A Thesis
for the Degree of Master of Science

Autophagy increased pro-inflammatory cytokines against *Listeria monocytogenes* infection in macrophage

리스테리아 감염에서 자가소화작용에 의한 대식세포의 염증성 사이토카인 증가

February 2017

By
Younggap Lim

School of Agricultural Biotechnology
Graduate School, Seoul National University

농 학 석 사 학 위 논 문

Autophagy increased pro-inflammatory cytokines against *Listeria monocytogenes* infection in macrophage

리스테리아 감염에서 자가소화작용에 의한 대식세포의 염증성 사이토카인 증가

지도교수 윤철희
이 논문을 농학석사 학위논문으로 제출함

2017년 02월

서울대학교 대학원
농생명공학부
임 영 갑

임영갑의 석사학위논문을 인준함
2017년 02월

위 원 장 한 승 현 (인)
부위원장 윤 철 희 (인)
위 원 박 병 철 (인)

Summary

Listeria monocytogenes, a Gram-positive intracellular pathogen, is a causative of listeriosis in both humans and animals. *L. monocytogenes* is known to evade host immunity, yet could be cleared by autophagy induction in macrophages. With this twist, we investigated the effect of autophagy inducing pro-inflammatory cytokines for protective immunity in bone marrow-derived macrophages (BMDMs) infected with *L. monocytogenes*. After the infection, autophagy induction by rapamycin reduced survival of intracellular *L. monocytogenes* while inhibition of autophagy enhances their survival. After 24 hours of *L. monocytogenes* infection, rapamycin-treated BMDMs produced interleukin (IL)-1β, IL-6, IL-12p40 higher than those of control group. As expected, autophagy inhibition reduced levels of cytokines compared with those of control. Treatment of heat killed *L. monocytogenes*, which acts as a TLR ligand without invasion, also showed a similar pattern with *L. monocytogenes* infection in BMDMs in terms of cytokine secretion, suggesting that modulation of pro-inflammatory cytokines by autophagy is TLR signaling dependent. The expression and nucleus translocation of interferon regulatory factor 5 (IRF5) that modulate IL-6 and IL-12 production, were increased in autophagy-induced BMDMs. In addition, degradation of IκBα, which is important for NF-κB activity, was increased by autophagy induction upon HKLM treated BMDMs. Taken together, rapamycin-induced autophagy enhances production of pro-inflammatory cytokines in BMDMs infected with *L. monocytogenes*.

Contents

List of Figures

List of Abbreviations

3-MA	3-methyladenine
ActA	Actin assembly-inducing protein
AMP	Adenosine 5′-monophosphate
AMPK	Adenosine 5′-monophosphate-activated protein kinase
APC	Antigen presenting cells
AV	Annexin V-FITC
Baf	Bafilomycin A1
BCG	Bacillus Calmette-Guérin
BHI	Brain heart infusion
BMDMs	Bone marrow derived macrophages
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
CMA	Chaperone-mediated autophagy
DAPI	4′,6-diamidino-2-phenylindole
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
HKLM	Heat-killed *Listeria monocytogenes*
HSV	Herpes simplex virus
IACUC	Institutional Animal Care and Use Committee
IFN	Interferon
IL	Interleukin
InlA	Internalin A
InlB	Internalin B
IRF5	Interferon regulatory factor 5
IκBα	Inhibitor of nuclear factor-kappa B α
L. monocytogenes	*Listeria monocytogenes*
LC3	Light chain 3 (of microtuble-associated protein 1)

LLO	listeriolysin O
LPS	Lipopolysaccharide
M. tuberculosis	*Mycobacterium tuberculosis*
MIF	Migration inhibitory factor
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
N.D.	Non detectable
N.S.	Not significant
NF-κB	Nuclear factor-kappa B
NI	Non-infection
NK cells	Natural killer cells
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinases
PlcA	phospholipase Phospholipase A
PlcB	phospholipase Phospholipase B
PVDF	Polyvinylidene fluoride
qRT-PCR	Real-time quantitative polymerase chain reaction
Rapa	Rapamycin
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
Th1 cells	Type 1 T helper cells
TLRs	Toll-like receptors
TMB	Tetramethylbenzidine
TOR	Target of rapamycin
TSC1	Tuberous sclerosis proteins 1
TSC2	Tuberous sclerosis proteins 2

I. Review of Literature

1. *Listeria monocytogenes* and its pathogenesis

1.1. Characteristics

Listeria monocytogenes is a rod-shaped Gram-positive, facultative anaerobic and intracellular bacterium which resides in the cytoplasm of the host cell (*1*). *L. monocytogenes* can colonize and invade into intestinal epithelial cells by interact with cell surface receptors called E-cadherin and Met (*2*). Its surface proteins internalin A (InlA) and internalin B (InlB) bind to E-cadherin and Met, respectively (*3*). Then, they are surrounded by vacuole but, *L. monocytogenes* can disrupt vacuole membrane by secretion of phospholipase A (PlcA), phospholipase B (PlcB), and the pore-forming toxin listeriolysin O (LLO) (*4*). As a result, *L. monocytogenes* releases into the cytoplasm and then propagates (*5*). Using actin assembly-inducing protein (ActA), *L. monocytogenes* polymerizes host F-actin so-called actin comet tails which allow motility to the bacteria in the cytoplasm (*6*)and cell-to-cell spread of the bacteria into neighboring cells (*1*) (Fig. 1).

1.2. Listeriosis

Listeriosis is a serious, life-threating illness caused by consuming food contaminated with *L. monocytogenes* (*7*) and, therefore, it is considered as a serious public health problem. Unfortunately, no vaccines are available at the moment against *L. monocytogenes* infection (*8*). *L. monocytogenes* can grow at

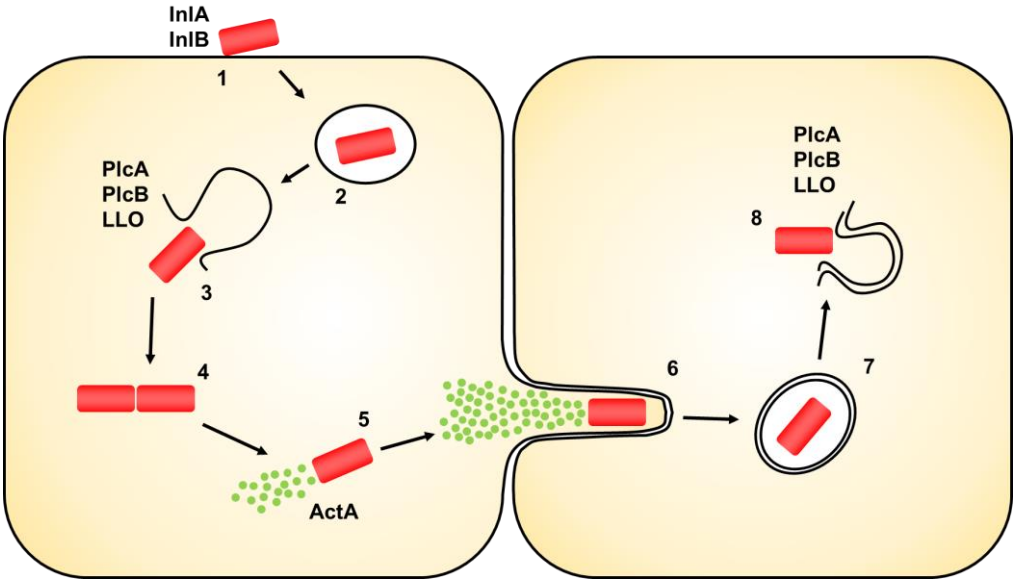


Figure 1. Life cycle of intracellular *L. monocytogenes*. 1. *L. monocytogenes* invades into host cell via InlA and InlB. 2. Internalized bacteria surrounded by vacuole. 3. Bacterial lysis of vacuole using PlcA, PlcB and LLO. 4. *L. monocytogenes* proliferates in the cytoplasm of the host cell. 5. *L. monocytogenes* polymerizes host actin by using ActA which allows motility to the bacteria in the cytoplasm. 6. *L. monocytogenes* penetrates into the neighboring cell using actin-based motility. 7. Internalized bacteria are surrounded by double-membraned vacuole. 8. Bacteria lysed vacuole using PlcA, PlcB and LLO then, re-start their life cycle.

low temperature as low as 4°C suggesting that refrigeration could not prevent bacterial growth (*9*). Listeriosis mainly affects the elderly, pregnant women, newborn babies, and people who are with weakened immune system (*8*). According to the Centers for Disease Control and Prevention (CDC), approximately 1,600 people suffer from listeriosis and 260 people die in the United States every year which sets listeriosis the third-leading cause of death (19%) among foodborne pathogenic infection. (*10, 11*). In addition, fatality rate of listeriosis is about 20-30% which overs *Salmonella* and *Clostridium* infection (*10*). Listeriosis can be treated with antibiotics. Since *L. monocytogenes* is intracellular pathogen, the antibiotics have to penetrate host cells and stably remain in the cytoplasm (*12*). Therefore, ampicillin, penicillin and amoxicillin are used to treat listeriosis (*13*). However, antibiotics treatment is often ineffective because the limited choice of antibiotics for the patients (*14*).

1.3. Pro-inflammatory cytokines and L. monocytogenes infection

Pro-inflammatory cytokines are important factor that activates various immune cells to clear *L. monocytogenes*. There are several pro-inflammatory cytokines crucial for susceptibility and clearance against *L. monocytogenes* infection *in vivo*: interleukin (IL)-1 (*15*), IL-6 (*16*), IL-12 (*17*), interferon (IFN)-γ (*18*).

IL-1β is one of the IL-1 family cytokine, which plays a role in inducing inflammatory response. It is mainly produced by monocytes and macrophages (*19*). Almost all pathogen-associated molecular patterns (PAMPs) induce IL-1β gene expression via TLR signaling pathway within 30 min (*20*). IL-1β is also

important for T cell-independent but IFN-γ dependent clearance of intracellular *L. monocytogenes* (*21-23*). IL-1β recruits neutrophils to the infection sites (*21*) and induces IL-12 production which stimulates IFN-γ production to natural killer cells (*23*) during *L. monocytogenes* infection. IL-1β also increases in blood leukocyte number, and macrophage responsiveness to IFN-γ (*21*). Therefore, IL-1β has a pivotal role in resistance against *L. monocytogenes* infection.

