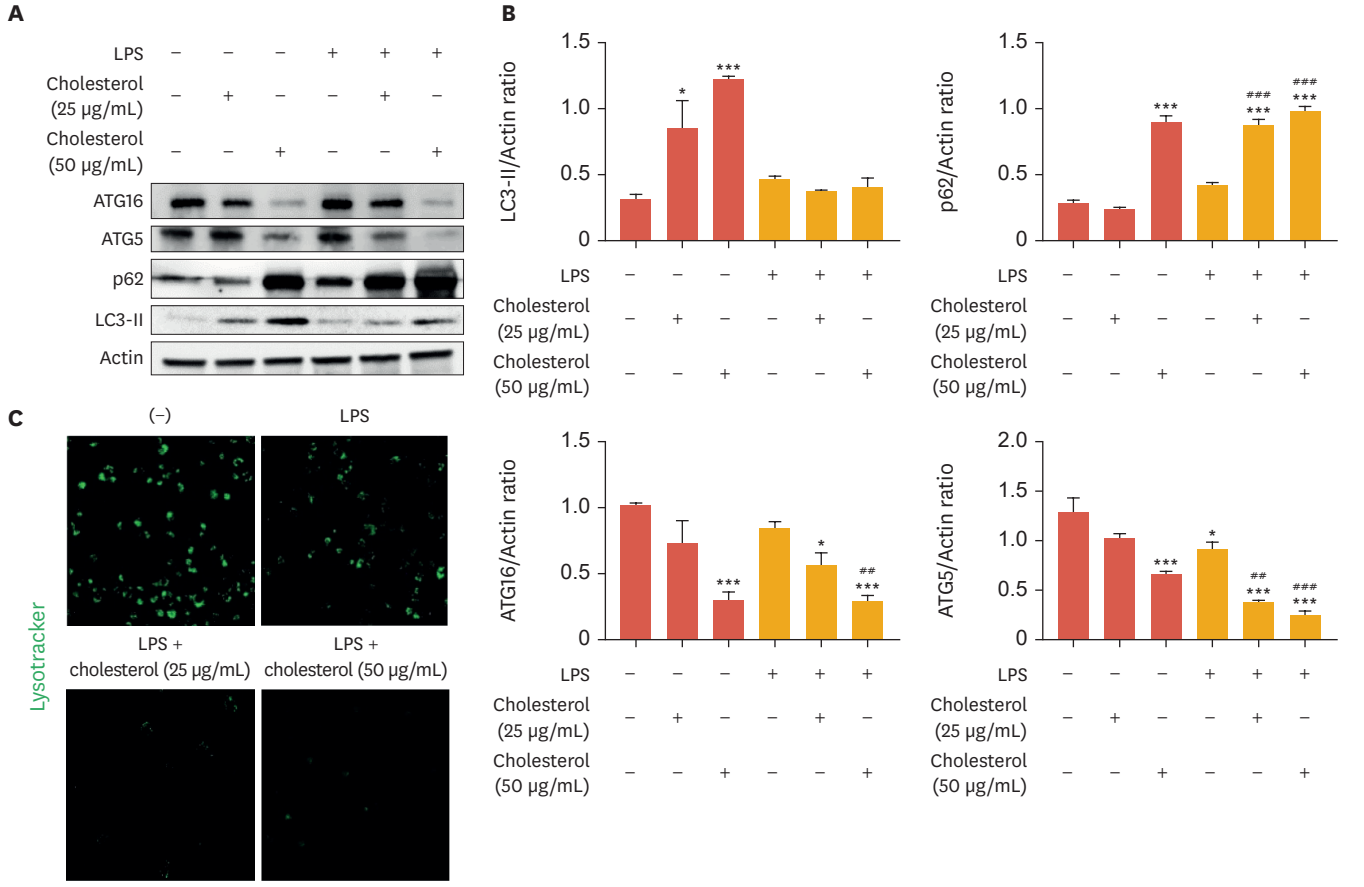

Fig. 2. High cholesterol induces alterations of autophagy-related molecules in LPS-primed macrophages. (A, B) By using western blotting, the bands (A) and graphs (B) for ATG16, ATG5, p62 and LC3-II protein levels in LPS-primed macrophages under high cholesterol (25 and 50 μg/mL) conditions. Data represented as quantified protein ratio. (C) To label acidic organelles such as lysosomes, lysotracker probes was used and a week green fluorescence was observed in LPS-primed macrophages under high cholesterol (25 and 50 μg/mL) conditions. (D, F) The bands and graphs for LAMP-1, cathepsin B, and cathepsin D protein levels in LPS-primed macrophages under high cholesterol (25 and 50 μg/mL) conditions. Data represented as quantified protein ratio. Values are presented as mean ± standard error of the mean (3–4 repeats).
LPS, lipopolysaccharide; ATG, autophagy-related gene; LAMP-1, lysosomal-associated membrane protein 1.
$^{*}p<0.05$, $^{***}p<0.001$ vs. untreated RAW 264.7 macrophages (without both treatments with LPS and cholesterol); $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. LPS-primed macrophages.
(continued to the next page)

markers, including LAMP-1, cathepsin B, and cathepsin D, were determined using western blot analysis (**Fig. 2D and E**). Treatment with 50 μg/mL cholesterol considerably reduced LAMP-1 protein levels. Although no significant difference was found in the levels of cathepsin B, the protein levels of pro-cathepsin D and cathepsin D heavy chain were altered in both LPS-primed macrophages and LPS-primed macrophages with cholesterol.

3. Autophagy inducers enhance high cholesterol-induced reduction of ATGs in LPS-primed macrophages

To examine whether autophagy inducers, such as rapamycin and torin, modulate cholesterol-induced alterations in ATGs, we performed western blot analysis (**Fig. 3**). After pretreatment with rapamycin or torin, LPS was treated to RAW 264.7 macrophages and cholesterol was incubated for 22 hours. Upon pretreatment with rapamycin, ATG7 levels increased significantly compared to those present in a vehicle-treated normal condition. In addition, LAMP-1, ATG7, and LC3-II protein levels increased in LPS-primed macrophages that were pre-treated with rapamycin compared to levels in macrophages lacking rapamycin pretreatment

https://doi.org/10.12997/jla.2023.12.2.189

JLA Journal of Lipid and Atherosclerosis

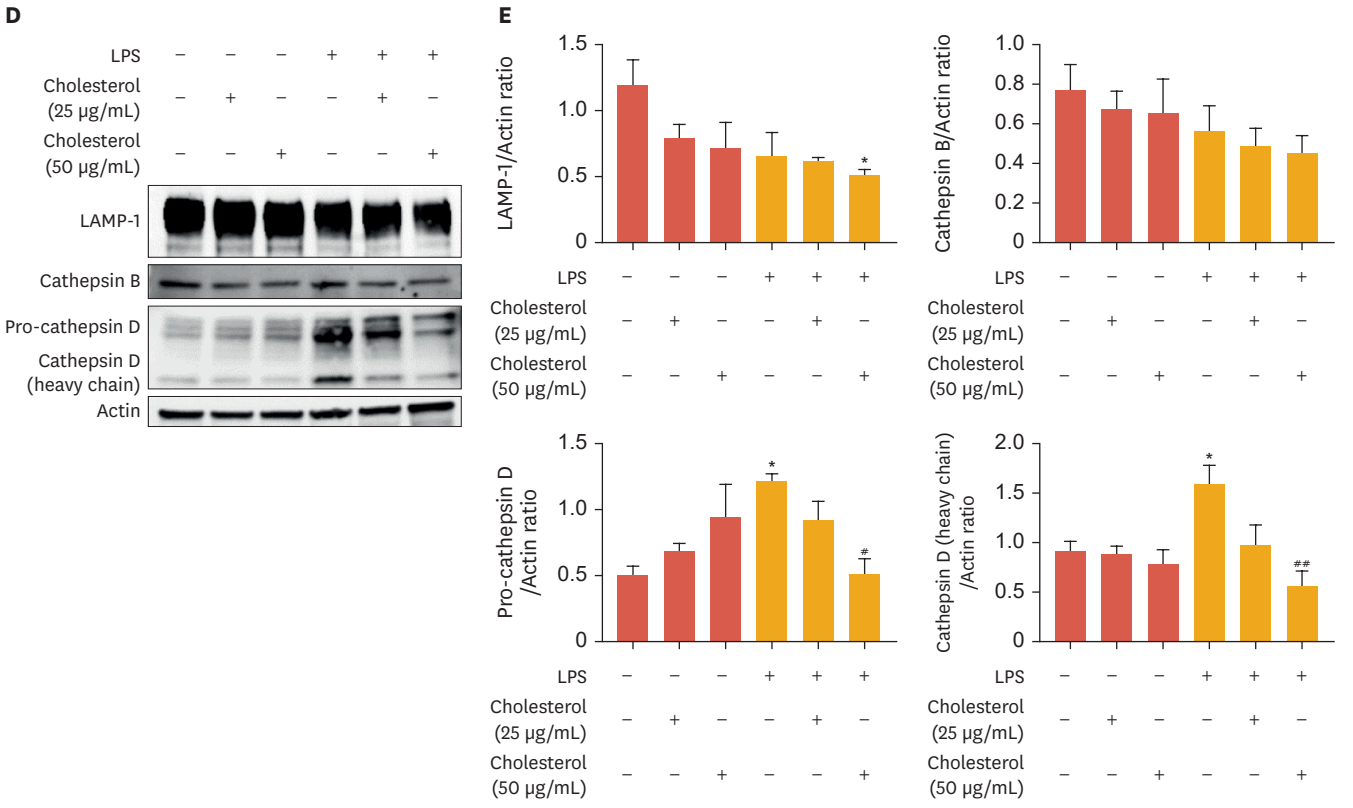

Fig. 2. (Continued) High cholesterol induces alterations of autophagy-related molecules in LPS-primed macrophages. (A, B) By using western blotting, the bands (A) and graphs (B) for ATG16, ATG5, p62 and LC3-II protein levels in LPS-primed macrophages under high cholesterol (25 and 50 μg/mL) conditions. Data represented as quantified protein ratio. (C) To label acidic organelles such as lysosomes, lysotracker probes was used and a week green fluorescence was observed in LPS-primed macrophages under high cholesterol (25 and 50 μg/mL) conditions. (D, F) The bands and graphs for LAMP-1, cathepsin B, and cathepsin D protein levels in LPS-primed macrophages under high cholesterol (25 and 50 μg/mL) conditions. Data represented as quantified protein ratio. Values are presented as mean ± standard error of the mean (3–4 repeats).
LPS, lipopolysaccharide; ATG, autophagy-related gene; LAMP-1, lysosomal-associated membrane protein 1.
*p<0.05, ***p<0.001 vs. untreated RAW 264.7 macrophages (without both treatments with LPS and cholesterol); #p<0.05, ##p<0.01, ###p<0.001 vs. LPS-primed macrophages.

under high cholesterol conditions (**Fig. 3A and B**). The p62 protein levels only decreased in LPS-primed macrophages, when compared to the levels in LPS-primed macrophages with rapamycin pretreatment (**Fig. 3A and B**), indicating restoration of autophagy activity. Torin pretreatment produced an effect similar to that observed with rapamycin pretreatment (**Fig. 3C and D**). Upon pretreatment with torin LAMP-1 levels increased significantly compared to those in vehicle-treated normal condition. The LAMP-1, ATG7, and LC3-II protein levels increased significantly in LPS-primed macrophages that were pre-treated with torin compared to levels without pretreatment with torin under high cholesterol conditions (**Fig. 3C and D**). The p62 protein levels decreased in LPS-primed macrophages with torin pretreatment, compared to those without pretreatment with torin (**Fig. 3C and D**).

4. Autophagy inducers regulate high cholesterol-induced cytokine secretion and cytotoxicity in LPS-primed macrophages

We assessed whether autophagy inducers modulate the high cholesterol-induced secretion of cytokines and cellular damage by performing ELISA and LDH assays (**Fig. 4**). Rapamycin