IL-6 is mainly produced by macrophages and monocytes at inflammatory region (*24*). It is a major cytokine in the serum of the mice infected with *L. monocytogenes* (*25*). Unlike IL-1β, IL-6 is the important cytokine for T cell-dependent clearance of intracellular *L. monocytogenes* (*26*). Recombinant IL-6 (rIL-6) injection to the mice enhance the resistance against listeriosis since IL-6 plays a pivotal role in the production of IFN-γ in T cells (*26*).

IL-12 is a heterodimeric cytokine composed of an alpha chain p35 and a beta chain p40 (*27*) It is known to be produced by dendritic cells and macrophages (*28*) then activates both innate and adaptive immunity against bacterial infection (*17*). The resistance against *L. monocytogenes* infection induced by IL-12 is mediated by IFN-γ production by natural killer (NK) cells and type 1 T helper (Th1) cells (*17*). IL-12 induces differentiation of naive T cells into Th1 cells upon *L. monocytogenes* infection (*29*).

IFN-γ is a type II interferon which has a pivotal role in inducing innate and adaptive immunity against viral and intracellular bacterial infection (*30*). During *L. monocytogenes* infection, pro-inflammatory cytokines which are produced by innate immune cells activate Th1 cells and induce IFN-γ production (*17, 23, 26*).

Therefore, induction of pro-inflammatory cytokines is important for the resistance against *L. monocytogenes* infection because that is needed to induce proper immune response for bacterial clearance.

2. Autophagy

2.1. Introduction

Autophagy is a highly conserved cellular recycling pathway throughout the multi-cellular kingdom from yeast to mammalian cells (*31*). It is a natural, but regulated, mechanism for cellular homeostasis degrading old or damaged organelles, macromolecules, misfolded proteins and re-using their building blocks for *de novo* synthesis (*32*). Since the identification of autophagy-related genes in yeast in the 1990s research scientists try to figure out the mechanism of autophagy (*33, 34*), which led to the award of the 2016 Nobel Prize in Physiology or Medicine to Japanese autophagy researcher Yoshinori Ohsumi. Autophagy allows cells to resist external stress such as nutrient starvation, insufficient growth factors and pathogen infections (*35*). Moreover, autophagy is involved in physiological cellular processes such as cellular development and differentiation (*36*).

There are three different types of autophagy: macroautophagy, microautophagy, chaperone-mediated autophagy (CMA) (*37*). In macroautophagy, cytoplasmic cargo engulfed by double membrane vacuole, called autophagosome, then fused with lysosome to form autophagolysosome (*38*). Autophagic cargo is degraded by lysosomal hydrolases in the autophagolysosome (*39*). In microautophagy, by

contrast, cytosolic components are directly taken up by the lysosome (*38*). In CMA, target molecules are translocated into the lysosome in a complex with chaperone proteins that are recognized by the lysosomal membrane receptor (*40*). Macroautophagy can be subdivided into xenophagy, mitophagy and pexophagy according to their cargo; xenophagy for selective microbe degradation by macroautophagy, pexophagy for peroxisome, mitophagy for mitochondria (*41, 42*). Hereafter, the term 'autophagy' indicates macroautophagy.

2.2. Signaling pathway in relation to autophagy

Basal levels of autophagy always occur in the cells to play an important role in housekeeping cellular homeostasis (*43*). However, autophagy is induced by nutrient deprivation that promotes survival of the cells (*44*). Mammalian target of rapamycin (mTOR) is a key molecule which senses nutrient levels and regulates cellular growth and autophagy. mTOR is activated by Akt, PI3-kinase and growth factor receptors (*45*). In nutrient rich condition, mTOR inhibits autophagy. Under nutrient deprivation or other cellular stress condition, mTOR is repressed by adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) which is activated by low ATP level (*46*). AMPK also activates tuberous sclerosis proteins 1 and 2 (TSC1/TSC2) complex which represses Rheb, a small GTPase that stimulates the phosphorylation of mTOR (*47*). Therefore, reduced mTOR activity by cellular stress induces autophagy. In addition, treatment of rapamycin can artificially inhibit mTOR activity, causing the induction of autophagy (*48*) (Fig. 2).

Autophagy begins with an isolation of the membrane, called phagophore, which is derived from endoplasmic reticulum (ER), Golgi apparatus or endosomes (*49*). Intracellular cargo, such as protein aggregates, damaged or old organelles and macromolecules, is recognized by SQSTM1/p62 then moved into phagophore (*50*). A phagophore sequesters the captured cargo and, elongation and closure of phagophore membrane formed double-membraned structure called autophagosome. Finally, autophagosome is fused with lysosome then, cargo degraded by lysosomal acid hydrolases in the autophagolysosome (*44*) (Fig. 3).

2.3. Regulatory role of autophagy in immunity

Autophagy can modulate various immune responses against pathogenic condition as following; (i) Autophagy directly eliminates intracellular pathogens, such as *Mycobacterium tuberculosis* (*51*) herpes simplex virus (HSV) (*52*) which is known as 'xenophagy'. (ii) Autophagy also regulates pro-inflammatory cytokine production in macrophages and DCs infected with pathogen or stimulated with PAMPs. For example, inhibition of autophagy upregulates IL-1α secretion in macrophages infected with *M. tuberculosis*. Therefore, in macrophage-specific autophagy gene knockout mice infected with *M. tuberculosis*, the bacteria burden is increased and the increased IL-1α production induces excessive Th17 response (*53*). (iii) Autophagy contributes to regular antigen presentation as well as cross-presentation in antigen presenting cells (APCs) then priming T cells (*54*). (iv) In addition, it is reported that induction of autophagy in APCs enhances memory response and vaccine efficacy of bacillus

Calmette-Guérin (BCG) (*55*). Taken together, regulation of autophagy upon pathogen infection affects not only pathogen clearance but also modulates innate and adaptive immune response.

2.4. Autophagy modulates cytokines production

It has been suggested that the production of several cytokines is regulated by autophagy (*56*), and IL-1β is one of the most widely studied cytokine. Autophagy degrades pro-IL-1β in the cytoplasm coincident with downregulation of cellular reactive oxygen species (ROS) level which is an essential component for inflammasome activation (*57*). Therefore, inhibition of autophagy prevents degradation of pro-IL-1β and activates inflammasome. As a result, secretion of IL-1β is increased by autophagy inhibition. However, it has been also suggested that autophagosome maturation is needed for IL-1β secretion via unconventional secretory pathway (*58*). Taken together, basal level of autophagy primarily inhibits IL-1β secretion by degradation of pro-IL-1β whereas induced autophagy increased IL-1β secretion via unconventional secretory pathway.

Regulatory role on the expression of other cytokines by autophagy is not as much studied as IL-1β. Expression of IL-6 is regulated by autophagy but that is controversial. IL-6 production is decreased by autophagy inhibition that reduces TLR downstream signaling molecules such as ERK, JNK, p38 and NF-κB in DCs infected with H1N1 (*59*). On the contrary, human peripheral blood mononuclear cells treated with autophagy inhibitor increased IL-6 production against *Borrelia burgdorferi* infection (*60*). Autophagy also regulates the expression of IL-12

family. Dendritic cells activated with LPS produce enhanced IL-12p70 but reduced IL-23 when autophagy was inhibited (*61, 62*). Macrophage migration inhibitory factor (MIF), which is pro-inflammatory cytokine then activates macrophages and T cells, is increased by autophagy inhibition in macrophages (*63*). Therefore, autophagy has pivotal roles for the regulation of inflammation by regulating cytokine expression.

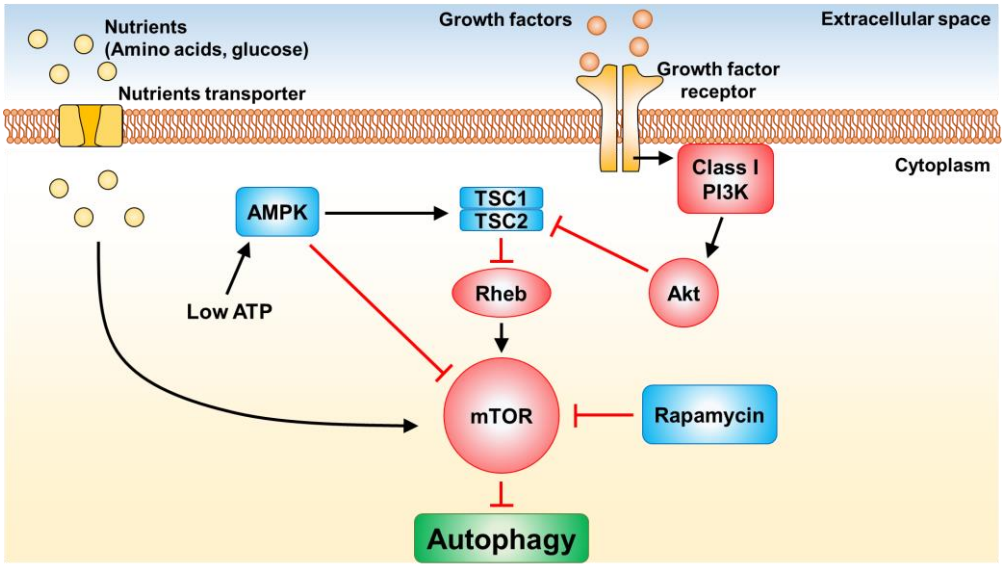


Figure 2. Signaling pathways involving autophagy modulation. In nutrient rich condition, class I PI3K and Akt activate mTOR which subsequently inhibits autophagy. Then, low level of intracellular ATP activates AMPK. Then, AMPK directly inhibits mTOR or activates TSC1/TSC2 complex which represses Rheb, which stimulates the phosphorylation of mTOR. Rapamycin can artificially inhibit mTOR activity, causing the induction of autophagy.

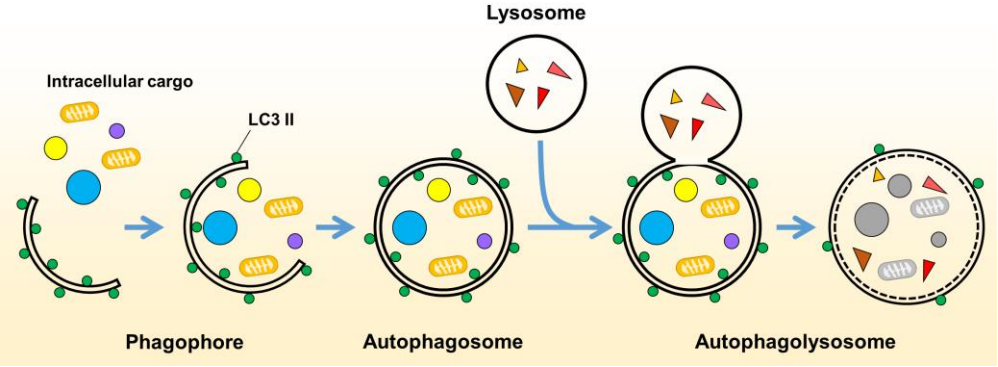


Figure 3. A schematic diagram for the formation and process of autophagy pathway. Autophagy begins with phagophore. Once start, intracellular cargo, such as protein aggregates, damaged or old organelles moved into phagophore. Then, phagophore sequesters the captured cargo and, elongation and closure of phagophore membrane formed double-membraned structure called autophagosome. Finally, autophagosome is fused with lysosome and then, cargoes are degraded by lysosomal acid hydrolases in the autophagolysosome.