https://doi.org/10.12997/jla.2023.12.2.189

JLA Journal of Lipid and Atherosclerosis

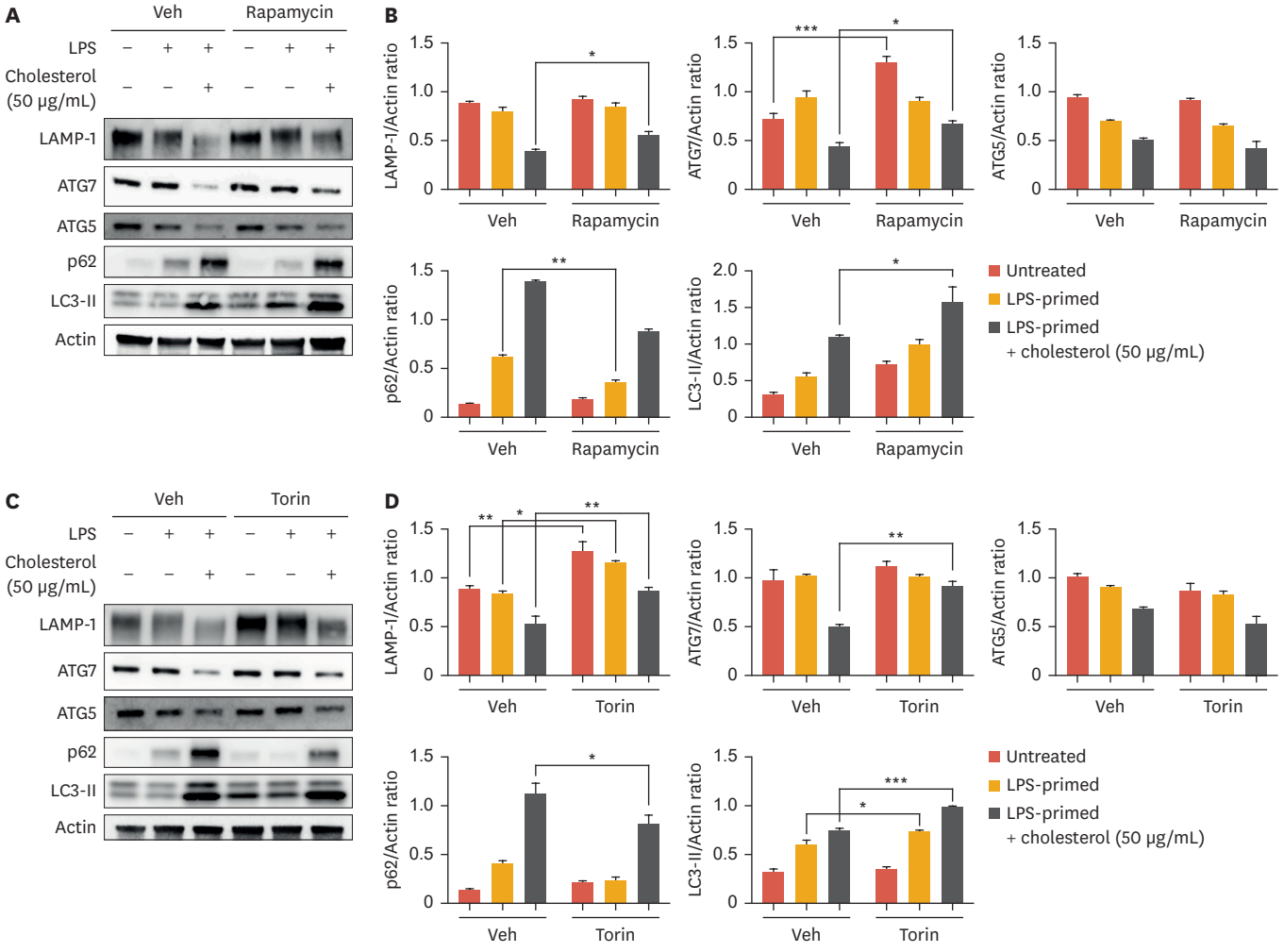

Fig. 3. High cholesterol induces reduction of autophagy-associated component protein levels after pretreatment with autophagy enhancers in LPS-primed macrophages. (A, B) Western blotting Gel bands (A) and graphical plot (B) of LAMP-1, ATG7, ATG5, p62, and LC3-II protein levels upon pretreatment with rapamycin in LPS-primed macrophages under the high cholesterol (50 μg/mL) condition. Data represented the quantified protein ratio. (C, D) Western blotting bands (C) and graphical plot (D) of LAMP-1, ATG7, ATG5, p62, and LC3-II protein levels upon pretreatment with torin in LPS-primed macrophages under high cholesterol (50 μg/mL) conditions. Data represented the quantified protein ratio. Values are presented as mean ± standard error of the mean (3–4 repeats).
LPS, lipopolysaccharide; ATG, autophagy-related gene; LAMP-1, lysosomal-associated membrane protein 1.
*p<0.05, **p<0.01, ***p<0.001 (vehicle control only vs. rapamycin only or torin only; LPS-primed macrophages with vehicle control vs. those with torin or rapamycin treatment; LPS-primed macrophages with vehicle control under high cholesterol conditions vs. those with rapamycin or torin treatment under high cholesterol conditions).

pretreatment significantly reversed the high cholesterol-induced release of IL-1β and IL-27 in LPS-primed macrophages, compared to that without pretreatment with rapamycin (**Fig. 4B and D**). Torin pretreatment reduced high cholesterol-induced secreted concentrations of IL-1α, IL-6, and IL-27 in LPS-primed macrophages (**Fig. 4A, C, and D**). Unexpectedly, the RANTES concentration was independent of treatment with autophagy inducers (**Fig. 4E**). Cell cytotoxicity assessed by the LDH assay revealed that rapamycin and torin pretreatment efficiently reversed high cholesterol-induced cellular toxicity in LPS-primed macrophages (**Fig. 4F**).

https://doi.org/10.12997/jla.2023.12.2.189

JLA Journal of Lipid and Atherosclerosis

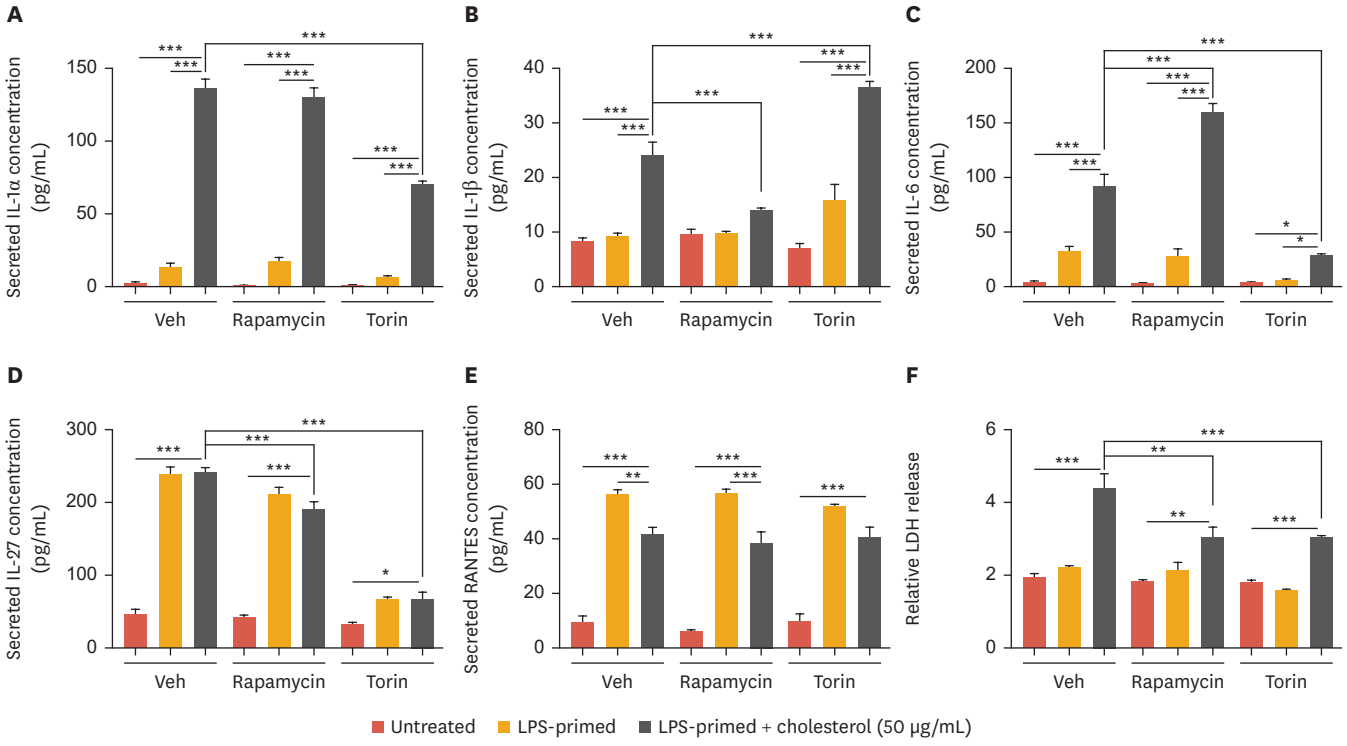

Fig. 4. Restoration of high cholesterol-induced cytokine secretion and cytotoxicity in LPS-primed macrophages. (A-E) Measurement of secreted IL-1α (A), IL-1β (B), IL-6 (C), IL-27 (D), and RANTES (E) levels by enzyme-linked immunosorbent assay after pretreatment with an autophagy enhancer, priming with LPS for 2 hours, and stimulation with high cholesterol concentration for 22 hours. (F) Cell cytotoxicity assessed by LDH release after pretreatment with an autophagy enhancer, priming with LPS for 2 hours, and stimulation with high cholesterol concentration for 22 hours. Values are presented as mean ± standard error of the mean (5–6 repeats).
LPS, lipopolysaccharide; IL, interleukin; LDH, lactate dehydrogenase.
*p<0.05, **p<0.01, ***p<0.001 vs. LPS-primed macrophage with cholesterol. **p<0.01, ***p<0.001 (LPS-primed macrophages with vehicle control vs. those with torin or rapamycin treatment; LPS-primed macrophages with vehicle control under high cholesterol vs. those with rapamycin or torin treatment under high cholesterol conditions).

DISCUSSION

In this study, cholesterol-laden macrophages showed an increased release of cytokines, including IL-1β, IL-1α, IL-6, IL-27, and RANTES, in the cytokine array. High cholesterol levels were toxic to macrophages. High cholesterol levels facilitate the impairment of autophagy machinery with abnormal levels of autophagosomal and lysosomal molecules. The autophagy inducers rapamycin and torin, improved autophagy dysfunction, and cytokine secretion from macrophages was influenced by rapamycin or torin treatment. Consequently, autophagy inducers alleviate cellular toxicity in macrophages under high cholesterol conditions. These results suggest that autophagy modulation may have cytokine secretory and cytotoxin regulatory effects in macrophages under metabolic disturbances, such as hypercholesterolaemia.