II. Introduction

Listeria monocytogenes is a causative pathogen of listeriosis in both humans and animals after ingesting food contaminated with *L. monocytogenes* (*7*). It is a rod-shaped intracellular gram-positive and facultative anaerobic bacterium which resides in the cytoplasm of the host cells (*1*). *L. monocytogenes* is considered as a serious public health problem because they can grow even at 4°C (*9*), therefore, refrigeration could not prevent their growth. It has been reported that 20-30% mortality of listeriosis is over those of *Salmonella* and *Clostridium* infection in the United States (*64*).

In general, intracellular pathogens have an escaping strategy which avoids host cell killing mechanism. *L. monocytogenes* expresses proteins such as listeriolysin O (LLO), phospholipase C (PLC), and actin assembly-inducing protein (ActA), act to escape the damage from host attack and to allow the bacteria to reside in the cytoplasm of the host cell (*65*). Counteract to this, autophagy could eliminate intracellular pathogens including *Mycobacterium tuberculosis* (*51*) and *L. monocytogenes* (*66*).

Autophagy is a conserved cellular recycling pathway throughout the multi-cellular kingdom from yeast to mammalian cells (*31*). It is important for a cellular homeostasis for the degradation of old or damaged organelles, macromolecules, misfolded proteins to be re-used for their building blocks (*32*). Recently many studies demonstrated that autophagy also plays an important role in the regulation of immune responses, such as pathogen degradation, antigen-presentation, and

pro-inflammatory cytokine production (*67*).

Interleukin (IL)-1β, IL-6 and IL-12 are crucial pro-inflammatory cytokines for resistance and clearance against *L. monocytogenes* infection (*15-17*). IL-1β is important cytokine for T cell-independent clearance of intracellular *L. monocytogenes* (*21-23*). IL-1β recruits neutrophils to the infection sites (*21*) and induces IL-12 that stimulates natural killer cells to produce IFN-γ (*23*) during *L. monocytogenes* infection. Also, IL-1β induced increase of blood leukocyte numbers, and macrophage responsiveness to IFN-γ (*21*). Recombinant IL-6 injection to the mice enhanced resistance against listeriosis since IL-6 plays a pivotal role in IFN-γ production in T cells upon *L. monocytogenes* infection (*26*). IL-12 induces type 1 T helper (Th1) cells differentiation and IFN-γ production by natural killer cells and Th1 cells against *L. monocytogenes* infection (*17, 29*). It appeared that pro-inflammatory cytokines are required for the protection against *L. monocytogenes* infection.

Autophagy can modulate production of IL-1β (*57*), IL-6 (*60*) and IL-12 (*61*) in macrophages or dendritic cells infected with pathogens or stimulated with TLR agonist. For example, autophagy inhibition reduced IL-6 and IL-12 production in macrophages stimulated with lipopolysaccharide (LPS), and dendritic cells infected with H1N1 virus (*57, 59*). Thus, inhibition of autophagy in DCs exhibited an impaired ability to induce H1N1 specific T-cell response. On the contrary, human peripheral blood mononuclear cells treated with autophagy inhibitor increased IL-6 production coincident with adaptive immune response against *Borrelia burgdorferi* infection (*60, 68*). Therefore, it is probable that

protective role of cytokines regulated by autophagy is dependent on type of infection and stimuli.

L. monocytogenes infection, however, it is unclear how autophagy modulates pro-inflammatory cytokines. Therefore, in this study, we examined whether autophagy regulates IL-1β, IL-6 and IL-12 production against *L. monocytogenes* infection in BMDMs and how they were controlled by autophagy.

III. Materials and methods

Mouse and bone marrow-derived macrophages

Six-week old C57BL/6 female mice were purchased from Orient bio Inc. (Korea). All experimental procedures in relation to mouse were approved by Institutional Animal Care and Use Committee (IACUC) at Seoul National University (Approval number: SNU-131025-2-4). In order to generate bone marrow-derived macrophages (BMDMs), bone marrow cells, isolated from femurs and tibias, were cultured in complete DMEM/F-12 medium containing DMEM/F-12 GlutaMAX (Thermo Fisher Scientific, USA), 10% L929 cell culture supernatant, 10% fetal bovine serum (FBS, Invivogen, USA), 50 μM β-mercaptoethanol (Thermo Fisher Scientific) and 100 U/ml streptomycin/penicillin (Invivogen) for 7 days.

Bacteria

Wild type *Listeria monocytogenes* strain 10403S was cultured in brain heart infusion (BHI; BD Biosciences, USA) media for 9 h at 250 rpm on a shaking incubator at 30°C. Bacteria culture suspension was harvested by centrifugation at 3,000 × g, for 10 min and thoroughly washed twice with sterile phosphate buffered saline (PBS). Bacteria count was estimated by measuring optical density at 600 nm as previously described (*69*). The number of bacteria infected to BMDMs was measured by colony-forming unit (CFU) assay through a serial

dilution and plating on BHI agar. Heat-killed *L. monocytogenes* (HKLM) was prepared by incubating bacteria at 60°C for 90 min in sterile PBS. The complete inactivation of *L. monocytogenes* was determined by plating on BHI agar.

Intracellular bacteria CFU assay

BMDMs were seeded on 96-well plates (5×10^4 cells per well). The cells were infected with 20 MOI of *L. monocytogenes* for 1 h at 37°C in humidified incubator with 5% CO_2. The cells were, then, washed three times with sterile PBS, and incubated for indicated time in the presence or absence of autophagy modulator. The cells were lysed with 100 µl of sterile distilled water. Cell lysates were diluted by 10-fold serial dilutions and plated on BHI agar. The plates were incubated for overnight at 37°C and visible colonies were counted.

Autophagy induction and inhibition

Autophagy was induced in BMDMs by treatment of 25 µg/ml rapamycin (LC Laboratories, USA). For autophagy inhibition, the cells were treated with 100 nM of bafilomycin A1 (Invivogen) or 10 mM of 3-Methyladenine (3-MA, Sigma-Aldrich, USA).

Western blot

BMDMs were washed with ice-cold PBS and lysed in RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitor cocktail (Thermo Fisher

Scientific). Same amount of protein extract was loaded in Tris-glycine polyacrylamide gel and electrophoresed. Then, the proteins were transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Bio-rad, USA) for 90 min at 4°C, and blocked with 5% skim milk in TBST (1 M Tris-HCl, 5 M NaCl, 10% Tween-20) for 1 h at room temperature. The blot was incubated with rabbit anti-LC3A/B IgG, anti-GAPDH IgG, anti-IκBα IgG (Cell signaling, USA), rabbit anti-IRF5 IgG (Abcam, UK) antibody overnight at 4°C. Subsequently, the membrane was washed and incubated with goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotechnology, USA) for 1 h at room temperature. The target protein was visualized with enhanced chemiluminescence system (GE Healthcare Life Sciences, USA), followed by analysis using ChemiDoc XRS (Bio-rad)

Confocal immunofluorescence microscopy

BMDMs were fixed with 100% methanol for 15 min at -20°C, and blocked with 5% FBS, 0.3% triton-100X for 1 h at room temperature. The cells were incubated with rabbit anti-LC3A/B or rabbit anti-IRF5 IgG antibody followed by staining with goat anti-rabbit IgG conjugated with Alexa flour 488 (Thermo Fisher Scientific) and 4′,6-diamidino-2-phenylindole (DAPI, Abcam) for nuclei. Images were captured using a confocal laser scanning microscope, (LSM700, Carl Zeiss, Germany).

Enzyme-linked immunosorbent assay (ELISA)

Mouse interleukin (IL)-1β, IL-6 and IL-12/23p40 in the culture supernatants

were measured by using DuoSet ELISA kits (R&D Systems, USA) according to the manufacturer's instruction. Briefly, 96-well microplate was pre-coated with capture antibody. After blocking with reagent diluent buffer (1% BSA in PBS) for at least 1 h at room temperature, supernatants of the cells and standard recombinant proteins were added in reagent diluent buffer and incubate 2 h at room temperature. Detection antibody was added to each well and incubated for 2 h at room temperature, followed by addition of the Streptavidin-HRP in reagent diluent buffer to each well. After incubation for 20 min at room temperature, tetramethylbenzidine (TMB; Millipore, USA) was added to develop the color and then the reaction was stopped by adding 2 N H_2SO_4. Optical density of each well was measured by VersaMax ELISA Microplate Reader (Molecular Devices, USA) at wavelength 450 nm.

RNA extraction and cDNA synthesis

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instruction. Briefly, BMDMs were treated with 1 ml of TRIzol reagent per 1-2 × 10^6 cells. Total RNA was isolated by the addition of 200 μl of chloroform followed by centrifugation at 12,000 × g for 15 min at 4°C. The aqueous phase was transferred into a new tube and 500 μl of isopropanol was added. Then, the samples were incubated for 10 min at room temperature for RNA precipitation and centrifuged at 12,000 × g for 10 min at 4°C. RNA pellet was obtained after washing with 75% ethanol and air dried for 5-10 min then resuspended with DEPC-treated water (Sigma-Aldrich). RNA

concentration was quantified with NanoDrop (Amersham Biosciences, USA) at A260. For complementary DNA (cDNA) synthesis, reverse-transcription polymerase chain reaction (PCR) was performed using M-MLV Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's instruction.