Metabolic overloads due to cholesterol uptake affect macrophage structure and functions, including their morphological extension, macrophage polarization (M1-like or M2-like), and efferocytosis.[15,16] Hypercholesterolaemia is responsible for cholesterol-laden macrophages, with cholesterol promoting the pro-inflammatory M1 phenotype in a glycolysis-dependent manner.[15,16] Autophagy plays an important role in regulating intracellular lipid droplets and lipid metabolism,[17] and controlling immune responses.[18] Autophagic abnormalities, including defects in autophagosome formation, also lead to lipid accumulation and cause

https://doi.org/10.12997/jla.2023.12.2.189

JLA Journal of Lipid and Atherosclerosis

excessive inflammation.[17,18] Structurally, autophagosomes by extension of phagophores fuse with lysosomes to degrade autophagic contents.[19] The lysosomal pathway of autophagy also plays an important role in the degradation of lipid droplet-stored triglycerides and cholesterol.[17] Lysosomal dysfunction or impairment results in foam cell formation.[20,21] Therefore, intact activated autophagy is essential for lipid metabolism and immune reactions in macrophages.[17,18] To generate autophagosomes, autophagic molecules, including ATGs, are required.[19] Compromised autophagy results in p62 accumulation.[22] In our study, under high cholesterol conditions, abnormally expressed autophagy-associated molecules, such as ATGs and LC3-II, increased in response to treatment with autophagy enhancers in macrophages. In addition, the increased p62 level was reduced upon treatment with autophagy enhancer torin. LAMP-1, a lysosomal marker and an indicator of lipophagy activation, was reduced in LPS-primed macrophages under high cholesterol conditions, indicating lysosomal dysfunction. In addition, cathepsin D levels were aberrantly altered, indicating abnormal changes in lysosomal hydrolases. These changes may contribute to the activation of inflammatory cytokines and cellular toxicity. When autophagy enhancers were used, abnormal LAMP-1 levels were reversed despite cholesterol loading, indicating that they ameliorated lysosomal impairment due to high cholesterol.

Under high cholesterol conditions, multiple cytokines, including G-CSF, GM-CSF, IL-1α, IL-1β, IL-6, IL-27, and RANTES, are secreted from macrophages. Previous studies have shown that macrophage foam cells in splenocytes exhibit elevated expression of G-CSF, which is involved in the production of neutrophils and bone-marrow monocytes.[3] Also, in atherosclerosis, GM-CSF plays an important role in differentiating blood-derived monocyte into macrophages, and furthermore, in forming cholesterol-laden foam cells.[23] The IL-1 family, which include IL-1α and IL-1β, is involved in the inflammatory response observed in obesity.[24] In addition, IL-1α and IL-1β production contributes to atherosclerotic plaque formation.[25] IL-6-mediated inflammatory responses are closely associated with cholesterol synthesis and metabolic diseases, such as atherosclerosis, dementia, and type 2 diabetes.[26] In the early atherosclerotic stages, RANTES is required for monocyte recruitment and lesion development.[27] Consistent with previous studies, our cytokine array results indicate that high cholesterol promotes the secretion of these cytokines in cholesterol-laden foam cells, and autophagy enhancers mostly reversed the upregulated secretion of these cytokines. Although RANTES was upregulated in LPS-primed macrophages under high cholesterol conditions, this upregulation was caused by LPS treatment rather than high cholesterol. RANTES levels were independent of pretreatment with rapamycin or torin. Since G-CSF and GM-CSF are involved in the differentiation of monocytes into macrophages, these experiments were excluded in this study.

This study has several limitations. First, we used only the RAW 264.7 cell line. Analyses utilising additional models incorporating different macrophage types, such as bone-marrow derived or peritoneal macrophages, would have provided more conclusive support to our findings. Second, we only performed *in vitro* experimentation on the RAW 264.7 cell line. The modulation of cytokine secretion and cytotoxicity via autophagy enhancer is yet to be validated with an *in vivo* model of hypercholesterolaemia, Third, we chose a high-dose cholesterol treatment regime to determine the detrimental effects of cholesterol. Cholesterol plays an important role in host homeostasis, and lower doses tend to have a protective effect; therefore, a higher dose (50 μg/mL cholesterol) was chosen for the purpose of our study.

In conclusion, high cholesterol-laden macrophages impaired autophagy. Autophagy enhancers regulate cytokine secretion in cholesterol-laden macrophages. In particular,

https://doi.org/10.12997/jla.2023.12.2.189

hypercholesterolaemia causes cholesterol-laden macrophages, which trigger an inflammatory immune response and cellular toxicity. Although autophagy is known to regulate inflammatory cytokines, this study suggests additional cytokines involving hypercholesterolaemia by cytokine array. The findings demonstrate the cytokine-regulating properties of autophagy enhancers under hypercholesterolaemic conditions. Targeting autophagy may be a novel approach to control metabolic disturbances, such as hypercholesterolaemia.