Real-time quantitative PCR

The real-time quantitative PCR (qRT-PCR) was carried out using a StepOne Plus real-time PCR system (Applied Biosystems, USA). The PCR reaction was carried out in 96-well reaction plate with 10 µl Power SYBR Green PCR master mix (Applied Biosystems), 0.5 µl primers, 1 µl cDNA template and 8 µl nuclease-free H_2O. Primers used were il-1beta: forward 5'-GAA GAG CCC ATC CTC TGT GA-3', reverse 5'-CGA CAG CAC GAG GCT TTT TT-3'; il-6: forward 5'-TTC CAT CCA GTT GCC TTC TTG-3', reverse 5'-AGG TCT GTT GGG AGT GGT ATC-3'; il-12p40: forward 5'-TCA TCA GGG ACA TCA TCA AAC C-3', reverse 5'-TGA GGG AGA AGT AGG AAT GGG-3'; irf5: forward 5'-CCT CAG CCG TAC AAG ATC TAC GA-3', reverse 5'-GTA GCA TTC TCT GGA GCT CTT CCT-3'; 50 cycles of PCR were performed in duplicate for each primer. Relative quantification was determined using the ΔΔCt method and normalized to expression of the housekeeping gene gapdh: forward 5'- CTC CAC TCA CGG CAA ATT CA -3', reverse 5'- GCC TCA CCC CAT TTG ATG TT -3'.

Flow cytometry

To measure cell viability, BMDMs detached from culture plate were stained with annexin V (BD Biosciences), propidium iodide (Sigma-Aldrich) at 4°C for 20 min in the dark. After staining, the cells were washed, and the intensity of the markers was examined by flow cytometry (FACS Canto II, BD Biosciences). All flow cytometric data were analyzed by using FlowJo software (Tree star, USA)

Statistics

The mean value ± standard deviation was determined for each group. For comparison of means between two groups, the data were analyzed using two-tailed paired student's t-test and considered statistically significant when p-value was less than 0.05. For multiple group comparison, one-way ANOVA was used. All statistical analysis was performed using GraphPad Prism 5 version 5.01 (GraphPad Software, USA)

IV. Results

1) Rapamycin treatment induced autophagy in BMDMs

Rapamycin, specific inhibitor of TOR signaling (*70*), is known as an inducer of autophagy. To examine which concentration of rapamycin properly induces autophagy in mouse BMDMs, the cells were treated with various concentration of rapamycin for 2 h. Rapamycin treatment induced LC3 conversion in a dose-dependent manner (Fig. 4A) as early as 30 min (Fig. 4B) without cell death (Supplementary Fig. 1). Using confocal immunofluorescence microscopy, we determined autophagosome formation and the result showed that the number of LC3 puncta in BMDMs was higher in rapamycin treatment group than the cells treated with DMSO (Fig. 4C and D). These results suggested that rapamycin induced autophagosome formation in BMDMs at the early time after the rapamycin treatment.

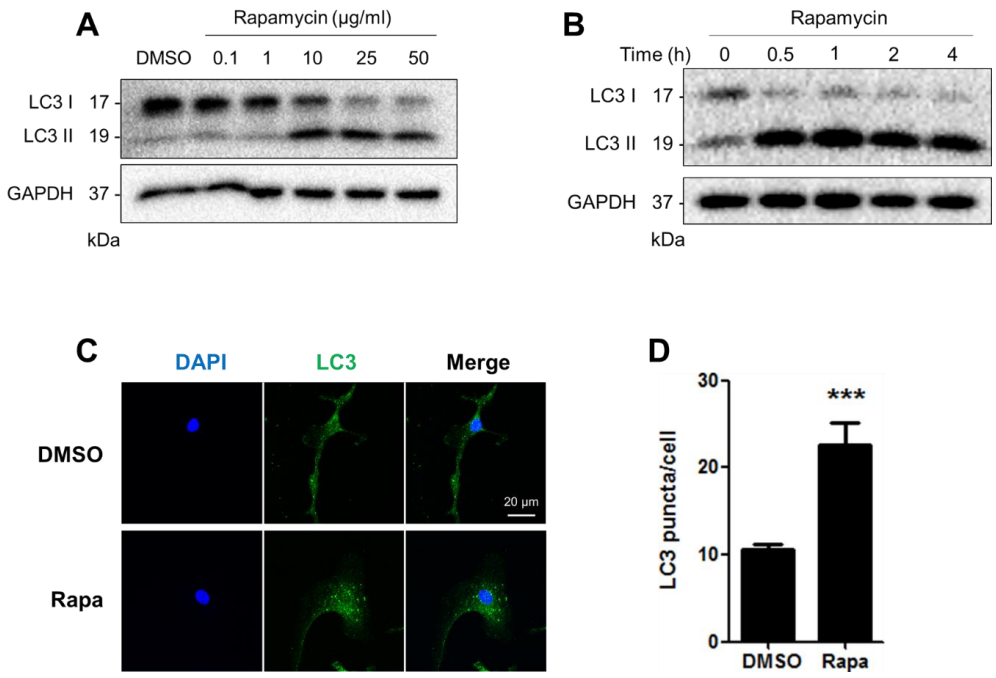


Figure 4. Rapamycin induces autophagy in BMDMs. In order to examine autophagy induction, BMDMs were treated with (A) indicated concentration of rapamycin for 2 h or (B) 25 μg/ml of rapamycin at indicated time points. Then LC3 conversion was visualized with rabbit anti-LC3A/B IgG antibody using western blot. (C and D) The cells were stained with rabbit anti-LC3A/B IgG antibody 4 h after the rapamycin treatment. (C) Using confocal immunofluorescence microscopy, formation of LC3 puncta was examined. (D) The number of LC3 puncta per cell was counted for at least ten different cells. Data represent mean ± S.D. for ten replicates. *** indicates $p < 0.001$.

2) L. monocytogenes infection induced pro-inflammatory cytokines in BMDMs

To determine optimal multiplicity of infection (MOI) for *L. monocytogenes* infection to produce pro-inflammatory cytokines, BMDMs were infected with at various MOI. IL-1β secretion peaked at MOI 20 (Fig. 5A) while IL-6 secretion reached to the steady state at MOI 10 (Fig. 5B). Interestingly, IL-12p40 secretion maximized at MOI 5 (Fig. 5C). Therefore, MOI 20 was chosen for further experiments.

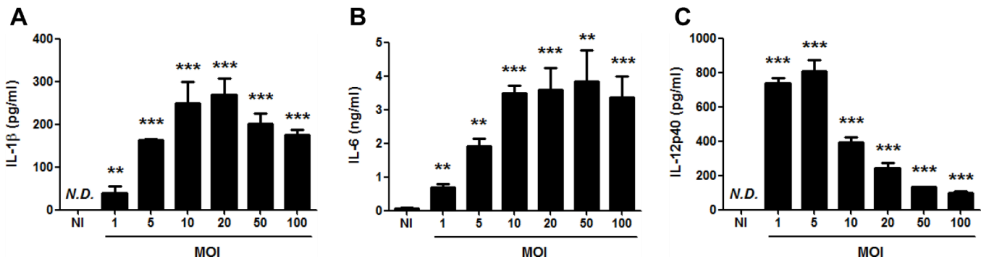


Figure 5. *L. monocytogenes* infection produces pro-inflammatory cytokines in BMDMs. BMDMs were infected with indicated MOI of *L. monocytogenes* for 1 h. At 24 h post infection, levels of (A) IL-1β, (B) IL-6, (C) IL-12p40 in the cell culture supernatants were measured by ELISA. Data represent mean ± S.D. from triplicate. NI: Non-infection. N.D.: Non Detectable. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3) L. monocytogenes infection induced autophagy in BMDMs

To examine whether *L. monocytogenes* infection induces autophagy in BMDMs, we examined LC3 conversion and puncta formation. After the infection, LC3 conversion was enhanced in a time-dependent manner (Fig. 6A). *L. monocytogenes* infection induced LC3 puncta formation in BMDMs regardless of autophagy induction (Fig. 6B), which was visualized using confocal microscopy (Fig. 6C). Taken together, *L. monocytogenes* infection induced autophagy in BMDMs.

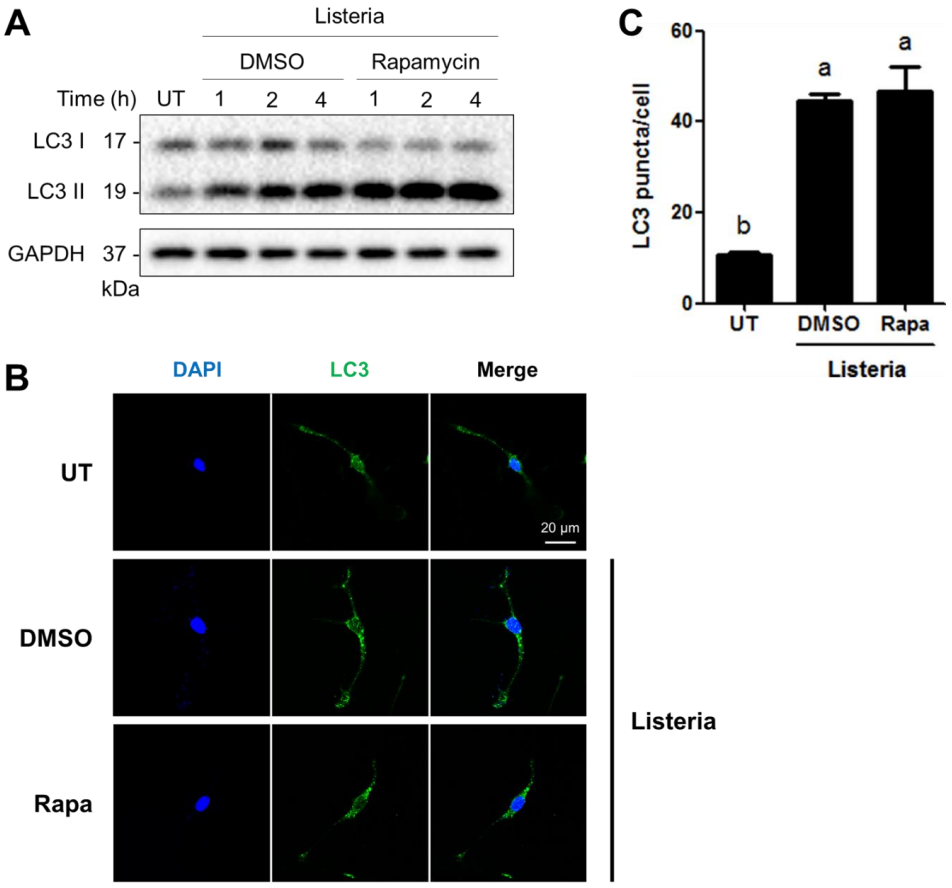


Figure 6. *L. monocytogenes* infection induced autophagy in BMDMs. BMDMs were infected with 20 MOI of *L. monocytogenes* for 1 h, followed by rapamycin treatment (25 μg/ml). (A) Immunoblot for LC3 conversion with rabbit anti-LC3A/B IgG antibody at indicated time point after the infection. LC3 puncta formation in BMDMs was examined (B) by confocal microscopy at 4 h post infection, and (C) the number of LC3 puncta per cell was counted. Data represent mean ± S.D. from five replicates. Different letters denote a significant difference at $p < 0.05$.

4) Induction of autophagy inhibited L. monocytogenes infection in BMDMs

Next, we examined whether autophagy induction could reduce *L. monocytogenes* survival in BMDMs. Rapamycin treatment inhibited *L. monocytogenes* survival in BMDMs infected with *L. monocytogenes* (Fig. 7A). On the contrary, autophagy inhibition using bafilomycin A1 enhanced survival of *L. monocytogenes* (Fig. 7B). These results demonstrated that autophagy reduces survival of *L. monocytogenes* in BMDMs.

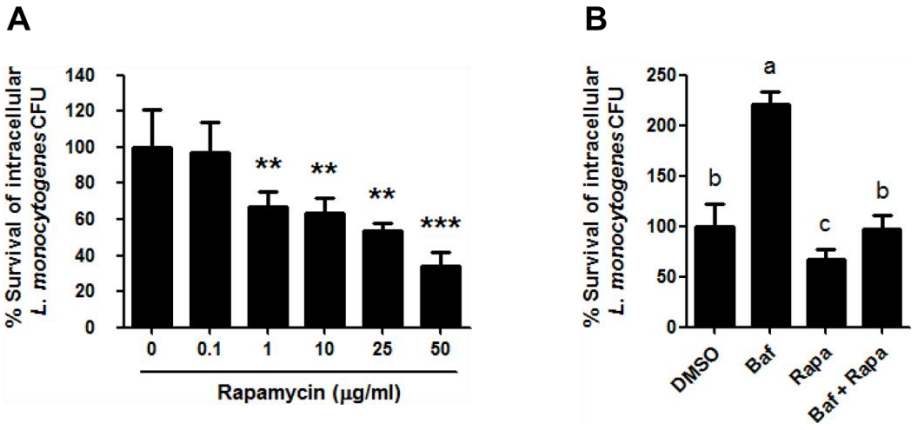


Figure 7. Autophagy reduced survival of *L. monocytogenes* in BMDMs. BMDMs were infected with 20 MOI of *L. monocytogenes* for 1 h. The cells were washed three times and further incubated with (A) indicated concentration of rapamycin (**, $p < 0.01$; ***, $p < 0.001$), and (B) rapamycin (25 μg/ml) and/or bafilomycin A1 (100 nM) for 4 h. The cells were lysed to determine survival of *L. monocytogenes* in BMDMs. Data represent mean ± S.D. for at least six wells. Different letters denote a significant difference at $p < 0.05$.

5) Rapamycin-induced autophagy increased pro-inflammatory cytokines BMDMs infected with L. monocytogenes

It has been suggested that autophagy could regulate the production of pro-inflammatory cytokines under inflamed condition including toll-like receptor (TLR) ligand stimulation and pathogenic infection (*71*). To determine autophagy induction regulates pro-inflammatory cytokine secretion against *L. monocytogenes* infection, BMDMs were treated with rapamycin and then infected with *L. monocytogenes*. Pre-treatment of rapamycin induced autophagy enhanced IL-1β (Fig. 8A), IL-6 (Fig. 8B) and IL-12p40 (Fig. 8C) and mRNA expression of those cytokines (Supplementary Fig. 2) in BMDMs infected with *L. monocytogenes*. These results suggest that rapamycin-induced autophagy enhanced the expression of pro-inflammatory cytokines in BMDMs infected with *L. monocytogenes*.

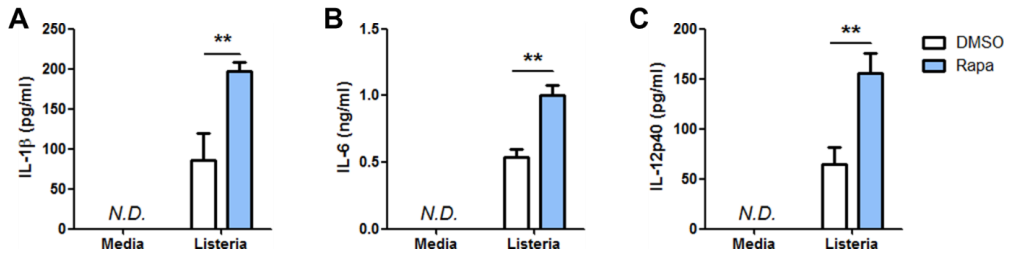


Figure 8. Rapamycin treatment increased pro-inflammatory cytokines in BMDMs infected with *L. monocytogenes*. BMDMs were incubated in the presence or absence of rapamycin (25 μg/ml) for 1 h, and then infected with 20 MOI of *L. monocytogenes* for 1 h. The cells were washed three times and incubated in the complete media for 24 h. Supernatants were harvested and the levels of (A) IL-1β, (B) IL-6 and (C) IL-12p40 were measured by ELISA. N.D.: Non Detectable. **, $p < 0.01$.

To validate whether expression of pro-inflammatory cytokines was increased by the induction of autophagy, we treated BMDMs with autophagy inhibitors, bafilomycin A1 and 3-MA. Bafilomycin A1 prevents autophagic flux and autophagosome maturation by inhibiting fusion between lysosomes and autophagosomes (*72*), whilst 3-MA inhibits the early stage of autophagy formation including the initiation of class III phosphatidylinositol 3-kinases (PI3K) signaling and autophagosome formation (*73*). No detectable IL-1β (Fig. 9A) was observed when the cells were treated with bafilomycin A1 whereas 3-MA treatment caused the increase of IL-1β production (Fig. 9D). Levels of IL-6 were not changed by autophagic flux inhibition (Fig. 9B) however, blocking the autophagosome formation decreased IL-6 (Fig. 6E). The results showed that inhibition of either autophagic flux (Fig. 9C) or autophagosomal maturation (Fig. 9F) reduced levels of IL-12p40. These results demonstrated that inhibition of autophagy reduces the expression of pro-inflammatory cytokines in BMDMs infected with *L. monocytogenes*.

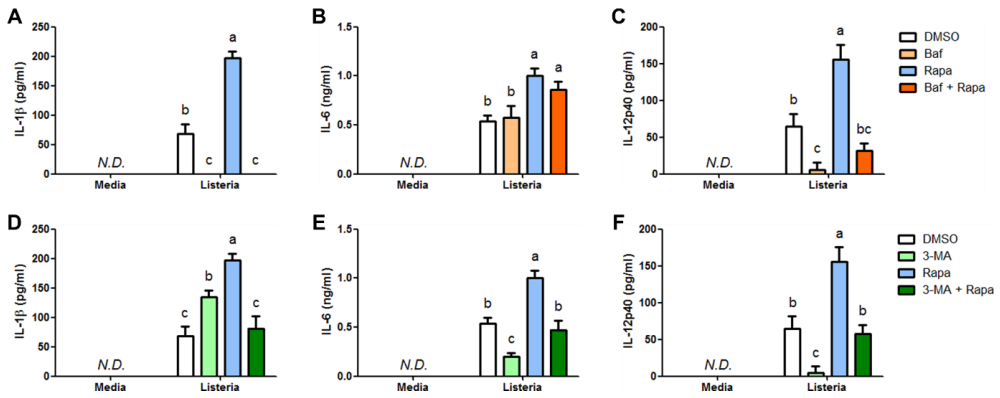


Figure 9. Autophagy inhibition reduced the expression of pro-inflammatory cytokines in BMDMs infected with *L. monocytogenes*. BMDMs were incubated with complete media containing rapamycin (25 μg/ml), bafilomycin A1 (100 nM) or 3-MA (10 mM) for 1 h, followed by *L. monocytogenes* infection for 1 h. In 3-MA + Rapa group, 3-MA was treated for 1 h before rapamycin treatment. Then, the cells were washed three times and incubated in complete media for 24 h. Supernatants were harvested and the levels of (A and D) IL-1β, (B and E) IL-6, and (C and F) IL-12p40 were measured by ELISA. N.D.: Non Detectable. Different letters denote a significant difference at $p < 0.05$.

6) Autophagy modulates pro-inflammatory cytokines in BMDMs treated with HKLM

Next, we examined whether the increased clearance of cytosolic *L. monocytogenes* by autophagy was related with the modulation of pro-inflammatory cytokines. Unlike live *L. monocytogenes*, heat-killed *L. monocytogenes* (HKLM) cannot invade into the cytosol. Therefore, we treated HKLM to BMDMs and examined cytokine production. The result showed that autophagic flux inhibition did not affect IL-6 production (Fig. 10A) whereas blocking of autophagosome formation reduced IL-6 production (Fig. 10C). On the other hand, both autophagic flux inhibition (Fig. 10B) and blocking of autophagosome formation (Fig. 10D) reduced levels of IL-12p40. Taken together, HKLM-induced IL-6 and IL-12p40 production was regulated by autophagy as similar as live *L. monocytogenes* infection.

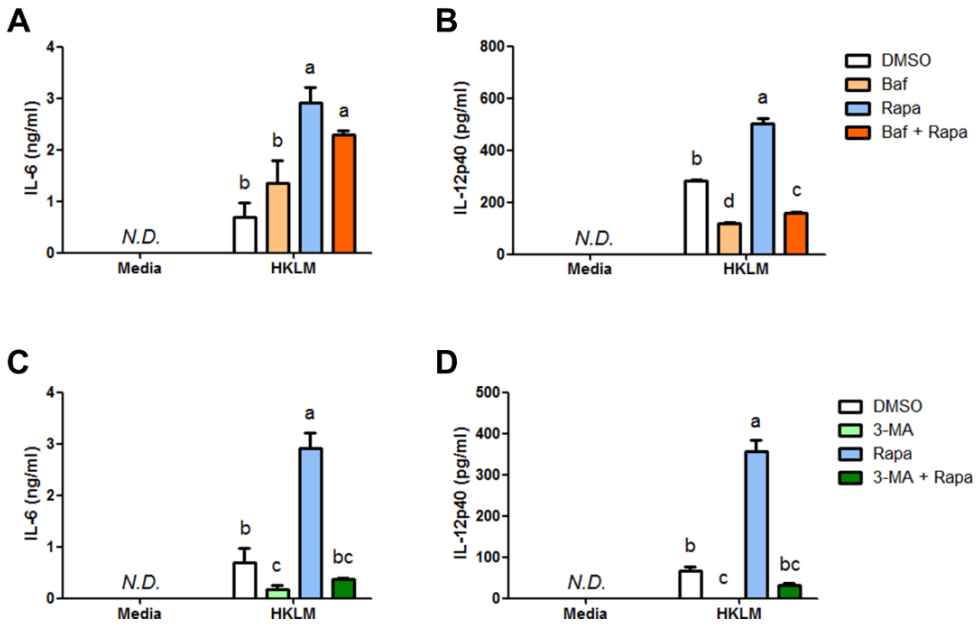


Figure 10. Autophagy modulates pro-inflammatory cytokine production in BMDMs treated with HKLM. BMDMs were treated with rapamycin (25 μg/ml), bafilomycin A1 (100 nM) or 3-MA (10 mM) for 1 h. In 3-MA + Rapa group, 3-MA was treated for 1 h before rapamycin treatment. Then, the cells were incubated in the presence or absence of HKLM. At 24 h after HKLM treatment, levels of (A and C) IL-6 and (B and D) IL-12p40 in the supernatants were measured by ELISA. N.D.: Non Detectable. Different letters denote a significant difference at $p < 0.05$.