REFERENCES

1. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. Lancet 2005;365:1415-1428.
PUBMED | CROSSREF
2. Alberti KG, Zimmet P, Shaw J; IDF Epidemiology Task Force Consensus Group. The metabolic syndrome--a new worldwide definition. Lancet 2005;366:1059-1062.
PUBMED | CROSSREF
3. Westerterp M, Murphy AJ, Wang M, Pagler TA, Vengrenyuk Y, Kappus MS, et al. Deficiency of ATP-binding cassette transporters A1 and G1 in macrophages increases inflammation and accelerates atherosclerosis in mice. Circ Res 2013;112:1456-1465.
PUBMED | CROSSREF
4. Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. Nat Rev Immunol 2015;15:104-116.
PUBMED | CROSSREF
5. Park SH. Regulation of macrophage activation and differentiation in atherosclerosis. J Lipid Atheroscler 2021;10:251-267.
PUBMED | CROSSREF
6. Persson J, Nilsson J, Lindholm MW. Cytokine response to lipoprotein lipid loading in human monocyte-derived macrophages. Lipids Health Dis 2006;5:17.
PUBMED | CROSSREF
7. Groh L, Keating ST, Joosten LA, Netea MG, Riksen NP. Monocyte and macrophage immunometabolism in atherosclerosis. Semin Immunopathol 2018;40:203-214.
PUBMED | CROSSREF
8. Angelovich TA, Hearps AC, Jaworowski A. Inflammation-induced foam cell formation in chronic inflammatory disease. Immunol Cell Biol 2015;93:683-693.
PUBMED | CROSSREF
9. Rubinsztein DC, Codogno P, Levine B. Autophagy modulation as a potential therapeutic target for diverse diseases. Nat Rev Drug Discov 2012;11:709-730.
PUBMED | CROSSREF
10. Liu K, Czaja MJ. Regulation of lipid stores and metabolism by lipophagy. Cell Death Differ 2013;20:3-11.
PUBMED | CROSSREF
11. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature 2011;469:323-335.
PUBMED | CROSSREF
12. Ko JH, Yoon SO, Lee HJ, Oh JY. Rapamycin regulates macrophage activation by inhibiting NLRP3 inflammasome-p38 MAPK-NFκB pathways in autophagy- and p62-dependent manners. Oncotarget 2017;8:40817-40831.
PUBMED | CROSSREF
13. Shappley RK, Spentzas T. Differential role of rapamycin and torin/KU63794 in inflammatory response of 264.7 RAW macrophages stimulated by CA-MRSA. Int J Inflamm 2014;2014:560790.
PUBMED | CROSSREF
14. Myerowitz R, Puertollano R, Raben N. Impaired autophagy: the collateral damage of lysosomal storage disorders. EBioMedicine 2021;63:103166.
PUBMED | CROSSREF
15. Yvan-Charvet L, Ivanov S. Metabolic reprogramming of macrophages in atherosclerosis: is it all about cholesterol? J Lipid Atheroscler 2020;9:231-242.
PUBMED | CROSSREF
16. O'Rourke SA, Neto NG, Devilly E, Shanley LC, Fitzgerald HK, Monaghan MG, et al. Cholesterol crystals drive metabolic reprogramming and M1 macrophage polarisation in primary human macrophages. Atherosclerosis 2022;352:35-45.
PUBMED | CROSSREF