7) Autophagy induction increased toll-like receptors downstream signaling in BMDMs

Interferon regulatory factor 5 (IRF5) is known as the one of the toll-like receptors (TLRs) downstream molecules and a main transcription factor regulates the expression of IL-6 and IL-12 cytokine family in dendritic cells and macrophages upon bacterial infection (*74*). Therefore, we examined the expression of IRF5 in BMDMs with or without autophagy induction. Rapamycin-induced autophagy increased IRF5 expression when compared with that of control group and blocking of autophagy by using bafilomycin A1 treatment reduced IRF5 expression at both mRNA and protein level (Supplementary Fig. 3A and B). In addition, we also confirmed nucleus translocation of IRF5. Rapamycin-induced autophagy increased IRF5 translocation compared with control group after *L. monocytogenes* infection (Fig. 11A). Furthermore, same as IRF5, nuclear factor-kappa B (NF-κB) is a main transcription factor which regulates pro-inflammatory cytokine production under TLRs stimulation (*75*). Inhibitor of nuclear factor-kappa B α (IκBα) degradation is needed for NF-κB activity because IκBα inhibits nuclear translocation of NF-κB (*76*). Therefore, we figured out IκBα degradation. Expression of IκBα was dramatically reduced by HKLM treatment within 15 min, and then it was restored Fig. 11B). However, rapamycin treatment inhibited the recovery of IκBα expression (Fig. 11B). These results suggest that rapamycin-induced autophagy could enhance the TLRs downstream signaling by increasing translocation of IRF5 and degradation of IκBα that modulates pro-inflammatory cytokine production in BMDMs.

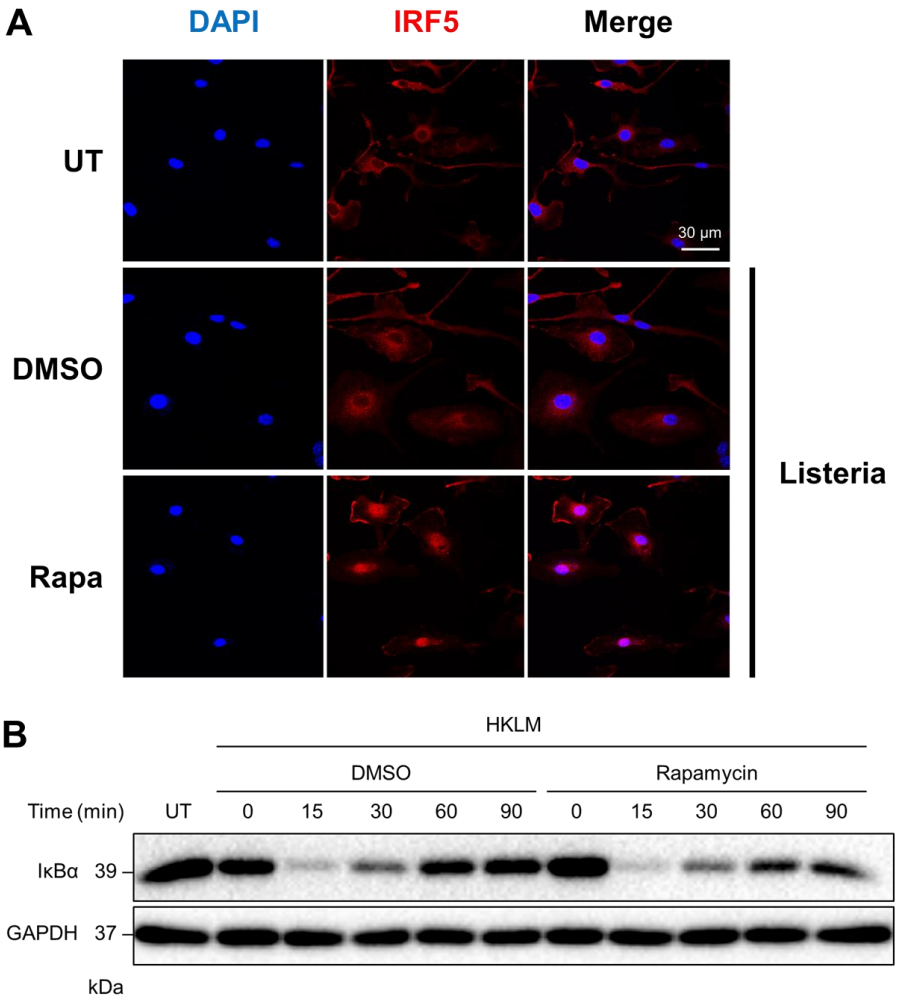
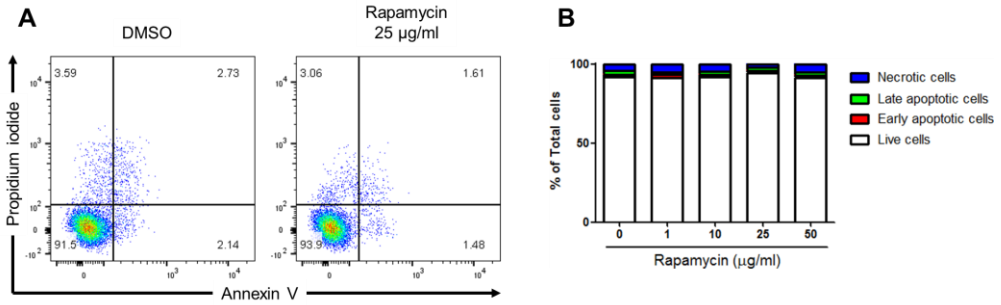
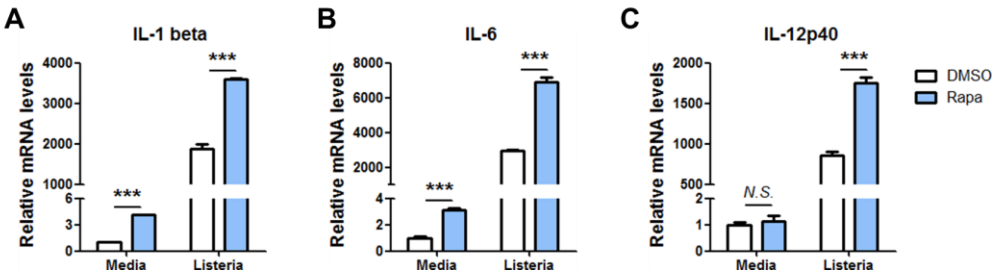


Figure 11. Rapamycin-induced autophagy could modulate nucleus translocation of IRF5 and degradation of IκBα in BMDMs. (A) Intracellular location of IRF5 in rapamycin treated BMDMs was examined by confocal microscopy at 1 h post infection. (B) Immunoblot for IκBα with rabbit anti-IκBα IgG antibody using BMDMs incubated with rapamycin for 1 h then treated with HKLM for indicated time points.

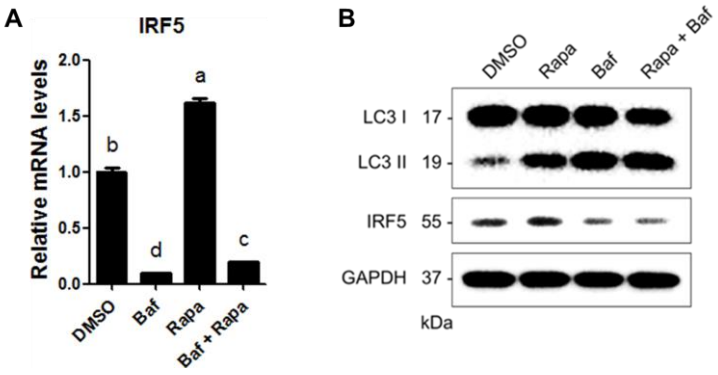
V. Supplementary Results



Supplementary Figure 1. Rapamycin treatment did not influence physiological status of BMDMs. BMDMs were treated with indicated concentration of rapamycin for 4 h. (A) Cells were stained with Annexin V-FITC (AV) and propidium iodide (PI), and the cell death was examined by using flow cytometry. (B) The percentage of Annexin V / PI positive or negative cells. Live cells: AV - / PI -, Early apoptotic cells: AV + / PI -, Late apoptotic cells: AV + / PI +, Necrotic cells: AV - / PI +.



Supplementary Figure 2. Rapamycin-induced autophagy enhanced pro-inflammatory cytokines mRNA expression in BMDMs infected with *L. monocytogenes*. BMDMs were treated with presence or absence of rapamycin (25 μg/ml) for 1 h, and then infected with 20 MOI of *L. monocytogenes* for 1 h. The cells were washed three times and incubated in the complete media for 6 h. mRNA of (A) IL-1β, (B) IL-6 and (C) IL-12p40 was measured by real-time qRT-PCR. Data represent mean ± S.D. for three replicates. ***, $p < 0.001$. N.S.: Not significant.



Supplementary Figure 3. Rapamycin-induced autophagy modulated expression of IRF5 in BMDMs. BMDMs were incubated with the presence or absence of rapamycin, or bafilomycin A1 for 4 h. Levels of (A) IRF5 mRNA expression was measured by qRT-PCR. Different letters denote a significant difference at $p < 0.05$. (B) The cell lysates were used for immunoblot for IRF5 with rabbit anti-IRF5 IgG antibody.

VI. Discussion

Autophagy is primarily a cellular recycling pathway, but it also plays a role in immunity such as eliminating intracellular pathogens (*51*) and modulating pro-inflammatory cytokine (*56*). Like other intracellular bacteria, growth of *Listeria monocytogenes* in the host cytoplasm can be restricted by autophagy (*66*). However, it is not clearly understood how autophagy modulates pro-inflammatory cytokine production in human and animals infected with *L. monocytogenes*. Therefore, in the present study, I examined whether autophagy induction modulates production of pro-inflammatory cytokine against *L. monocytogenes* infection in bone marrow-derived macrophages (BMDMs).