17. Dong H, Czaja MJ. Regulation of lipid droplets by autophagy. Trends Endocrinol Metab 2011;22:234-240.
PUBMED | CROSSREF
18. Festa BP, Barbosa AD, Rob M, Rubinsztein DC. The pleiotropic roles of autophagy in Alzheimer's disease: from pathophysiology to therapy. Curr Opin Pharmacol 2021;60:149-157.
PUBMED | CROSSREF
19. Cheon SY, Kim H, Rubinsztein DC, Lee JE. Autophagy, cellular aging and age-related human diseases. Exp Neurobiol 2019;28:643-657.
PUBMED | CROSSREF
20. Jerome WG. Lysosomes, cholesterol and atherosclerosis. Clin Lipidol 2010;5:853-865.
PUBMED | CROSSREF
21. Mori M, Itabe H, Higashi Y, Fujimoto Y, Shiomi M, Yoshizumi M, et al. Foam cell formation containing lipid droplets enriched with free cholesterol by hyperlipidemic serum. J Lipid Res 2001;42:1771-1781.
PUBMED | CROSSREF
22. Moreau K, Luo S, Rubinsztein DC. Cytoprotective roles for autophagy. Curr Opin Cell Biol 2010;22:206-211.
PUBMED | CROSSREF
23. Lappalainen J, Yeung N, Nguyen SD, Jauhiainen M, Kovanen PT, Lee-Rueckert M. Cholesterol loading suppresses the atheroinflammatory gene polarization of human macrophages induced by colony stimulating factors. Sci Rep 2021;11:4923.
PUBMED | CROSSREF
24. Ghanbari M, Momen Maragheh S, Aghazadeh A, Mehrjuyan SR, Hussen BM, Abdoli Shadbad M, et al. Interleukin-1 in obesity-related low-grade inflammation: from molecular mechanisms to therapeutic strategies. Int Immunopharmacol 2021;96:107765.
PUBMED | CROSSREF
25. Jiang X, Wang F, Wang Y, Gisterå A, Roy J, Paulsson-Berne G, et al. Inflammasome-driven interleukin-1α and interleukin-1β production in atherosclerotic plaques relates to hyperlipidemia and plaque complexity. JACC Basic Transl Sci 2019;4:304-317.
PUBMED | CROSSREF
26. Omoigui S. The interleukin-6 inflammation pathway from cholesterol to aging--role of statins, bisphosphonates and plant polyphenols in aging and age-related diseases. Immun Ageing 2007;4:1.
PUBMED | CROSSREF
27. Jongstra-Bilen J, Tai K, Althagafi MG, Siu A, Scipione CA, Karim S, et al. Role of myeloid-derived chemokine CCL5/RANTES at an early stage of atherosclerosis. J Mol Cell Cardiol 2021;156:69-78.
PUBMED | CROSSREF