Notable findings in this study are as follow; (i) Rapamycin-induced autophagy enhanced production of IL-1β, IL-6 and IL-12 in BMDMs infected with *L. monocytogenes*. (ii) Such cytokine production appeared to be differentially regulated by autophagy. (iii) Pro-inflammatory cytokines in BMDMs treated with heat-killed *L. monocytogenes* (HKLM) were increased by autophagy suggesting TLR2-dependent manner. (iv) Nuclear translocation of IRF5 and degradation of IκBα were enhanced by induction of autophagy. Taken together, induction of autophagy upregulates pro-inflammatory cytokines production in BMDMs infected with *L. monocytogenes*.

Autophagy induction increased pro-inflammatory cytokines in macrophages infected with *L. monocytogenes*. It is reported that autophagy induction increased pro-inflammatory cytokines in dendritic cells (DCs) infected with respiratory

syncytial virus (RSV) and H1N1 (*59, 77*). In addition, T cell priming of macrophages and DCs infected with Bacillus Calmette-Guérin (BCG) was increased by autophagy induction (*55*) resulting increase of protective immunity. On the contrary, in case of *Borrelia burgdorferi* infection, autophagy induction reduced pro-inflammatory cytokine and adaptive immune response (*60, 68*). It is important to note that regulation of cytokines and protective immunity by autophagy induction depend on type of stresses (i.e., infection, nutrients and stimulants and etc.) and duration of infection.

The secretion of IL-1β was increased by blocking of autophagosome formation (Fig. 9D) whilst the inhibition of autophagosome maturation reduced IL-1β secretion (Fig. 9A). It has been suggested in the IL-1β secretion that autophagosome formation and autophagosome maturation have an opposite effect. IL-1β secretion increased by the inhibition of autophagosome formation, because autophagy could degrade pro-IL-1β in the cytoplasm (*57*). Whereas autophagosome maturation required for IL-1β secretion could be achieved via unconventional secretory pathway (*58*). In addition, bafilomycin A1 is also acting as the inhibitor of vacuolar-type H^+-ATPase, which inhibits acidification of lysosome (*78*), which is necessary for NALP3 inflammasome activation (*79*). Therefore, our results suggested that bafilomycin A1 inhibited IL-1β secretion by blocking unconventional secretory pathway, and at the same time, the activation of NALP3 inflammasome. Therefore, depending on which step of the autophagy pathway is blocked, IL-1β secretion is modulated differently in BMDMs infected with *L. monocytogenes*.

It has been reported that HKLM does not invade into the cells but can stimulate TLR (*80*). Thus, we have used HKLM to examine whether TLR signaling is associated with increased cytokine production in autophagy-induced macrophages. HKLM-induced cytokines in BMDMs are increased by autophagy induction in the present study proves the possibility that enhanced pro-inflammatory cytokines by autophagy induction was dependent on TLR signaling pathway. Although additional experiments are needed using knock-out system, increased pro-inflammatory cytokine in autophagy-induced BMDMs seemingly depends on TLR signaling.

In this study, I examined autophagy induction enhanced not only IRF5 expression and nuclear translocation but also IκBα degradation which could enhance production of pro-inflammatory cytokines. IRF5 is one of the TLR downstream molecules and a main transcription factor which regulates the expression of IL-6 and IL-12 (*74*). It was noting that there are no reports showing the relationship between IRF5 and autophagy. Recently, it has been revealed that other IRF family, such as IRF8 and IRF7, and autophagy was closely related with pathogen infection (*59, 81*). For instance, nucleus translocation of IRF7, transcription factor which is also a downstream molecule of TLR signal inducing type-1 interferon gene expression, was down regulated by autophagy inhibition in DCs infected with H1N1 (*59*). In addition, there are reports on relationship between autophagy and NF-κB signaling (*82*). Upon TNF-α stimulation, IκBα was degraded in the autophagosome and blockade of autophagy inhibited IκBα degradation coincidently with low NF-κB expression (*76*). Therefore, it is

important to further define the exact mechanism in the future how IRF5 expression and nucleus translocation was upregulated by autophagy induction upon *L. monocytogenes* infection.

In conclusion, rapamycin-induced autophagy reduced survival of intracellular *L. monocytogenes* and increased pro-inflammatory cytokine production in BMDMs infected with *L. monocytogenes*.

VII. Literature Cited

1. L. G. Tilney, D. A. Portnoy, Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, Listeria monocytogenes. *J Cell Biol* **109**, 1597-1608 (1989).
2. S. Dramsi, C. Kocks, C. Forestier, P. Cossart, Internalin-mediated invasion of epithelial cells by Listeria monocytogenes is regulated by the bacterial growth state, temperature and the pleiotropic activator prfA. *Mol Microbiol* **9**, 931-941 (1993).
3. J. Pizarro-Cerda, P. Cossart, Bacterial adhesion and entry into host cells. *Cell* **124**, 715-727 (2006).
4. K. Hybiske, R. S. Stephens, Exit strategies of intracellular pathogens. *Nat Rev Microbiol* **6**, 99-110 (2008).
5. M. Hamon, H. Bierne, P. Cossart, Listeria monocytogenes: a multifaceted model. *Nat Rev Microbiol* **4**, 423-434 (2006).
6. M. Bonazzi, M. Lecuit, P. Cossart, Listeria monocytogenes internalin and E-cadherin: from bench to bedside. *Cold Spring Harb Perspect Biol* **1**, a003087 (2009).
7. W. F. Schlech, 3rd *et al.*, Epidemic listeriosis--evidence for transmission by food. *N Engl J Med* **308**, 203-206 (1983).
8. R. F. Lamont *et al.*, Listeriosis in human pregnancy: a systematic review. *J Perinat Med* **39**, 227-236 (2011).
9. R. Bortolussi, Listeriosis: a primer. *CMAJ* **179**, 795-797 (2008).
10. E. Scallan *et al.*, Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis* **17**, 7-15 (2011).

11. J. L. Self *et al.*, Notes from the Field: Outbreak of Listeriosis Associated with Consumption of Packaged Salad - United States and Canada, 2015-2016. *MMWR Morb Mortal Wkly Rep* **65**, 879-881 (2016).
12. F. S. Southwick, D. L. Purich, Intracellular pathogenesis of listeriosis. *N Engl J Med* **334**, 770-776 (1996).
13. M. E. Temple, M. C. Nahata, Treatment of listeriosis. *Ann Pharmacother* **34**, 656-661 (2000).
14. H. Hof, An update on the medical management of listeriosis. *Expert Opin Pharmacother* **5**, 1727-1735 (2004).
15. E. A. Havell, L. L. Moldawer, D. Helfgott, P. L. Kilian, P. B. Sehgal, Type I IL-1 receptor blockade exacerbates murine listeriosis. *J Immunol* **148**, 1486-1492 (1992).
16. S. A. Dalrymple *et al.*, Interleukin-6-deficient mice are highly susceptible to Listeria monocytogenes infection: correlation with inefficient neutrophilia. *Infect Immun* **63**, 2262-2268 (1995).
17. C. S. Tripp, M. K. Gately, J. Hakimi, P. Ling, E. R. Unanue, Neutralization of IL-12 decreases resistance to Listeria in SCID and C.B-17 mice. Reversal by IFN-gamma. *J Immunol* **152**, 1883-1887 (1994).
18. A. F. Kiderlen, S. H. Kaufmann, M. L. Lohmann-Matthes, Protection of mice against the intracellular bacterium Listeria monocytogenes by recombinant immune interferon. *Eur J Immunol* **14**, 964-967 (1984).
19. M. D. Wewers, H. A. Dare, A. V. Winnard, J. M. Parker, D. K. Miller, IL-1 beta-converting enzyme (ICE) is present and functional in human alveolar macrophages: macrophage IL-1 beta release limitation is ICE independent. *J Immunol* **159**, 5964-5972 (1997).
20. R. Schindler, P. Ghezzi, C. A. Dinarello, IL-1 induces IL-1. IV. IFN-

gamma suppresses IL-1 but not lipopolysaccharide-induced transcription of IL-1. *J Immunol* **144**, 2216-2222 (1990).

21. H. W. Rogers, C. S. Tripp, R. D. Schreiber, E. R. Unanue, Endogenous IL-1 is required for neutrophil recruitment and macrophage activation during murine listeriosis. *J Immunol* **153**, 2093-2101 (1994).
22. H. W. Rogers *et al.*, Interleukin 1 participates in the development of anti-Listeria responses in normal and SCID mice. *Proc Natl Acad Sci U S A* **89**, 1011-1015 (1992).
23. C. A. Hunter, R. Chizzonite, J. S. Remington, IL-1 beta is required for IL-12 to induce production of IFN-gamma by NK cells. A role for IL-1 beta in the T cell-independent mechanism of resistance against intracellular pathogens. *J Immunol* **155**, 4347-4354 (1995).
24. E. Gyotoku *et al.*, The IL-6 family cytokines, interleukin-6, interleukin-11, oncostatin M, and leukemia inhibitory factor, enhance mast cell growth through fibroblast-dependent pathway in mice. *Arch Dermatol Res* **293**, 508-514 (2001).
25. E. A. Havell, P. B. Sehgal, Tumor necrosis factor-independent IL-6 production during murine listeriosis. *J Immunol* **146**, 756-761 (1991).
26. Z. Liu, R. J. Simpson, C. Cheers, Role of IL-6 in activation of T cells for acquired cellular resistance to Listeria monocytogenes. *J Immunol* **152**, 5375-5380 (1994).
27. L. W. Collison, D. A. Vignali, Interleukin-35: odd one out or part of the family? *Immunol Rev* **226**, 248-262 (2008).
28. G. Trinchieri, Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* **3**, 133-146 (2003).
29. C. S. Hsieh *et al.*, Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* **260**, 547-549

(1993).

30. J. R. Schoenborn, C. B. Wilson, Regulation of interferon-gamma during innate and adaptive immune responses. *Adv Immunol* **96**, 41-101 (2007).
31. F. Reggiori, D. J. Klionsky, Autophagy in the eukaryotic cell. *Eukaryot Cell* **1**, 11-21 (2002).
32. Z. Yang, D. J. Klionsky, An overview of the molecular mechanism of autophagy. *Curr Top Microbiol Immunol* **335**, 1-32 (2009).
33. D. J. Klionsky, R. Cueva, D. S. Yaver, Aminopeptidase I of Saccharomyces cerevisiae is localized to the vacuole independent of the secretory pathway. *J Cell Biol* **119**, 287-299 (1992).
34. K. Takeshige, M. Baba, S. Tsuboi, T. Noda, Y. Ohsumi, Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J Cell Biol* **119**, 301-311 (1992).
35. B. Levine, J. Yuan, Autophagy in cell death: an innocent convict? *J Clin Invest* **115**, 2679-2688 (2005).
36. B. Levine, D. J. Klionsky, Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* **6**, 463-477 (2004).
37. N. Mizushima, Autophagy: process and function. *Genes Dev* **21**, 2861-2873 (2007).
38. M. Kundu, C. B. Thompson, Macroautophagy versus mitochondrial autophagy: a question of fate? *Cell Death Differ* **12 Suppl 2**, 1484-1489 (2005).
39. R. D. Leone, R. K. Amaravadi, Autophagy: a targetable linchpin of cancer cell metabolism. *Trends Endocrinol Metab* **24**, 209-217 (2013).
40. A. M. Cuervo, E. Wong, Chaperone-mediated autophagy: roles in disease and aging. *Cell Res* **24**, 92-104 (2014).

41. F. Reggiori, D. J. Klionsky, Autophagic processes in yeast: mechanism, machinery and regulation. *Genetics* **194**, 341-361 (2013).
42. Y. Feng, D. He, Z. Yao, D. J. Klionsky, The machinery of macroautophagy. *Cell Res* **24**, 24-41 (2014).
43. S. Jin, Autophagy, mitochondrial quality control, and oncogenesis. *Autophagy* **2**, 80-84 (2006).
44. D. Glick, S. Barth, K. F. Macleod, Autophagy: cellular and molecular mechanisms. *J Pathol* **221**, 3-12 (2010).
45. D. M. Sabatini, mTOR and cancer: insights into a complex relationship. *Nat Rev Cancer* **6**, 729-734 (2006).
46. R. J. Shaw, LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. *Acta Physiol (Oxf)* **196**, 65-80 (2009).
47. K. Inoki, Y. Li, T. Xu, K. L. Guan, Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev* **17**, 1829-1834 (2003).
48. Y. Kamada *et al.*, Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol* **150**, 1507-1513 (2000).
49. S. A. Tooze, T. Yoshimori, The origin of the autophagosomal membrane. *Nat Cell Biol* **12**, 831-835 (2010).
50. S. Pankiv *et al.*, p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* **282**, 24131-24145 (2007).
51. M. G. Gutierrez *et al.*, Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* **119**, 753-766 (2004).
52. A. M. Yakoub, D. Shukla, Autophagy stimulation abrogates herpes simplex virus-1 infection. *Sci Rep* **5**, 9730 (2015).

53. E. F. Castillo *et al.*, Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. *Proc Natl Acad Sci U S A* **109**, E3168-3176 (2012).
54. V. L. Crotzer, J. S. Blum, Autophagy and its role in MHC-mediated antigen presentation. *J Immunol* **182**, 3335-3341 (2009).
55. C. Jagannath *et al.*, Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. *Nat Med* **15**, 267-276 (2009).
56. J. Harris, Autophagy and cytokines. *Cytokine* **56**, 140-144 (2011).
57. J. Harris *et al.*, Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation. *J Biol Chem* **286**, 9587-9597 (2011).
58. N. Dupont *et al.*, Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta. *EMBO J* **30**, 4701-4711 (2011).
59. F. Zang *et al.*, Autophagy is involved in regulating the immune response of dendritic cells to influenza A (H1N1) pdm09 infection. *Immunology* **148**, 56-69 (2016).
60. K. Buffen *et al.*, Autophagy modulates Borrelia burgdorferi-induced production of interleukin-1beta (IL-1beta). *J Biol Chem* **288**, 8658-8666 (2013).
61. C. Peral de Castro *et al.*, Autophagy regulates IL-23 secretion and innate T cell responses through effects on IL-1 secretion. *J Immunol* **189**, 4144-4153 (2012).
62. A. Said, S. Bock, T. Lajqi, G. Muller, G. Weindl, Chloroquine promotes IL-17 production by CD4+ T cells via p38-dependent IL-23 release by monocyte-derived Langerhans-like cells. *J Immunol* **193**, 6135-6143 (2014).
63. J. P. Lee *et al.*, Loss of autophagy enhances MIF/macrophage migration

inhibitory factor release by macrophages. *Autophagy* **12**, 907-916 (2016).

64. C. Centers for Disease, Prevention, Vital signs: Listeria illnesses, deaths, and outbreaks--United States, 2009-2011. *MMWR Morb Mortal Wkly Rep* **62**, 448-452 (2013).

65. N. E. Freitag, G. C. Port, M. D. Miner, Listeria monocytogenes - from saprophyte to intracellular pathogen. *Nat Rev Microbiol* **7**, 623-628 (2009).

66. B. F. Py, M. M. Lipinski, J. Yuan, Autophagy limits Listeria monocytogenes intracellular growth in the early phase of primary infection. *Autophagy* **3**, 117-125 (2007).

67. B. Levine, N. Mizushima, H. W. Virgin, Autophagy in immunity and inflammation. *Nature* **469**, 323-335 (2011).

68. K. Buffen *et al.*, Autophagy suppresses host adaptive immune responses toward Borrelia burgdorferi. *J Leukoc Biol* **100**, 589-598 (2016).

69. A. L. Koch, Turbidity measurements of bacterial cultures in some available commercial instruments. *Anal Biochem* **38**, 252-259 (1970).

70. T. Noda, Y. Ohsumi, Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J Biol Chem* **273**, 3963-3966 (1998).

71. R. Sumpter, Jr., B. Levine, Autophagy and innate immunity: triggering, targeting and tuning. *Semin Cell Dev Biol* **21**, 699-711 (2010).

72. A. Yamamoto *et al.*, Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct Funct* **23**, 33-42 (1998).

73. Y. T. Wu *et al.*, Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III

phosphoinositide 3-kinase. *J Biol Chem* **285**, 10850-10861 (2010).

74. A. Takaoka *et al.*, Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* **434**, 243-249 (2005).

75. G. Zhang, S. Ghosh, Toll-like receptor-mediated NF-kappaB activation: a phylogenetically conserved paradigm in innate immunity. *J Clin Invest* **107**, 13-19 (2001).

76. A. Colleran *et al.*, Autophagosomal IkappaB alpha degradation plays a role in the long term control of tumor necrosis factor-alpha-induced nuclear factor-kappaB (NF-kappaB) activity. *J Biol Chem* **286**, 22886-22893 (2011).

77. S. Morris *et al.*, Autophagy-mediated dendritic cell activation is essential for innate cytokine production and APC function with respiratory syncytial virus responses. *J Immunol* **187**, 3953-3961 (2011).

78. T. Yoshimori, A. Yamamoto, Y. Moriyama, M. Futai, Y. Tashiro, Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J Biol Chem* **266**, 17707-17712 (1991).

79. V. Hornung *et al.*, Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* **9**, 847-856 (2008).

80. P. K. Shihab *et al.*, TLR2 and AP-1/NF-kappaB are involved in the regulation of MMP-9 elicited by heat killed Listeria monocytogenes in human monocytic THP-1 cells. *J Inflamm (Lond)* **12**, 32 (2015).

81. M. Gupta *et al.*, IRF8 directs stress-induced autophagy in macrophages and promotes clearance of Listeria monocytogenes. *Nat Commun* **6**, 6379 (2015).

82. A. Trocoli, M. Djavaheri-Mergny, The complex interplay between autophagy and NF-kappaB signaling pathways in cancer cells. *Am J Cancer Res* **1**, 629-649 (2011).

VIII. Summary in Korean

리스테리아 모노사이토제니스(*Listeria monocytogenes*)는 세포 내에서 기생하는 그람 양성 세균으로 사람과 동물에서 감염형 식중독인 리스테리아증을 일으킨다. 자가소화작용(autophagy)의 유도에 의해서 세포 내 기생하는 리스테리아를 제거 할 수 있다는 것이 보고되었다. 또한, 자가소화작용은 병원성 세균 감염과 TLR자극에 의한 염증성 사이토카인(pro-inflammatory cytokine)의 발현을 조절할 수 있다. 하지만, 리스테리아 감염에서 자가소화작용이 염증성 사이토카인의 발현을 조절한다는 것에 대한 연구는 부족한 실정이다. 따라서, 본 연구에서는 리스테리아에 감염된 골수 유래 대식세포(bone marrow-derived macrophages)에서 자가소화작용의 유도가 염증성 사이토카인의 발현에 미치는 영향에 대해서 연구하였다. 기존 연구 결과와 동일하게 자가소화작용의 유도는 골수 유래 대식세포에 생존하는 리스테리아를 감소시켰다. 또한, 자가소화작용의 유도가 리스테리아에 감염된 골수 유래 대식세포에서 염증성 사이토카인인 interleukin (IL)-1β, IL-6, IL-12p40의 발현을 증가시키는 것을 확인하였다. 이러한 염증성 사이토카인의 발현 조절이 toll-like receptor (TLR)2 자극에 의한 것임을 확인 하기 위해, 열 사멸된 리스테리아를 골수 유래 대식세포에 처리하여 자가소화작용에 의한 사이토카인의 발현을 확인 한 결과 IL-6와 IL-12p40의 발현이 증가하였다. 따라서 TLR2 자극에 의한 염증성 사이토카인의 발현은 자가소화작용에 의해 조절된다고 유추할 수 있다. 그리고, 자가소화작용이 염증성 사이토카인의 발현에 핵심적인

역할을 하는 TLR 하위 신호전달 분자인 interferon regulatory factor 5 (IRF5)와 nuclear factor-kappa B (NF-κB)의 활성에 미치는 영향을 확인하였다. 자가소화작용의 유도에 의해 골수 유래 대식세포에서 IRF5의 발현과 핵으로의 이동이 증가되었으며, NF-κB가 핵으로 이동하는 것을 억제하는 IκBα의 발현이 감소되는 것을 확인하였다. 따라서, 이러한 IRF5와 IκBα의 변화가 자가소화작용에 의한 염증성 사이토카인의 발현 증가에 중요한 역할을 하는 것으로 나타났다.

결과적으로, 리스테리아에 감염된 골수 유래 대식세포에서 자가소화작용의 유도가 염증성 사이토카인의 발현을 증가시키며, 이러한 염증성 사이토카인의 증가에 의해 리스테리아를 제거하는데 영향을 미치는 추가적인 면역반응의 활성을 유도할 수 있을 것이다.